Valorisation of brewers' spent grain: enzymatic hydrolysis in the production of xylooligosaccharides

UNIVERSITY *of the* WESTERN CAPE

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I declare that the thesis entitled "**Valorisation of brewers' spent grain: enzymatic hydrolysis in the production of xylooligosaccharides**" is my own work, that it has not been submitted for any degree or examination in any other university, and that all sources I have used have been indicated and acknowledged by complete references.

Signature:

Date: February 2023

Contents

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List of abbreviations

AX	Arabinoxylan
AXOS	Arabino-xylooligosaccharides
BS	Barley straw
BSG	Brewers' spent grain
DP	Degree of polymerisation
dm	Dry matter content
Glc-OS	Glucooligosaccharides
GH	Glycoside-hydrolase
kg	Kilograms
LHW	Liquid hot water
L	Litres
U	One unit
PBSG	Pure pale malt BSG
RSSE	Residual solids after steam explosion fraction
SCFA	Short chain fatty acids
SE	Steam explosion
SEL	Steam explosion liquid
WBSG	Weiss malt BSG
X2	Xylobiose
X≥7	Xyloheptaose and above
XOS	Xylooligosaccharides
X5	Xylopentaose
X4	Xylotetraose
X3	Xylotriose

List of figures

List of tables

Abstract

The beverage industry constitutes approximately 26% of all food wastes, making it one of the largest contributors in this waste segment. By utilising waste or by-products from agriculture and food production in manufacturing value added compounds, the concepts of waste mitigation and green chemistry can contribute to establishing a circular bio-economy. In a biorefinery, bio-catalytic, thermal, chemical and physical techniques are used to extract valuable compounds from food and agricultural wastes. Brewers' spent grain (BSG) is an ideal candidate for such a biorefinery approach. This high moisture, nutrient-rich by-product from beer production is either disposed of in landfills or used as an animal feed. However, high value products, such as xylooligosaccharides (XOS) can be extracted from BSG, thereby valorising this brewery waste. Xylooligosaccharides are sought after for their ability to function as a low caloric sweetener while exhibiting prebiotic effects in stimulating the growth of probiotic bacteria in the mammalian gut.

This study sought to produce high XOS yields from BSG and to develop a basic process that could be used in a biorefinery. To achieve this aim, mechanical screw press dewatering and steam explosion pretreatment was used as a means of fractionation of BSG prior to enzymatic hydrolysis of selected fractions with an endoxylanase. Screw press dewatering reduced the moisture content of fresh BSG from 79.0% to 73.2% (w/w). During moisture reduction, water-soluble and insoluble components, such as glucooligosaccharides (Glc-OS), starch, lignin and protein from the fresh BSG were transferred to the press liquid fraction which, as a waste stream, can be upcycled into various food products. Steam explosion of pressed BSG solids fractionated the remaining hemicellulose component by partially solubilising it. After separating the steam explosion slurry by centrifugation, the solubilised hemicellulose (35.3%) was recovered in a steam explosion liquid fraction (SEL), with a significant portion of the hemicellulose component (53.6%) remaining in the solid residue fraction (RSSE). The steam explosion process produced an XOS yield of 27.6%, calculated as the mass of XOS in the SEL as a percentage of the initial xylan, which was mostly comprised of xylopentaose (X5) and xyloheptaose and above (X≥7).

The SEL fraction was then subjected to enzymatic hydrolysis using the commercial xylanase Pentopan Mono BG (1; 5; 25 and 50 U.ml SEL^{-1}) to depolymerise the solubilised hemicellulose oligomers with the aim of producing XOS with a degree of polymerisation (DP) of 2 to 6. Time–dependent enzymatic hydrolysis of SEL using an enzyme dosage 50 $U.ml^{-1}$ SEL for 3 hrs, resulted in X2-6 and X2-3 that respectively corresponded to yields of 51.9% and 40.5% as a percentage of the theoretical xylan due to the depolymerisation of X≥7.

The remaining hemicellulose in RSSE was enzymatically solubilised into longer chained XOS DP ≥7 and concurrently depolymerised into shorter XOS chain lengths (DP 2-6) using high solids enzymatic hydrolysis (Pentopan Mono BG 25; 100; 500; 1000 and 1500 U.g $RSSE^{-1}$) at an industrially relevant solids loading of 25% (w/w). During enzymatic hydrolysis of RSSE, different dosages respectively enabled maximum yields for solubilisation and depolymerisation. At 100 $U.g^{-1}$ RSSE for 24 hrs, 41.1% of the available hemicellulose was solubilised mainly into XOS and arabino-oligosaccharides (Ar-OS) with minimal monomer formation. However, a much higher dosage of 1000 $U.g^{-1}$ was necessary to achieve the maximum depolymerisation yield for X2-3 of 13.9%, due to possible product inhibition and mass transfer problems at a higher solids loading.

The fractionation and enzymatic hydrolysis process was able to produce yields of 47.9% for XOS, 16.1% for Ar-OS, 23.9% for X2-6, and 20.4% for X2 and X3 as a percentage of hemicellulose. Therefore, the aim of establishing a viable strategy for BSG valorisation in a biorefinery process by creating a basic process to produce xylooligosaccharides, was achieved. However, with further improvements to the process such as, using fresh SEL, and optimisation of solids loadings for high solids enzymatic hydrolysis of RSSE, higher XOS yields can likely be obtained.

Chapter 1

Introduction

South Africa leads the continent in producing 32 million hectolitres of beer annually (Maqhuzu *et al*., 2019). Brewers' spent grain is an abundant, valuable and inexpensive by-product in the beer-brewing industry made up of the residue collected after the mashing process (Lynch *et al*., 2016). Its main component is malted barley grain (*Hordeum vulgare L*.) and barley husks (Fărcaş *et al.*, 2017; Lynch *et al*., 2016). Brewers' spent grain represents up to 85% of the by-products generated during the beer-making process. Annually, approximately 39 million tonnes are produced globally. South Africa's largest beer producer, South African Breweries (SAB) produces approximately 460 tonnes of BSG a day (Swart *et al.*, 2021).

The majority of all BSG, is used as animal feed, or is disposed of in landfills (Mussatto, 2014). However, its nutrient-rich composition combined with its high moisture content of 75% to 85% (w/w) usually results in rapid microbial spoilage and, therefore, restricts the storage of wet BSG to approximately three days at ambient temperatures (Sterling and Karlijn, 2020; Robertson *et al.*, 2010b). Hence, a logistical challenge is presented to breweries to distribute BSG to farmers prior to spoilage. The accumulation of large amounts of such biologically instable food and agricultural wastes in landfills poses an ecological threat due to imminent greenhouse gas emissions, an altered soil quality, phytotoxicity and pollution of ground water, which occurs as a consequence of the decomposition of organic matter (Nayak and Bhushan, 2019). Furthermore, drying of such large amounts of high moisture wastes prior to disposal is energetically expensive (Mussatto *et al.*, 2006). Thus, it is imperative to find various ways of exploiting waste streams such as BSG for its useful components in industry to mitigate the need for disposal and create additional revenue streams.

Brewers' spent grain has been shown to be a suitable feedstock for a range of applications (Lynch *et al*., 2016; Mussatto, 2014). For example, BSG flour has been widely used during food production for bakery products and can be incorporated into meat products (Garrett *et al.*, 2021; Mussatto, 2014; Özvural *et al.*, 2009; Finley *et al.,* 1976). Aqueous BSG solutions have shown to be successful as a strategy for bioremediation in the adsorption of toxic metals such as, lead and cadmium, and of dyes used in textile and paper industries (Silva *et al.*, 2004; Low *et al.*, 2000). Furthermore, BSG has also been used in energy production as a substrate for biogas (Weger *et al.,* 2017; Sežun *et al.*, 2011) and ethanol production (White *et al.*, 2008). Other industrially important chemicals such as, laccase and polyphenols, hydroxycinnamic acids and α-amylase have been successfully produced through

fermentation using BSG as a carbon source due to its components which makes it suitable for microbial growth (Tišma *et al.*, 2018; Bartolomé *et al.*, 2003; Francis *et al.*, 2002).

Brewers' spent grain is composed of non-starch polysaccharides including cellulose (ca. 19.7% w/w) and hemicellulose (ca. 24.4% w/w), as well as starch (ca. 6.7% w/w), protein (ca. 18.6% w/w), Klason lignin (ca. 14.5%), lipids and phenolic compounds (Lynch *et al.*, 2016; Robertson *et al.*, 2010a). Hemicellulose is the predominant fraction of the dry weight of the BSG because after mashing of the barley grain large amounts of starch and protein are removed from the grain (Robertson *et al.*, 2010a). The hemicellulose component mainly consists of a xylan backbone substituted with arabinan side chains and acetyl groups, which can be hydrolysed into shorter xylan chains containing 2 to 20 xylose residues known as xylooligosaccharides (XOS) (Otieno and Ahring, 2012). Xylooligosaccharides with a degree of polymerisation (DP) of 2-6 are known to exhibit prebiotic effects (Sajib *et al.*, 2018; Gómez *et al.*, 2015). Upon consumption by probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* species, short chain fatty acids (SCFA) are produced which provide a range of health benefits (Rajendran *et al.*, 2017). This includes protection against pathogens, a reduction in cholesterol synthesis, the stimulation of blood flow in the colon, and protection against colon cancer development (Rajendran *et al.*, 2017; Aachary *et al.*, 2015). As consumers are becoming more health conscious, the market for prebiotics is expected to increase from 3.6 billion Euros in 2017 to 6.6 billion Euros in 2023 (Markets and markets, 2018). Xylooligosaccharides are therefore emerging as a value added compound due to their prebiotic potential (Sajib *et al.*, 2018) and organoleptic properties (Kumar *et al.*, 2012), making it suitable for use as a functional food product (Lynch *et al.*, 2016; Carvalho *et al.*, 2013).

The extraction of XOS typically involves a process of fractionation of hemicellulose using hydrothermal technologies, such as liquid hot water treatment and steam explosion (SE), at temperatures ranging between 121 to 200 °C and at residence times of between 10 and 50 min, as a pretreatment to enable greater accessibility of enzymes to active sites (Zhu *et al.,* 2022; Álvarez *et al.,* 2021; Bhatia *et al.,* 2020; Swart *et al.,* 2020; Ravindran *et al*., 2018; Huang *et al.*, 2017; Kemppainen *et al*., 2016; Carvalheiro *et al.*, 2004). Such technologies facilitates the partial release of XOS by a process of autohydrolysis, whereby, acetyl groups are released from the xylan backbone lowering the pH, which causes the degradation of xylan (Otieno and Ahring, 2012). After the pretreatment process and the separation of the slurry into two fractions; residual solids and autohydrolysis liquor, XOS is recovered in the autohydrolysis liquor fraction at yields of between 21.1% and 75.3% of XOS (as a weight percentage of the initial xylan) depending on the feedstock composition, starting solids concentration, reaction condition and the efficiency of SE as it relates to these combined factors, as well as the degradation

of xylose into furfural (Álvarez *et al*., 2021; Swart *et al*., 2020). Previously, using barley straw at a low solids concentration of 10% (w/w dried mass) as a feedstock a XOS yield of 62.9% was obtained after SE (Álvarez *et al*., 2021). Moreover, using pure pale malt BSG (PBSG) and Weiss malt BSG (WBSG) at a solids concentration of 15% (w/w dried mass), respective XOS yields of 50% and 21.1% were obtained after SE (Swart *et al*., 2020). Coupled with mechanical screw press dewatering as the method for efficiently removing moisture from fresh BSG prior to SE, higher XOS yields of 75.3% and 73.1% for PBSG and WBSG (25% w/w dried mass) were respectively obtained after SE. Through the usage of a screw press, starch and protein was removed from the fresh BSG and transferred to the press liquid fraction. This contributed to the enrichment of the hemicellulose component in the pressed BSG solids and subsequently resulted in higher XOS yields after SE (Swart *et al*., 2020). The XOS yielded in the liquid fraction after hydrothermal pretreatment is typically in its longer form, made up of more than 6 xylose residues (Álvarez *et al*., 2021; Bhatia *et al*., 2020; Vegas *et al*., 2008; Kabel *et al*., 2002).

To obtain low molecular weight XOS with a DP of 2 to 6, enzymatic hydrolysis is used to depolymerise the longer XOS molecules found in the autohydrolysis liquor (Álvarez *et al*., 2021; Su *et al*., 2021; Bhatia *et al*., 2020; Lian *et al*., 2020; Huang *et al*., 2017; Gómez *et al.*, 2015; Vegas *et al*., 2008). According to Alvarez *et al.*, (2021), prior to enzymatic hydrolysis of autohydrolysis liquor, the XOS DP 2-6 was 35.7% of the initial xylan. When a complex enzyme cocktail made up of endo-β-(1,4)-D-xylanase, α-L-arabinofuranosidase, acetylxylan esterase and feruloyl esterase was applied, the yield increased to 58.8%. In addition to complex enzyme cocktails, enzymes used for depolymerisation of longer XOS molecules are endo-β-(1,4)-xylanase preparations with low β-xylosidase activity, which allows for less xylose formation by minimising the cleaving off of xylose residues from the end of the xylan chain (Otieno and Ahring, 2012). Moreover, endo-β-(1,4)-xylanases from the glycoside-hydrolase (GH) families 10 and 11 that have an affinity for DP 4-6 and DP ≥4, respectively, are also used in the production of low molecular weight XOS, using autohydrolysis liquor as a substrate (Saini *et al*., 2022; Álvarez *et al*., 2018; Gomez *et al*., 2015; Carvalho *et al*., 2013; Vegas *et al*., 2008). Enzymes from the family GH 10 typically produces XOS with a DP of 2-3, while those from GH 11 produces XOS with the a DP of 4-6 (Vegas *et al*., 2008). Furthermore, research has shown that after hydrothermal pretreatment, yields of XOS (DP 2-6) of ca. 41.2-58.8% (w/w percentage of the initial xylan) can be achieved with the enzymatic hydrolysis of the liquid fraction, making it the most suitable substrate for low molecular weight XOS production (Álvarez *et al*., 2021; Su *et al*., 2021; Bhatia *et al.*, 2020; Lian *et al*., 2020; Huang *et al*., 2017; Gómez *et al.*, 2015). In lignocellulosic *Miscanthus,* ca. 52.6% of the initial xylan was released as XOS, of which ca. 80.8% was depolymerised to low molecular weight XOS (DP 2 and 3) and ca. 5.3%, released as xylose in the autohydrolysis liquor (Bhatia *et al.*, 2020). However, ca.

42.1% of the xylan remained in the solid residue after autohydrolysis and was considered to be less accessible, yet could be released by a commercial xylanase (Novozymes endoxylanases NS22083).

Due to the extensive hydrolysis of hemicellulose during autohydrolysis, smaller amounts of xylan (23.5-42.1%) is typically recovered in the residual solids of various lignocellulosic feedstocks (Zhu *et al.*, 2022; Alvarez *et al.*, 2021; Bhatia *et al.*, 2020; Huang *et al.*, 2017). However, as seen previously in BSG, ca. 83.9% of xylan remained in the residual solids after SE at 200 °C for 10 min (Kemppainen *et al.*, 2016). Some attempt has been made to liberate such residual xylan using enzymatic hydrolysis. Firstly, at a solids loading of 20% (w/w dried BSG), Kemppainen *et al.*, (2016) found that using an enzyme cocktail (made up of 60% cellobiohydrolase I, 10 % cellobiohydrolase II, 15% endoglucanase, 12% xylanase and 3% betaglucosidase) resulted in a hydrolysate consisting of 59% carbohydrates of which 66% was glucose, 20% was xylose and 7% was arabinose. Secondly, at low solids loadings of 1% to 10%, literature shows that the hydrolysis of residual xylan from wheat straw, *Miscanthus*, rye grass, rye grass pulp and corn powder after hydrothermal treatment into low molecular weight XOS was attainable (Precup *et al.*, 2022; Bhatia *et al.*, 2020; Dotsenko *et al.*, 2018; Aachary and Prapulla, 2009). However, little to no attempt has been made to liberate the residual xylan at high solids loading for the production of XOS using the solid residue of BSG after hydrothermal treatment as a substrate.

It is clear that potentially many streams can be created during the fractionation of BSG through screw press dewatering, SE pretreatment and enzymatic hydrolysis. To attain whole-waste exploitation of BSG, there is a need for more investigation into valorisation of these fractions. Therefore, this study sought to produce XOS by applying a mild SE pretreatment to screw pressed BSG for partial hemicellulose release (**Figure 1.1.**). Then, using an endoxylanase, both the liquid and residual solid fractions obtained after SE pretreatment was enzymatically hydrolysed for the purpose of depolymerisation of longer chained XOS in the liquid fraction and for the solubilisation of xylan in the residual solids.

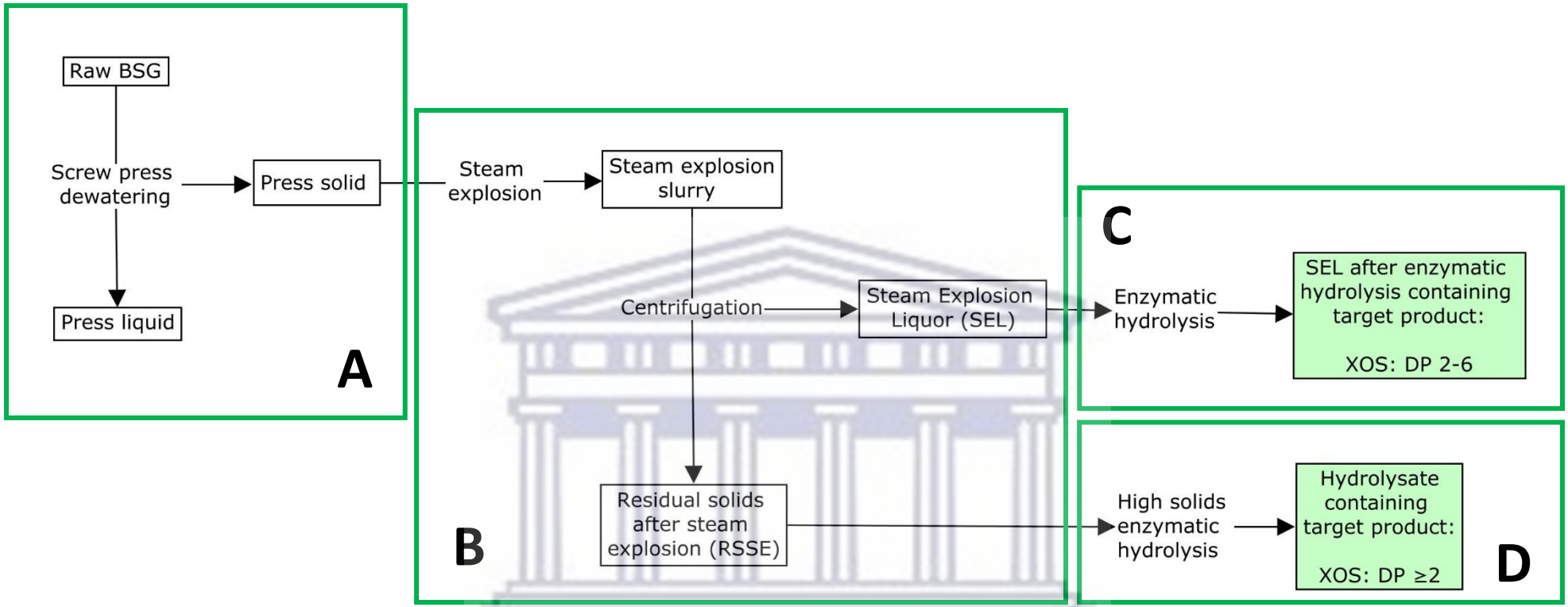

Figure 1.1. Layout of BSG processing steps used in this study. Screw press dewatering and steam explosion pretreatment followed by enzymatic hydrolysis of steam explosion liquor and solid residue after steam explosion producing the target product in green. **A.** Screw press dewatering of raw BSG which produced the press liquid and press solid fraction. **B.** Steam explosion of the pressed solids, after which a slurry was formed and separated by centrifugation into a steam explosion liquor and a residual solid fraction. **C.** Enzymatic hydrolysis of the steam explosion liquor into low molecular weight XOS target product. **D.** High solids enzymatic hydrolysis of the residual solids obtained after steam explosion producing XOS as the target product.

Chapter 2:

Literature Review

2.1. Waste-management and biomass valorisation

In South Africa, it is estimated that 10.2 million tonnes of food waste is generated annually throughout the food supply chain from agricultural production to the consumer (DEFF and CSIR, 2021). It is further estimated that 2.6 million tonnes are generated during processing and packaging. These wastes typically contain a high moisture content, a volatile microbial population and organic loading, which promotes microbial activity (Nayak and Bhushan, 2019). Previously, agro-food industries in developing countries did not have a large scope for waste-management. Therefore, prior to 2006, wastes were disposed by allowing it to dry and decay, burying, burning, and by disposal at sea (Onu and Mbohwa, 2021). These methods of disposal lead to environmental problems such as, bad odour, an altered soil quality, the pollution of ground water, phyto-toxicity, air pollution and toxicity to aquatic life (Nayak and Bhushan, 2019). Since then, waste-management concepts have evolved to include the reuse and recycling of agro-food wastes or by-products by employing valorisation techniques.

The green chemistry concept focusses on efficiently utilising sustainable resources, waste minimisation and circumventing the use of toxic or hazardous reagents during the manufacturing of chemical products (Sheldon, 2016). While resources used are not always from renewable sources, this is preferred. By utilising waste or by-products from agriculture and food production in creating value added compounds, the concepts of waste mitigation, and green chemistry can be linked, resulting in a circular bio-economy. This is known as the biorefinery concept in which, biomass feedstocks such as, barley straw, wheat straw, rice husk and brewers' spent grain are processed by efficiently applying bio-catalytic, thermal, chemical and physical techniques for extraction of valuable compounds (Álvarez *et al.* 2021; Sajib *et al.* 2018; Huang *et al.* 2017; Vegas *et al.* 2008).

According to Baiano (2014), the largest contributor to food waste is the drinks industry, which contributes to approximately 26% of food wastes. Brewers' spent grain is a by-product of the beer brewing process consisting of fibre, protein, lipids and ash, and has low value as an unprocessed food-grade feedstock (Lynch *et al.*, 2016). The annual global production of BSG is estimated to be approximately 39 million tonnes. In Europe and the United States respectively, around 3.4 and 4.5 million tonnes of BSG is produced annually (Mitri *et al.*, 2022). As the country's largest producer of beer, South African Breweries (SAB) produces approximately 460 tonnes of BSG per day. Disposal of BSG can be problematic due to the negative environmental impacts such as, ecological effects of

dumping at landfill sites and energy consumption and the associated emissions of incineration (Assandri *et al.*, 2021). Furthermore, compared to other lignocellulosic feedstocks, BSG has a low market price of ca. 40 USD per tonne and is mainly supplied to farmers as an animal feed. However, the BSG supply can often outweigh the demand, which implies that further technology development is required to add value to this resource while simultaneously preventing the brewery by-product from being disposed (Lynch *et al.,* 2016; Buffington, 2014).

Due to its high hemicellulose component of 19.2-41.9% (w/w dried BSG), there is much potential for adding value by producing XOS using a biorefinery concept (Lynch *et al.,* 2016). Xylooligosaccharides are oligomers with a degree of polymerisation (DP) of 2 to 20 that are produced by the hydrolysis of xylan through thermal, chemical and enzymatic methods which are commonly used in conjunction with each other (Otieno and Ahring, 2012). Furthermore, XOS are sought-after as a functional food due to its prebiotic abilities and health benefits, cryoprotective properties and its ability to function as a low caloric sweetener (Su *et al.*, 2020; Sajib *et al.*, 2018; Lynch *et al.*, 2016; Gómez *et al.*, 2015).

2.2. Production of brewers' spent grain

Beer brewing occurs in three main stages namely, mashing, boiling and fermentation (**Figure 2.1. A**) (Lynch *et al.*, 2016). Prior to mashing, the barley grain undergoes malting, which includes the controlled germination of barley grain by steeping and drying in a kiln at to a moisture content of 4-4.5% (Wang *et al.*, 2004). In the mashing stage, with added water, malted barley undergoes various biochemical reactions. The malted starch is converted to fermentable (maltose and maltotriose) and non-fermentable (dextrins) sugars in a process of saccharification involving endogenous amylase. Proteins are partially converted to soluble proteins, polypeptides and amino acids through enzymatic degradation as well (Fărcaş *et al.*, 2017). This stage of the process results in a liquid fraction known as wort and the solid fraction, which consists mainly of the outer layers of the barley grain and is known in the industry as BSG (**Figure 2.1. B**). After filtration in the lauter tun, the wort is transferred to a brewing kettle, hops is added and the mixture is boiled (Lynch *et al.*, 2016). The liquid extract is then separated from the spent hops, and is fermented with yeast to form beer (Fărcaş *et al.*, 2017). The main by-products of this process are therefore, BSG making up 85% of the brewing by-products, with spent hops (trub) and spent yeast making up the remaining 15% (Mussatto *et al*., 2006). From 100 litres of beer produced, approximately 25 kg of wet BSG is generated (Mitri *et al.*, 2022).

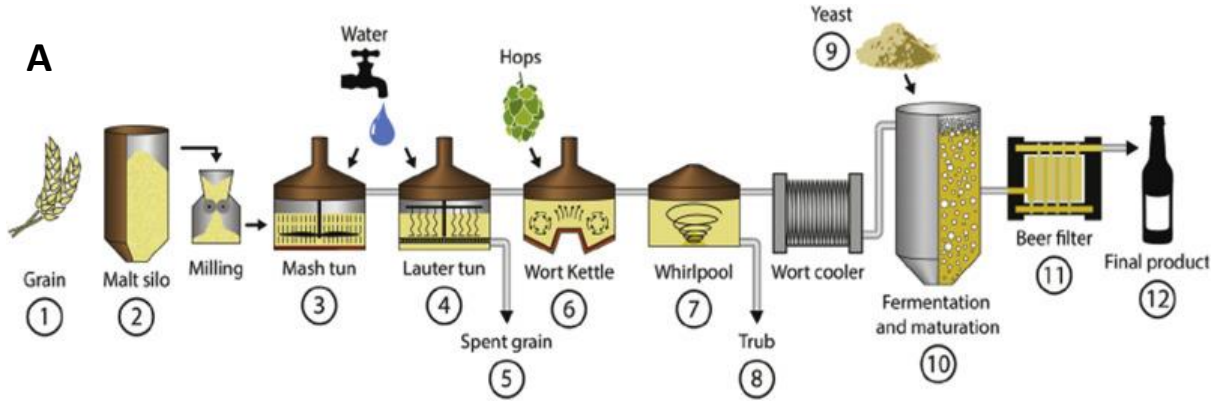

Figure 2.1. A: Overview of the beer brewing process (Fangel *et al*., 2018). B: Brewers spent grain from SAB, Newlands.

2.3. Components of brewers' spent grain

Brewers' spent grain has a moisture content of 75-85% (w/w) with an insoluble fraction (15-25% w/w) mainly consisting of the barley husk, pericarp and seed coat, along with smaller amounts of starchy endosperm and cell walls of the aleurone layer, which may differ due to variations in the mashing regime from one brewery to the next (Swart *et al.*, 2020; Lynch *et al.*, 2016). Furthermore, BSG composition is also affected by the harvesting time, cereal variety and beer type (Lynch *et al.,* 2016; Robertson *et al.*, 2010a). However, it has been reported that the profile of non-starch polysaccharides (hemicellulose, lignin and cellulose) remain relatively similar between breweries making up a large percentage (approximately 50-68% of the dried BSG) (Robertson *et al.*, 2010a). Since hemicellulose forms part of the lignocellulosic matrix, having a high amount of lignocellulose, which contains high amounts of hemicellulose is beneficial for the production of XOS.

2.3.1. Chemical composition

2.3.1.1. Hemicellulose

Hemicelluloses are a group of complex amorphous heteropolysaccharides that are classified according to their main sugar residue for example xylans, arabinans, mannans and glucans (Wyman *et al.,* 2005). Brewers' spent grain has a hemicellulose content of 18.9% to 29.6% (w/w) and in rare cases, as much as 40.0% and above (Swart *et al.*, 2020; Lynch *et al.*, 2016; Meneses *et al.*, 2013; Waters *et al.*, 2012; Robertson *et al.,* 2010b; Celus *et al*., 2006; Kanauchi *et al*., 2001). Compared to the ranges obtained for feedstocks such as, rice straw, wheat straw, barley straw, oat straw and rice husks which have a hemicellulose content of between 16.1% and 23.4%, BSG is slightly higher (Mussatto, 2014). The function of the hemicellulose matrix is to complement the cell wall by strengthening the rigidity of the cellulose microfibril through its interaction with cellulose and lignin (Wyman *et al*., 2005). Furthermore, adhesion between hemicellulose, cellulose and lignin is provided by hydrogen bonds, covalent bonding and van der Waals forces attributed to side chains in the xylan backbone, ultimately forming a recalcitrant crosslinked polymer referred to as lignocellulose (Otieno and Ahring, 2012).

The xylan backbone is made up of β-1,4-linked D-xylose units and is, therefore, a source of xylose and XOS (Sajib *et al.*, 2018; Gomez *et al*., 2015; Otieno and Ahring, 2012). These components are liberated through fractionation and hydrolysis of the hemicellulose component facilitated by thermal, chemical and enzymatic hydrolysis, or a combination thereof. The hemicellulose component of BSG is predominantly made up of arabinoxylans (AX), which make up 14.8% (w/w) of dried BSG (Sajib *et al.*, 2018). It contains a xylan backbone substituted with single α-L-arabinofuranosyl residues, which are branched as side groups and may also be attached to glucuronic acid or ferulic acid residues (**Figure 2.2.**). The xylan backbone is hydrolysed by xylanases. More specifically, endo-1,4-β-xylosidase which cleaves within the xylan chain to form XOS and by β-xylosidase which cleaves off the end of the xylan chain to form xylose monomers (Otieno and Ahring, 2012). Moreover, the hydrolysis of α-L-arabinofuranosyl, glucuronic acid and ferulic side chain residues are achieved by α-L-arabinofuranosidase, α-D-glucuronidase and ferulic acid esterase, respectively, which expands the accessibility of xylanases to their active sites, resulting in improved xylan hydrolysis (Álvarez *et al.*, 2021; Otieno and Ahring, 2012). Furthermore, the xylan backbone is also substituted with acetyl groups which play a major role in hemicellulose breakdown during autohydrolysis.

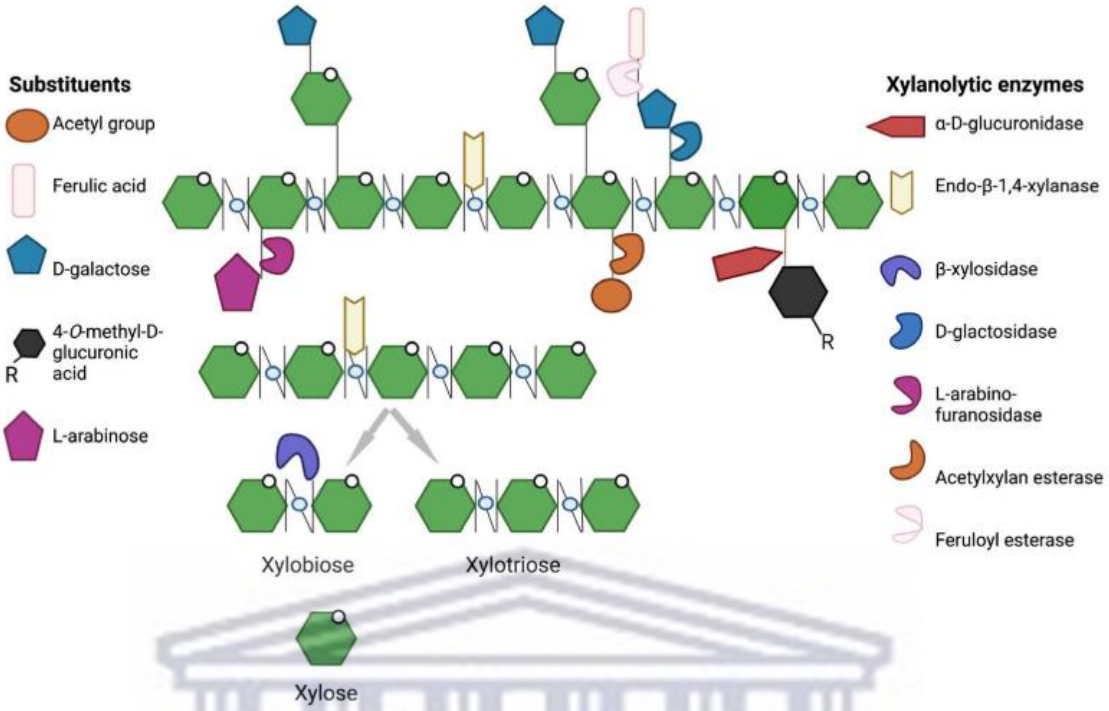

Figure 2.2. Structure of hemicellulose and sites of enzymatic hydrolysis (Kruger and den Haan, 2022).

2.3.1.2. Lignin

Lignin is a major constituent of lignocellulosic biomass, making up approximately 10-28% of dried BSG (Lynch *et al.,* 2016). It is a polyphenolic constituent in plants made up of three monomers including coniferyl alcohol, *p*-coumaryl alcohol, and sinapyl alcohol, which binds the cellulose-hemicellulose matrix (Lynch *et al.,* 2016). Hemicellulose and lignin are linked through ester bonds, for example acetyl groups are bound to xylan chains in the hemicellulose through ester linkages on the one hand and to lignin on the other (Otieno and Ahring, 2012). Thus, lignin removal may become essential in enhancing accessibility of enzymes to hemicellulose during valorisation of lignocellulosic biomass for the production of xylan-derived XOS (Fuso *et al.,* 2021).

2.3.1.3. Cellulose

Cellulose is a polymer with a crystalline, unbranched structure consisting of D-glucose monomers, which are linked by β-1, 4-glycosidic bonds, with neighbouring chains linked through hydrogen bonds. It is a structural component of the cell walls of vascular plants, many algae and oomycetes that stabilises the cell wall by allowing it to become rigid and tough (McNamara *et al.,* 2015). In BSG, the cellulose content is approximately 12-25% (w/w dried BSG) and compared to other agro-industrial by-products such as spent wheat straw (450 $g.kg^{-1}$), rice husks (367 $g.kg^{-1}$) and oat straw (398 $g.kg^{-1}$) the cellulose content is lower in barley husks (214 $g.kg^{-1}$) (Lynch *et al.,* 2016; Mussatto, 2014). Furthermore, the content of cellulose is generally lower than that of hemicellulose in BSG compared

to other by-products (Lynch *et al.,* 2016). For example, in profiling the composition of BSG of 10 breweries, Robertson *et al.*, (2010a) found that the mean arabinoxylan made up ca. 24.4% (w/w) of the dried BSG, while the mean cellulose was 19.7% with little variance.

2.3.1.4. Starch

Starch found in the endosperm of barley consists of D-glucose monomers connected by α-1,4-glycosidic bonds and acts as a carbohydrate store for plants (Lynch *et al.*, 2016). Most of the starch component is enzymatically removed during the mashing process due to endogenous amylases inherent in the grain. However, it has been demonstrated that residual starch makes up 2-12% of the dry weight of BSG depending on variation between breweries and beer type (Lynch *et al.*, 2016; Robertson *et al.*, 2010a). For example, BSG from ale malt contained 9.8% of residual starch, while that of lager malt contained only 3.6%. To minimise contaminating components during the production of XOS, starch is usually removed by a range of pretreatment steps including enzymatic hydrolysis of the amylose using amylase enzymes (Sajib *et al.*, 2018), aqueous treatments and non-isothermal water extractions (Gómez *et al.*, 2015), as well as screw press dewatering (Swart *et al.*, 2020). Therefore, to save on costs related to pretreatments for starch removal and downstream purification of the XOS fraction, sourcing BSG from breweries with a more efficient mashing regime would be preferred.

2.3.1.5. Protein and amino acids

Protein makes up approximately 20% (w/w) of dried BSG with some variation (Lynch *et al*., 2016). For example, Robertson *et al.*, (2010a) has reported that BSG obtained from lager breweries has a higher protein content (22.3%) than that of ale-producing breweries (14.9%) due to the variety of barley used and the higher temperature at which ale malt is kilned. Although malting leads to proteolytic degradation causing a reduction of about half the hordein (a glycoprotein found in the endosperm of barley) content in the barley grain, hordeins remain the most abundant protein in BSG (Lynch *et al.*, 2016). According to Robertson *et al.*, (2010a), the predominant amino acids detected were glutamic acid, proline, leucine and aspartic acid. In addition, amongst the essential amino acids, leucine, phenylalanine, valine and lysine were the most abundant. During the production of XOS using BSG as the feedstock, protein derived products end up in hydrolysates after hydrothermal treatment, necessitating a refining process (Gómez *et al.*, 2015). Altogether, the high moisture content and nutrient-rich chemical composition renders the BSG susceptible to microbial spoilage which will be discussed in the following section (Robertson *et al.,* 2010b).

2.3.2. Microbiological composition and spoilage

Brewers' spent grain is prone to rapid deterioration by microbial attack from resident bacteria and moulds present in the harvested cereal grain, limiting its use as a food-grade feedstock (Robertson *et al.,* 2010a). It was found that the naturally-associated microbiome of fresh BSG sampled a few hours after mashing, consisted mainly of low amounts of aerobic mesophilic and thermophilic bacteria betweeen 10^2 and 10^3 microorganisms.g^{-1} fresh BSG (Robertson *et al.*, 2010b). In another study, the concentration of aerobic mesophilic and thermophilic bacteria, predominantly spores, for which the mashing temperatures would select, was found to be between 10^2 and 10^7 microorganisms.g^{-1} fresh BSG (Robertson *et al.,* 2010a). In addition, microorganisms, such as *Pseudomonas spp*. remained below the limit of detection (10^2) while yeasts and moulds were detected in slightly larger amounts (below $10^{4.56}$ micro-organisms.g^{-1} fresh BSG). Thus, at the point of production, BSG was considered microbiologically stable and acceptable for use in food. Hereafter, the microbiome rapidly changed due to the proliferation of anaerobes and microaerophilic bacteria.

The imminent deterioration of wet BSG restricts exploitation and causes a threat for storage, transport, downstream processing, and general applications such as, the use of BSG as a food-grade feedstock. Thus, it is important to consider post-production storage conditions and its effect on microbial proliferation. According to Robertson *et al.,* (2010b), at 20 °C microbial populations reached their maximum by day 5. Mesophilic bacteria increased to ca. 10^6.g^{-1} fresh BSG, while thermophilic bacteria increased to ca. 10^8.g^{-1} fresh BSG. Therefore, for its use as a food-grade feedstock, BSG must be utilised prior to this 5 day limit. For samples stored at 4 °C, naturally associated aerobic mesophilic and thermophilic bacteria remained below 10^4.g^{-1} fresh BSG during the first week of storage. After a 16-day storage period, they proliferated to below 10^6.g^{-1} fresh BSG. However, at this point their growth had not entered stationary phase and had, therefore, been slowed down indicating that storing BSG at 4 °C is suitable only for short term storage. Moreover, samples that were respectively frozen and autoclaved showed no evidence of microbial activity after 16 days of storage indicating that these methods may be appropriate for long term storage. However, autoclaving resulted in compositional changes such as, losses of starch, ferulic acid and highly branched arabinoxylan. Furthermore, although refrigerating, freezing and autoclaving are proven to inhibit microbial proliferation to various extents on a lab scale, these methods are not feasible for industry-scale operations due to the associated costs (Robertson *et al.,* 2010b; Mussatto *et* al., 2006). Therefore, larger volumes of BSG should be used as a fresh feedstock obtained on the day of production. Furthermore, to eliminate logistical complications including, transport and storage, and to carry out economically viable processes for BSG valorisation a small biorefinery for BSG processing should ideally be annexed to a brewery (Swart *et al.*, 2021).

2.4. Potential applications of brewers' spent grain in industry

Brewers' spent grain can potentially be used in industry as a feedstock for biotechnological processes, bioremediation, energy production and food production (Lynch *et al*., 2016; Mussatto, 2014). Its physical and chemical properties are well suited to support the growth of microorganisms, which is beneficial for the production of industrially important chemicals through fermentation (Tišma *et al.*, 2018; Bartolomé *et al.*, 2003; Francis *et al.*, 2002; Wang *et al.*, 2001; Sim and Oh, 1990). In addition, the fibrous nature of BSG makes it suitable for use in the production of paper products and cellulose nanofibre, which can lead to a range of applications in commercial products (Berglund *et al.*, 2016; Mussatto *et al.*, 2008). It was also demonstrated that aqueous BSG solutions could be used in the adsorption of toxic metals such as lead and cadmium, and of dyes used in textile and paper industries, which reveals a potential to alleviate environmental threats (Silva *et al.*, 2004; Low *et al.*, 2000). Another potential application of BSG which has been explored, is the production of energy in the form of biogas (Weger *et al.,* 2017; Sežun *et al.*, 2011) and ethanol (White *et al.*, 2008). Although the composition, availability and low cost of BSG may be advantageous for such industrial applications, processes may still be challenged by its short shelf life in terms of storage and transportation issues which will cause production costs to increase. Higher value products should therefore be sought out to offset these costs.

2.4.1. Application of brewers' spent grain in feed and food

At little to no additional cost, BSG is commonly used as animal feed for cattle and pigs since it contains all the essential amino acids for animal nutrition and has been shown to increase milk production in cows, decrease the fat content of the milk and increase weight gain (Kaur and Saxena, 2004; Belibasakis and Tsirgogianni, 1996; Huige, 2006). Furthermore, BSG flour is widely used in bakery products, such as breads, biscuits, cakes, waffles, tortillas and a wide variety of snacks (Garrett *et al.*, 2021; Mussatto, 2014; Finley *et al.,* 1976). Additionally, BSG flour can be incorporated into meat products such as frankfurters and smoked sausages or be used as a vegetarian alternative to meat products (Özvural *et al.*, 2009; Finley *et al.,* 1976). The use of BSG flour in such products is suitable because it retains water, decreases cooking losses and produces a high-fibre and low-fat meat product, while decreasing the amount of synthetic antioxidant additives (Özvural *et al.*, 2009).

2.4.2. Health benefits of brewers' spent grain

Upon consumption, BSG provides major health benefits to humans. Compounds, such as arabinoxylan, β-glucans, essential amino acids, protein, lignin and phenolic compounds improves nutritional value when supplemented into foods (Lynch *et al.,* 2016). Arabinoxylan, as well as its oligomeric forms XOS

and arabino-xylooligosaccharides (AXOS) are considered to be a major component of dietary fibre originating from the hemicellulose component (Sajib *et al.*, 2018; Moure *et al.*, 2006; Mandalari *et al.*, 2005). β-glucan is a minor fibre component of BSG, which increases gastrointestinal viscosity, reducing the reabsorption of bile acids and increasing the synthesis of bile acids from cholesterol, leading to an overall reduction in cholesterol and risk of coronary heart disease (Steiner *et al.,* 2015; Truswell, 2002). Lignin is another component of dietary fibre and is considered to be an inert compound within the gastrointestinal tract that remains unaffected by the gut microflora. However, through a colonic model it was demonstrated that a lignin-rich fraction of BSG allowed bifidobacteria to survive longer than when it was provided with a glucose substrate (Niemi *et al.,* 2013). Additionally, the lignin component has proved to be a valuable source of phenolic acids, which accumulate in the husks and cell walls of barley grain (Mussatto, 2014). When phenolic extracts from BSG, which was rich in ferulic acid and *p*-coumaric acid was examined, it was found that it significantly reduced DNA damage caused by hydrogen peroxide, with some extracts providing more protection than the ferulic acid control (Mccarthy *et al.,* 2013). Furthermore, the health benefits of BSG ingestion included an accelerated transit time in the gut, increased faecal weight and fat excretion, alleviating constipation and diarrhoea, and decreased gallstone incidence (Mussatto, 2014).

2.5. Xylooligosaccharides and its prebiotic potential

Xylooligosaccharides are obtained by the hydrolysis of xylan into smaller oligomeric molecules with a degree of polymerisation (DP) of 2-20 after which they are named xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and so forth (Lian *et al.*, 2020; Sajib *et al.*, 2018). Although commercial XOS products usually have a DP of between 2 to 4, molecules with a DP of up to 20 are still considered to be XOS (Poletto *et al.,* 2020). Xylooligosaccharides are linked by β-(1,4) bonds and can be substituted with various side groups such as acetyl groups, uronic acid and arabinofuranosyl residues, which give it a branched structure (Coelho *et al.,* 2016). These chemical properties allow XOS to have excellent stability over a wide range of conditions with pHs of 2.3 to 8 and temperatures up to 120 °C (Contesini *et al.*, 2019; Carvalho *et al.*, 2013). This is advantageous over fructooligosaccharides which were shown to be decomposed by up to 10% (w/w) at 100 °C, at pHs of 2, 3 and 7, while the decomposition of XOS remained undetected or below 4% (Courtin *et al.*, 2009). Due to its stability at low pHs, XOS are able to pass through the stomach and into the large intestine and are, therefore, classified as non-digestible oligosaccharides (Contesini *et al.*, 2019). Xylooligosaccharides are non-carcinogenic, 30-40% as sweet as sucrose, and low in calories, making it suitable for low-calorie diet products (Kumar *et al.*, 2012; Carvalho *et al.*, 2013). Commercial XOS are sold either as a powder or syrup at various concentrations and are the most competitively priced of

all the oligosaccharides due to a low dosage of only 1.4 – 2.8 g per day being required to achieve a prebiotic effect (Cardoso *et al.*, 2021; Otieno and Ahring 2012).

The prebiotic potential of XOS has been well documented throughout literature (Grootaert *et al.*, 2007). Xylooligosaccharides have been proven to stimulate increased levels of probiotics such as, *Lactobacilli* and *Bifidobacteria* (**Table 2.1.**), in some cases to a greater extent than other prebiotics, such as fructooligosaccharides and other oligosaccharides (Sajib *et al.*, 2018; Gómez *et al.*, 2015; Carvalho *et al.*, 2013; Moure *et al.*, 2006). The prebiotic effectiveness and biological activity of XOS are dependent on their degree of polymerisation (DP). Xylooligosaccharides with a DP of 2 and 3 were shown to be preferred by probiotic bacteria because they consume these molecules much faster than XOS with a DP of 5 and 6 (Gómez *et al.*, 2015; Gullón *et al.*, 2008; Moura *et al.*, 2007). Furthermore, Gullón *et al.*, (2008) showed that in a XOS mixture, 84% xylobiose (X2), 90% xylotriose (X3), 83% xylotreaose (X4) and 71% xylopentaose (X5) was consumed by *B. adolescentis* CECT 5781, indicating that more X2, X3 and X4 was consumed than X5. In addition, X2 and X3 were also consumed faster, which altogether showed the preference of X2 and X3 consumption. Upon consumption, probiotic bacteria then produce short chain fatty acids (SCFA), which provide protection against pathogens, reduces cholesterol synthesis, stimulates colonic blood flow, enhances muscular contractions and protects against colon cancer development (Rajendran *et al.*, 2017; Aachary *et al.*, 2015). As an added benefit, XOS have antioxidant activity, anti-allergy, antimicrobial, anti-infection and anti-inflammatory effects along with properties such as cytotoxic activity and immunomodulatory action amongst other health benefits (Adamberg *et al.*, 2014; Nabarlatz *et al.*, 2007).

Table 2.1: Probiotic strains which utilise xylooligosaccharides

Probiotic sample origin	Probiotic strain(s)	XOS utilisation/ SCFA production	Reference
Single bacterial strains	*Lactobacillus brevis*	Growth sustained by XOS; 10.13 mg/ml SCFA produced	Sajib *et al.*, 2018
	Bifidobacterium adolescentis	Growth somewhat sustained by XOS; 3.2 mg/ml SCFA produced	
Human faecal inoculum	-	BSG AXOS- 50% purity - consumed during fermentation; 0.7 mg/ml SCFA produced	
Human faecal inoculum	-	89.3% XOS consumed during fermentation; 125.04 mM SCFA produced	Gómez *et al.*, 2015
Single bacterial strains	*Bifidobacterium breve 46*	Growth sustained by GOS-FOS-XOS mixture	Adamberg *et al.*, 2014
Single bacterial strains	*Bifidobacterium adolescentis* CECT 5781.	Growth sustained by XOS with 77% of XOS consumed	Gullón *et al.*, 2008
Single bacterial strains	*Lactobacillus brevis; Bifidobacterium adolescentis*	Growth sustained by XOS	Moura *et al.*, 2007

Xylooligosaccharide utilisation and probiotic activity represented by consumption of XOS or whether growth of probiotics were sustained by XOS and the concentration of SCFA (short chain fatty acids) produced by probiotics. GOS: glucooligosaccharides. FOS: Fructooligosaccharides.

2.6. Xylooligosaccharide production through fractionation and enzymatic hydrolysis of lignocellulosic biomass

*2.6.1. Mechanical dewatering of brewers' spent grain (refer to **Figure 1.1. A**)*

Mechanical dewatering methods are commonly used for reducing moisture in BSG (Milew *et al*., 2022; Akermann *et al*., 2020; Swart *et al.*, 2020; Bjerregaard *et al*., 2019; Weger *et al*., 2017; Machado *et al*., 2016; Finley *et al.,* 1976). Drying BSG to a shelf-stable moisture content of approximately 10% (w/w) is not always industrially feasible using methods such as, oven drying at 60 °C and freeze-drying due to process-related disadvantages such as, the generation of unpleasant flavours, toasting and burning of the grain, high energy requirements and high capital costs for equipment (Mussatto *et al.*, 2006). However, employing a mechanical dewatering step coupled with traditional drying technologies may save on such expenses (Machado *et al.*, 2016; El-Shafey *et al.*, 2004). Mechanical dewatering systems used in the moisture reduction of BSG includes a screw press, rotary drum press, friction press, hydraulic basket press and a membrane filter press (**Table 2.2**.). A screw press (**Figure 2.3.**) involves loading the wet biomass into a feeder area where it is transported to a compression zone, which in the current study, is comprised of a single screw. The bottle neck of biomass between the compression zone and extruder (**Figure 2.3. C and D**) results in a pressure build-up. This action then causes the biomass to become compressed and moisture to seep out of the compression zone (Yan and Modigell,

2012). The biomass is thus, fractionated into a press liquid and a press solid, which exits the screw press through an opening onto which various extruder configurations can be attached (Figure 2.1. **D**). By using a screw press, the moisture content of BSG can be reduced by between 10% and 22.2% depending on the configuration (Swart *et al.*, 2020; Weger *et al*., 2017; Weger *et al.*, 2014). In doing so, water-soluble and insoluble components are also removed, which contributes to the enrichment of cellulose and hemicellulose in the press solids (Swart *et al.*, 2020; Lopez *et al.*, 2012).

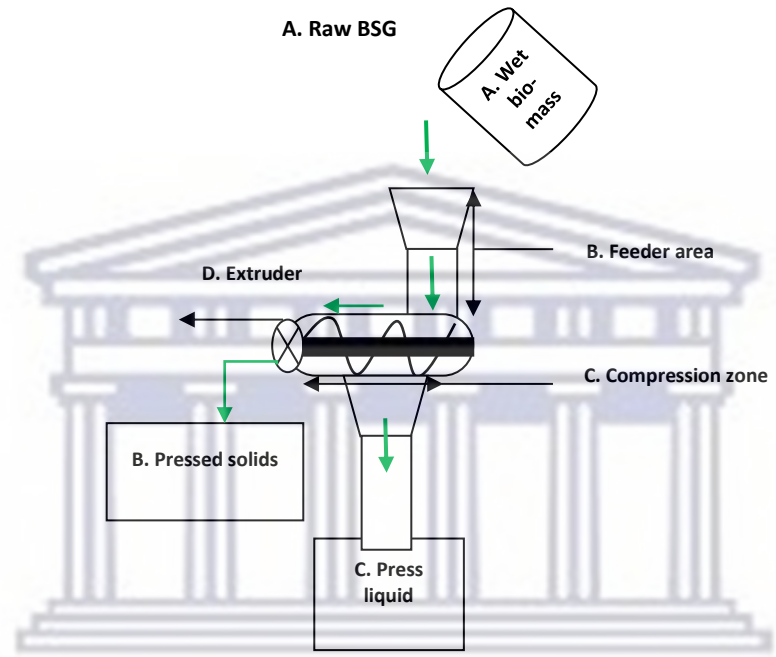

Figure 2.3. Screw press operation adapted from Yan and Modigell, (2012)**.** The flow of biomass and the resultant fractions through the screw press indicated by green arrows.

The mechanical dewatering methods illustrated in **Table 2.2.** were shown to reduce the moisture content of BSG by 9% to 29% (w/w), which is slightly more than what had previously been achieved using a screw press (Milew *et al*., 2022; Akermann *et al*., 2020; Bjerregaard *et al*., 2019; Machado *et al*., 2016; El-Shafey *et al*., 2004). Membrane filter pressing was employed by El-Shafey *et al*., (2004) and Machado *et al*., (2016) for the production of a shelf-stable BSG cake to be used as animal feed or for its potential use in biotechnological applications. The process used by El-Shafey *et al*., (2004) had 13 steps in each cycle which resulted in 2.6 kg of press cake with a moisture content of 21% (w/w). Hereafter, Machado *et al*., (2016) adapted the process to a less involved 10 step process which was more feasible. Furthermore, Bjerregaard *et al*., (2019) used a rotary drum press, which has similar advantages over the membrane filter press as the screw press. Firstly, the rotary drum press and the screw press is a continuous process, so it is more appropriate for large volumes of feedstock of 120 kg and 282-795 kg, respectively (Swart *et al.*, 2020; Bjerregaard *et al.*, 2019) . Secondly, some valorisation

processes only require mild dewatering. For example, according to Swart *et al.*, (2020), when comparing moisture contents of 10, 68, 75 and 85% (w/w), the XOS yield obtained after steam explosion treatment, with BSG as a feedstock, was the highest using a moisture content of 75% (w/w). Therefore, with some configuration optimisation a screw press or a rotary drum would be appropriate for moisture reduction of large amounts of BSG.

Table 2.2. Types of presses other than a screw press used in the dewatering of BSG

Press type	Starting moisture content (% w/w)	Mass (kg) or volume (L) of BSG processed	Final moisture content (% w/w)	Reference
Hydraulic basket press	-	-	60-65%	Milew *et al.*, 2022
Friction press	75	ca. 34 kg	65%	Akermann *et al.*, 2020
Rotary drum press	81	120.3 kg	65%	Bjerregaard *et al.*, 2019
Membrane filter press	76	-	After membrane filter pressing with filtration and squeezing: 55-62%. After further 4h vacuum drying: 18-25%	Machado *et al.*, 2016
Membrane filter press	80	-	After cold squeezing: 71%. After further hot squeezing: 51% After further vacuum drying: 21%	El-Shafey *et al.*, 2004
Type 3A Davenport press	-	ca. 170 L	ca. 70%	Finley *et al*., 1976

2.6.1.1. Effect of mechanical press dewatering on brewers' spent grain composition

As a consequence of moisture reduction of the fresh BSG, apart from the press cake, an additional product stream in the form of press liquid is generated (*refer to* **Figure 1.1. A**). Using the different mechanical press systems mentioned, approximately 1-10.0% (w/w) solids can be found in the press liquid (Milew *et al.*, 2022; Bjerregaard *et al.*, 2019; Weger *et al.*, 2017; Finley *et al.,* 1976). Using a screw press, Weger *et al.*, (2014) demonstrated that 55% of the protein content was transferred to the press liquid and was used as a substrate for biogas production. By further fractionation of the press liquid using a centrifuge, it can once more be split into two product streams, an insoluble solid fraction otherwise known as the 'pellet' and clarified liquid fraction or 'supernatant', which has been proven to be of use in various applications due to its respective chemical compositions (Milew *et al.*, 2022; Akermann *et al.*, 2020; Bjerregaard *et al.*, 2019; Weger *et al.*, 2014; Finley *et al.,* 1976). The

insoluble solids are comprised of dietary fibre (3.5-14.3%), carbohydrates (29.7%), protein (21.0-54.3%) and fat (8.4%) (Swart *et al.*, 2020; Bjerregaard *et al.*, 2019; Finley *et al.,* 1976). Finley *et al.,* (1976) demonstrated that this high protein fraction can be used as a vegetable based protein to be used as a meat extender. The authors also showed that the clarified press liquid containing 86.4% soluble sugars and 5.9% protein, could be recycled back into the wort without affecting beer quality. However, more recently, Akermann *et al.*, (2020) demonstrated that this clarified press liquid fraction combined with yeast extract from Brewers' yeast could be used as a substrate for lactate production. Furthermore, Milew *et al.*, (2022) compared the recovery of BSG components in press liquid from a hydraulic basket press used in their study, and a rotary drum press used in another. They reported that as a percentage of the mass of the component in fresh BSG, 2.0% of solids was recovered in the hydraulic basket press liquid, which indicates that the fraction was largely made up of water-soluble components. Therefore, large amounts of disaccharides (51.7%) and glucose (24.8%) was recovered, and smaller amounts (3.8-0.7%) of protein, soluble polysaccharides (starch, dextrin, β-glucan), and ash was recovered, making it a suitable feedstock for applications such as, fermentation and the recycling of the press liquid into the wort (Milew *et al.*, 2022; Akermann *et al.*, 2020; Finley *et al*., 1976). Using a rotary drum press, 21.7% of BSG solids were recovered in the press liquid as fine particles, 100-300 µm in size (Bjerregaard *et al.*, 2019). In addition, high recovery rates were found for protein (24.9%), soluble polysaccharides (24.7%), and disaccharides (35.7%) making this press liquid fraction suitable for biogas production, and with further centrifugation, meat extenders, as demonstrated by Weger *et al.*, (2014) and Finley *et al.,* (1976).

Due to the transfer of water-soluble and insoluble components in the press liquid fraction, the composition of the pressed solids, which is of great interest in this study, differs from the original BSG (Swart *et al.*, 2020). Swart *et al.*, (2020) noted that by decreasing the moisture content from 85% to 75%, higher XOS yields were achieved during hydrothermal treatment with steam explosion. The removal of starch and protein may become beneficial to downstream XOS production from auto hydrolysis liquors by resulting in a purer XOS fraction (Gómez *et al.*, 2015). According to Lopez *et al.*, (2012), after screw press treatment of BSG, the moisture content was reduced from 79% to 65%. In addition, the fibre (including hemicellulose) content of the press solids increased from 10.8% to 18.0%. Moreover, Swart *et al.*, (2020) demonstrated that when reducing the moisture content of Weiss malt BSG (WBSG) from 85% to 75%, using screw press treatment, the protein and starch content decreased. For example, with WBSG, the protein content decreased from 24.3% (w/w dried BSG) to 21.8% and the starch content decreased from 12.9% (w/w dried BSG) to 9.2%. With PBSG, the protein content decreased from 21.5% (w/w dried BSG) to 18.2% and the starch content decreased from 4.1% (w/w dried BSG) to 2.8%. As a result of the reduction in protein and starch by screw press dewatering, the

authors noted an increase in hemicellulose of from 18.9% to 21.8% in WBSG, and 20.8% to 22.7% in PBSG which contributed to a higher XOS yield after steam explosion.

*2.6.2. Steam explosion as an autocatalytic hydrothermal treatment of brewers' spent grain and other lignocellulosic biomass feedstocks in the production of xylooligosaccharides (refer to **Figure 1.1. B**)*

Steam explosion (SE) is typically used as a pretreatment step during the valorisation of lignocellulosic biomass (Álvarez *et al.*, 2021; Bhatia *et al.*, 2020; Kemppainen *et al.*, 2016). It is carried out in a pressurised reactor with the feedstock placed inside. The temperature and pressure in the reactor increases with the direct injection of steam (Kemppainen *et al.*, 2016). During SE, the process of "autocatalysis" or "autohydrolysis" takes place, in which high temperatures cause the deacetylation of xylan in the hemicellulose complex (Carvalho *et al.*, 2013). Acetyl groups are released from xylan to form acetic acid, which lowers the pH of the medium causing the breakdown and release of xylan and arabinan from cellulose and lignin (Otieno and Ahring, 2012).

Studies have demonstrated that high XOS yields of ca. 21.1-78.0% can be achieved through the autohydrolysis of various barley–type lignocellulosic feedstocks at various dry matter concentrations (**Table 2.3**) (Álvarez *et al.,* 2021; Swart *et al.,* 2020; Ravindran *et al*., 2018; Gomez *et al.,* 2015; Carvalheiro *et al.*, 2004; Nabarlatz *et al.,* 2007; Parajo *et al.*, 2004). For example, using SE at 180 °C for 10 min, Swart *et al.*, (2020) found that at a dry matter content (dm) of 15% (w/w) in fresh pure pale malt BSG (PBSG) the XOS yield (calculated as the mass fraction of XOS of the initial xylan in the BSG feedstock) was 50.0%. After increasing the dm to 25% (w/w) prior to SE using screw press treatment, the XOS yield increased to 75.3%. Using fresh barley/wheat-straw BSG (Weiss malt WBSG) at 15% (w/w) dm, Swart and co-workers obtained a XOS yield of 21.1% after SE, whereas at a higher dm of 25% (w/w), the XOS yield of BSG was 73.1%. The authors noted that the higher XOS yields achieved after SE using 25% (w/w) dm may have been due to the reduced buffering capacity, reduced bulk density, increased steam penetration and improved heating patterns as a result of the increasing the dm by 10%. However, at an even higher dm of 32%, the XOS yields remained lower than at 25% dm with yields of 64.8% and 48.3% for PBSG and WBSG, respectively. Therefore, 25% dm was found to be the optimal moisture content for XOS production through steam explosion, which implies that some form of moisture reduction in fresh BSG would be appropriate.

Table 2.3. Summary of component and XOS yields achieved through autohydrolysis of lignocellulosic feedstocks

Feedstock	Solids loading (% w/w dm)	Autohydrolysis conditions	Component yield (% w/w)	XOS yield (%)	Reference
Barley straw	10	SE at 180 °C; 30 min	Xylan yield: ca. 76.5	ca. 62.9 (% of initial xylan)	Álvarez *et al.*, 2021
Pure pale malt BSG (PBSG)	15	SE at 180 °C; 10 min	-	50.0	Swart *et al.*, 2020
Weiss malt BSG (WBSG)	15		-	21.1	
PBSG	25		Hemicellulose yield: 76.2	75.3	
WBSG	25		Hemicellulose yield: 77.8	73.1	
PBSG	32		-	64.8	
WBSG	32		-	48.3	
WBSG	15	LHW at 180 °C; 5 min	-	65.0	
WBSG	15	LHW at 180 °C; 15 min	-	78.0	
WBSG	25	LHW at 180 °C; 5 min	-	65.3	
BSG	50	SE using autoclave at 121 °C; 30 min	Xylan yield: ca. 19.8	-	Ravindran *et al.*, 2018
BSG	30.8	SE 200 °C; 10 min	Xylan yield: ca. 53.6	-	Kemppainen *et al.*, 2016
Starch-extracted BSG	11.1	Non-isothermal autohydrolysis; 200 °C	-	77.0	Gomez *et al.*, 2015
BSG	11.1	LHW at 190 °C; 5 min	Xylan yield: ca. 76	61.0	Carvalheiro *et al.*, 2004
Barley straw	5.7	LHW at 179 °C; 23 min	-	ca. 43.0 (% of original hemicellulose)	Nabarlatz *et al.*, 2007
Barley husk	Suspended in water	LHW 145-190 °C	-	27.1	Parajo *et al.*, 2004
Miscanthus	9.1	SE at 200 °C; 10 min	Xylan yield: ca. 50.9	ca. 52.6 (% of initial xylan)	Bhatia *et al.*, 2020
Eucalyptus wood	20	SE at 200 °C; 10 min	-	ca. 20.3 (% of initial xylan)	Cebreiros *et al.*, 2021
Poplar	10	Autohydrolysis 170 °C; 50 min	Xylan yield: ca. 65.4	36.2 (% of initial xylan)	Zhu *et al.*, 2022
Waste wheat straw	9.1	Autohydrolysis 180 °C; 40 min	Hemicellulose yield: 76.3	ca. 36.9 (% of initial xylan)	Huang *et al.*, 2017

Xylan, galactan, mannan yield (XGM)
Autohydrolysis using liquid hot water treatment (LHW)
Steam explosion (SE)

Literature has shown that high xylan and hemicellulose solubilisation yields of 50.9-77.8% (w/w) were obtained after autohydrolysis using various technologies, reaction conditions and feedstocks (**Table 2.3**) resulting in a significant amount of the available xylan being solubilised into XOS (Zhu *et al.,* 2022; Álvarez *et al.,* 2021; Bhatia *et al.,* 2020; Swart *et al.,* 2020; Huang *et al.*, 2017; Kemppainen *et al*., 2016; Carvalheiro *et al.*, 2004). For example, using screw pressed BSG at 25% (w/w) dm as a feedstock, Swart *et al.,* (2020) noted that SE conditions of 180 °C for 10 min was the best suited condition due to 76.2% and 77.8% of hemicellulose being solubilised for PBSG and WBSG and the high XOS and arabino-oligosaccharide (Ar-OS) yields that were achieved. In addition, at this condition the formation of monomers and degradation products were kept to a minimum compared to higher temperatures and longer treatment times. Moreover, Álvarez *et al.,* (2021) used SE with conditions of 180 °C for 30 min as a pretreatment to enzymatic hydrolysis of the autohydrolysis liquor. The authors found that using barley straw (BS) at 10% (w/w) dm produced a yield of 62.9% of XOS as a fraction of the initial xylan with ca. 76.5% of the xylan being hydrolysed overall. Various iterations of autohydrolysis technology, reaction conditions and feedstock can be applied in obtaining the highest hemicellulose and XOS yields. Optimisation for such reactions for moisture content, feedstock type, and reaction is therefore an imperative step in defining the most suitable process and conditions.

The XOS that is yielded by the autohydrolysis process are water-soluble and is thus, found in the liquid fraction obtained after autohydrolysis (Zhu *et al.,* 2022; Álvarez *et al.,* 2021; Bhatia *et al.,* 2020; Swart *et al.,* 2020; Huang *et al.*, 2017; Vegas *et al.*, 2008). A large portion of this fraction is characterised by longer chained XOS due to low yields (< 10% calculated as the weight percentage of total XOS) of xylobiose (X2) a xylotriose (X3) that are generally obtained (Álvarez *et al.,* 2021; Swart *et al.,* 2020; Vegas *et al.*, 2008). In addition to XOS being obtained, the solubilisation of hemicellulose results in monomer formation. However, a higher arabinose content than xylose is found in the liquid fraction since arabinan is hydrolysed to a greater extent due to its branched nature which allows for it to be prefferentially cleaved off as opposed to the xylan backbone (Swart *et al.,* 2020; Kemppainen *et al*., 2016). When the monomeric constituents of hemicellulose are further degraded, they form furfural which is present in relatively low yields of approximately <0.1% to 0.5% as a weight percentage of dry BSG (Swart *et al.,* 2020). Furthermore, starch and cellulose constituents are quite prevalent in this fraction in the form of glucooligosaccharides (Vegas *et al.*, 2008). Overall, using the liquid fraction obtained after autohydrolysis, longer XOS molecules can be hydrolysed by xylanase enzymes into commercially relevant low molecular weight XOS with prebiotic activity (Zhu *et al.,* 2022; Álvarez *et al.,* 2021; Bhatia *et al.,* 2020; Huang *et al.*, 2017). However, degradation products and other contaminating components may decrease enzyme activity due to unproductive binding and steric hindrance.

Other autohydrolysis technologies employed for the production of XOS (summarised in **Table 2.3**.) from BSG or barley-type feedstocks include liquid hot water, isothermal- and non-isothermal autohydrolysis and using an autoclave to replicate SE (Swart *et al*., 2020; Ravindran *et al*., 2018; Gomez *et al*., 2015; Nabarlatz *et al*., 2007; Carvalheiro *et al.*, 2004; Parajo *et al*., 2004). Liquid hot water (LWH) reactions are typically carried out in a stirred batch or Parr reactor containing feedstocks and water. Using LHW, high XOS yields of 61.0% to 78.0% as a percentage of the initial xylan have been achieved using BSG (Swart *et al.,* 2020; Gomez *et al.,* 2015; Carvalheiro *et al.*, 2004). However, this autohydrolysis technique typically requires a feedstock with a low dm of between 5.7-15.0% (w/w) which is achieved by adding deionised water in order to allow for adequate mixing with a stirring arm (Nabarlatz *et al*., 2007; Carvalheiro *et al.*, 2004; Parajo *et al*., 2004; Swart *et al.,* 2020). Therefore, small quantities of 125 g and 300 g of feedstock can be treated per run in a 2 L and 10 L reactor, respectively (Nabarlatz *et al*., 2007; Carvalheiro *et al.*, 2004). Literature has also shown that at slight increments in the dm at similar temperatures and reaction times (**Table 2.3**), higher yields can be achieved (Swart *et al.,* 2020; Gomez *et al.,* 2015; Nabarlatz *et al*., 2007; Carvalheiro *et al.*, 2004). Furthermore, Ravindran *et al*., (2018) recreated a lab scale SE process in an autoclave in which small amounts of BSG (5 g) at 50% (w/w) dm in conical flasks were subjected to conditions of 121 °C for 30 min. The pressure was rapidly dropped by opening the pressure valve which subjected the BSG to an "explosion", resulting in a low xylan yield of ca. 19.8%.

Autohydrolysis has also been shown to produce competitive XOS yields from other lignocellulosic biomass sources (**Table 2.3**). However, lower yields of 20.3% to 52.6% were achieved using feedstocks such as, poplar, eucalyptus wood, *Miscanthus* and wheat straw, compared to BSG (Zhu *et al.,* 2022; Cebreiros *et al.*, 2021; Bhatia *et al.,* 2020; Huang *et al*., 2017). This is likely due to the hemicellulose concentration of the various feedstocks which is typically higher in BSG.

Compared to LHW and other technologies previously reported in the production of XOS from BSG, SE is more feasible due to the injection of saturated steam which brings about autohydrolysis by penetrating the material. Therefore, SE does not require a low dm to enable stirring. In addition, SE has been shown to utilise larger feedstock loadings of 1 kg per run which equates to ca. 320 g of dried material (Swart *et al.*, 2020). Moreover, the explosive decompression promotes the disruption of fibres resulting in an increased surface area of components in the residual solids creating better accessibility for enzymes to substrates in both the autohydrolysis liquor and the residual solids (Moreno *et al.*, 2019).

2.6.2.1. Effect of autohydrolysis on the composition of residual lignocellulosic material

As a result of xylan hydrolysis by autohydrolysis, the residual solids of lignocellulose is significantly altered as it leaves behind a lignin-cellulose-rich fraction (Zhu *et al.*, 2022; Alvarez *et al.*, 2021; Bhatia *et al.*, 2020; Kemppainen *et al.*, 2016). Using SE conditions of 200 °C for 10 min on BSG, Kemppainen *et al.*, (2016) showed that the xylan concentration decreased from 16.6% (w/w dry matter) in the fresh BSG to 7.7% after SE. It was also noted that arabinan decreased from 7.0% in the fresh BSG to only 2.1% after steam explosion. Yet, the mass balance revealed that a large amount of xylan (83.9%) and arabinan (82.2%) remained in the solid residue after SE pretreatment due to the fact that mainly protein was targeted during autohydrolysis rather than hemicellulose (**Table 2.4.**). However, studies (summarised in **Table 2.4**) which utilised various other feedstocks such as, poplar, barley straw, *Miscanthus* and waste wheat straw, showed that smaller amounts (ca. 23.5% to 42.1%) of xylan was recovered after autohydrolysis. Furthermore, because of more extensive xylan hydrolysis, high recovery rates of glucan (ca. 89.9% to 96.8%) and lignin (ca. 83.3% to 99.4%) were observed. Therefore, the recovery of the various components resulted in the altered compositions of the residues after autohydrolysis. For example, in untreated barley straw, the composition of xylan, glucan and acid-insoluble lignin as a percentage of the dried mass of the feedstock was ca. 22.1%, 32.9% and 16.8%, respectively (Alvarez *et al.*, 2021). After SE pretreatment the composition of the respective components were ca. 9.6%, 55.7% and 30.8%, indicating that the solids were enriched with glucan and lignin. In a biorefinery with focus on XOS production, high xylan recovery in autohydrolysis liquor is beneficial for enabling access of endoxylanases to active sites for efficient depolymerisation (Otieno and Ahring, 2012). However, a significant proportion of xylan may still remain in the residue depending on the feedstock used and the efficacy of autohydrolysis, which creates the opportunity for further solubilisation of the residual solids using enzymatic hydrolysis (Precup *et al.*, 2022; Bhatia *et al.*, 2020; Kemppainen *et al.*, 2016).

Table 2.4. Component recovery of xylan, arabinan, glucan and lignin in the residual solids of feedstocks after autohydrolysis

Feedstock	Autohydrolysis conditions	Xylan recovery	Arabinan recovery	Glucan recovery	Lignin recovery	Reference
Poplar	Autohydrolysis 170 °C; 50 min	34.6	-	96.2	83.3	Zhu *et al.*, 2022
Barley straw	SE at 180 °C; 30 min	23.5	-	91.8	99.4*	Alvarez *et al.*, 2021
Miscanthus	SE at 200 °C; 10 min	42.1	-	89.8	96.9	Bhatia *et al.*, 2020
Waste wheat straw	Autohydrolysis 180 °C; 40	23.7[a]	-	96.8[b]	-	Huang *et al.*, 2017
BSG	SE at 200 °C; 10 min	83.9[c]	82.2[c]	89.8[c]	-	Kemppainen *et al.*, 2016

Recovery calculated as the mass percentage of the initial component remaining in the solid residue (% w/w).
*Acid-soluble lignin
[a] Hemicellulose
[b] Cellulose
[C] calculated from remaining percentage of component after solubilisation

2.6.3. Enzymatic hydrolysis of pretreated brewers' spent grain and other lignocellulosic biomass feedstocks

*2.6.3.1. Xylooligosaccharide production by enzymatic hydrolysis of the liquid fraction obtained after hydrothermal pretreatment (refer to **Figure 1.1. C**)*

After hydrothermal treatment of BSG and other lignocellulosic biomass, XOS yields of ca. 20.3-62.9% (w/w) as a percentage of initial xylan have been achieved (Álvarez et al., 2021; Cebreiros et al., 2021; Bhatia et al., 2020; Huang *et al*., 2017). As previously described, the liquid fraction after pretreatment is rich in soluble xylan in the form of XOS, with a large portion of the XOS in the form of longer chains (DP≥7) (Álvarez *et al*., 2021; Bhatia *et al*., 2020; Vegas *et al*., 2008; Kabel *et al*., 2002). According to various reports (summarised in **Table 2.5**), following pretreatment, high yields of low molecular weight XOS can be achieved with the enzymatic hydrolysis of the liquid fraction, making it the most suitable substrate for low molecular weight XOS production (Álvarez *et al*., 2021; Su *et al*., 2021; Bhatia *et al*., 2020; Lian *et al*., 2020; Huang *et al*., 2017; Gómez *et al.*, 2015). For example, using autohydrolysis liquor obtained from de-starched BSG as a substrate, Gómez *et al.*, (2015) showed that after enzymatic hydrolysis with Shearzyme 2X (Novozymes-Spain) the largest portion of XOS had a DP of 2-6. In another study, Álvarez *et al*., (2021) used barley straw as a feedstock, which was subjected to SE at 180 °C for 30 min as a pretreatment. Hereafter, the yield of XOS DP 2-6 was 35.7% of the initial xylan. The liquid fraction after pretreatment was treated with an enzyme cocktail made up of a xylanase and accessory enzymes separately supplied by Megazyme International (Bray, Ireland). The enzyme

cocktail consisted of endo-β-(1, 4)-xylanase, α-L-arabinofuranosidase, acetylxylan esterase and feruloyl esterase activities. After enzymatic hydrolysis, the yield of XOS DP 2-6 increased to 58.8% with the highest concentration of XOS DP 2 being obtained. This indicated that longer chained XOS were successfully hydrolysed into shorter chained XOS. Moreover, after pretreatment with autohydrolysis at 180 °C for 40 min, Lian *et al.*, (2020) obtained a XOS DP 2-6 yield of ca. 28.0% and following enzymatic hydrolysis the yield increased to ca. 41.2%.

Table 2.5. Low molecular weight XOS production from autohydrolysis liquor from various lignocellulosic feedstocks using enzymatic hydrolysis

Lignocellulosic material	Pretreatment conditions	Enzyme	DP and yield of XOS (% w/w of initial xylan)		Reference
BSG	Starch extraction; Autohydrolysis at 180 °C; 12.2 min	Shearzyme 2X	Total XOS 80.6% HPSEC chromatogram showed the highest area observed was ca. DP2-6		Gómez *et al.*, 2015
Barley straw	SE at 180 °C; 30 min	Endo-β-(1,4)-xylanase, α-L-arabinofuranosidase, acetylxylan esterase and feruloyl esterase	DP 2-6	ca. 58.8	Álvarez *et al.*, 2021
Poplar sawdust	Autohydrolysis 170 °C; 50 min	Endo-β-(1,4)-xylanase	DP 2-6 DP 2-3	44.6 34.9	Su *et al.*, 2021
Micanthus (Mx2779)	SE at 200 °C; 10 min	Endo-xylanases NS22083 and NS22002	DP 2-3	42.5	Bhatia *et al.*, 2020
Xylan from corn straw	Autohydrolysis 180 °C; 40 min	Xylanase with low β-xylosidase activity	DP 2-6 DP 2-3	41.2 26.5	Lian *et al.*, 2020
Waste wheat straw	LWH at 180 °C; 40 min	Endo-β-(1,4)-xylanase	DP 2-3	ca. 27.5	Huang *et al.*, 2017

HPSEC: High performance size exclusion chromatography

Furthermore, as summarised in **Table 2.5.**, XOS with a DP of 2 and 3 namely xylobiose (X2) and xylotriose (X3) with stronger prebiotic activity, have been produced from autohydrolysis liquors (Gullón *et al.*, 2008). Bhatia *et al.*, (2020) obtained an XOS DP 2 yield of ca. 5.2% after SE at 200 °C for 10 min, after enzymatic hydrolysis with commercial endoxylanases (NS22083 and NS22002 supplied by Novozymes) they obtained a yield of ca. 38.0%. Su *et al.*, (2021) obtained an X2 and X3 yield of 16.7% after autohydrolysis of poplar sawdust 170 °C for 50 min, which increased to 34.9% after enzymatic hydrolysis with an endoxylanase. Therefore, higher yields of ca. 41.2-58.8% (w/w % of initial xylan) are generally obtained for XOS (DP 2-6) whereas, for XOS (DP 2 and 3) which typically have greater prebiotic activity, the yield as a percentage of the initial xylan is lower (26.5-42.5% w/w). Furthermore, the highest yield of XOS DP 2-6 was obtained using the autohydrolysis liquor from barley straw treated with an enzyme cocktail (Álvarez *et al.*, 2021). In essence, various lignocellulosic

feedstock types could produce satisfactory low molecular weight XOS yields. However, to improve the yield of the XOS which are bound to side chains in the hemicellulose matrix, accessory enzymes are necessary.

In general, during the production of XOS, enzyme cocktails should have low β-xylosidase activity to minimise monomer formation (Kabel *et al*., 2002; Vázquez *et al*., 2002). In addition, xylanases belonging to glycoside-hydrolase (GH) families 10 and 11 are widely studied and are commonly employed for depolymerisation of longer chained XOS in autohydrolysis liquors (Saini *et al*., 2022; Álvarez *et al*., 2018; Gomez *et al*., 2015; Carvalho *et al*., 2013; Vegas *et al*., 2008). As observed by Vegas *et al*., (2008), Shearzyme 2X, a GH 10 enzyme supplied by Novozymes-Spain, produced a higher percentage of X2 and X3 compared to the GH 11 Pentopan Mono BG (Novozymes-Spain), which produced a wider DP range of X2 to X5. However, Shearzyme 2X displayed slower reaction kinetics and produced more xylose and X≥7 than Pentopan Mono BG indicating its affinity for X4-6 as a substrate. Furthermore, researchers have also opted for enzyme cocktails with various enzyme activities, which displayed synergy with endoxylanases, thereby increasing yields of short chain XOS by providing more efficient hydrolysis of longer XOS chains. Enzymes that cleave branched molecules increases the production of low DP XOS by providing more accessibility of xylanases to the xylan backbone. For example, Álvarez *et al*., (2018) found that using a combination of an endo-β-(1,4)-xylanase, α-L-arabinofuranosidase, and a feruloyl esterase resulted in a yield of 7.8% XOS (DP 2 and 3) (% w/w dried barley straw) and later Álvarez *et al*., (2021) found that using an acetylxylan esterase prior to an endo-β-(1,4)- xylanase, α-L-arabinofuranosidase, and feruloyl esterase enzyme cocktail, produced an XOS yield (DP 2-6) of 13% (w/w dried BSG). Moreover, in a previous study, α-L-arabinofuranosidase activity was not only found to increase arabinose release, but also increased xylose release from the xylan chain, which led to the conclusion that higher levels of substitution with arabinose causes the xylan chain is to be more resistant to hydrolysis by xylanases (Xiros *et* al., 2011).

*2.6.3.2. Xylooligosaccharide production by enzymatic hydrolysis of the solid fraction after hydrothermal pretreatment (refer to **Figure 1.1. D**)*

Although the residual solids after hydrothermal treatment consists of considerably less hemicellulose, studies summarised in **Table 2.6**. demonstrated that there is still potential for further solubilisation and concurrent depolymerisation through low solids loadings enzymatic hydrolysis resulting in XOS concentrations of 1.23 to 3.7 $g.L^{-1}$ (Precup *et al*., 2022; Aachary and Prapulla, 2009). Precup *et al*., (2022) showed that at a solids loading of 10% (w/w), 1.23 $g.L^{-1}$ of XOS DP 3 and 4 was liberated from

wheat straw residue after enzymatic hydrolysis using a crude endo-β-(1-4)-xylanase preparation from *Trichoderma viride.* Using Cellic® CTec2, a commercial blend containing high xylanolytic and cellulolytic activity, the authors obtained a lower concentration of XOS DP 3 and 4 of 0.59 $g.L^{-1}$ and 0.78 $g.L^{-1}$ after hydrothermal pretreatment at 160 °C for 15 min and 180 °C for 15 min, respectively (Chekushina *et al*., 2013). Such low concentrations are typical in low solids enzymatic hydrolysis reactions. Conversely, these concentrations correspond to high component yields. For example, using steam pretreated corncob powder at 3% (w/w) solids loading, Aachary and Prapulla, (2009) reported an XOS concentration of ca. 3.7 $g.L^{-1}$ which corresponded to a yield of ca. 77%. At 1% (w/w) solids loading of SE pretreated *Miscanthus*, a 21.9% XOS yield as a percentage of the initial xylan was obtained by Bhatia *et al.*, (2020). These concentrations and yields are sizably lower than those achieved by enzymatic hydrolysis of the autohydrolysis liquor. For example, approximately 23 $g.L^{-1}$ of XOS DP 2-6 (Álvarez *et al.*, 2021) and 52.6% (w/w) of the initial xylan (Bhatia *et al.*, 2020) was previously achieved. Furthermore, higher XOS concentrations of 4.80-13.60 $g.L^{-1}$ have been obtained by Dotsenko *et al.*, (2018) after hydrothermal pretreatment and enzymatic hydrolysis of wheat straw and rye grass. However, to achieve these concentrations, approximately 96% of water had to be evaporated. Low solids loadings (lower than 15% w/w) are, therefore, not a feasible strategy for XOS production a biorefinery since the energy requirements and costs involved in the removal large amounts of water are high.

Furthermore, little to no research has been done to date on the potential of XOS production from lignocellulosic biomass using high solids loadings enzymatic hydrolysis. The advantage of lignocellulosic biomass conversion at high solids loadings is that the reaction volume decreases, which reduces the required equipment volume, and with less free water, the final product concentration increases, requiring less downstream separation steps (da Silva *et al*., 2020). Due to the reduced water consumption, less wastewater is generated. Additionally, energy demands for agitation, heating and cooling steps decrease (Geng *et al.,* 2015). Overall, a reduction in capital and operational costs is observed, which is beneficial for industrial purposes (da Silva *et al.,* 2020). On the other hand, technical problems arise when the amount of free water is reduced. These challenges include poor mixing as well as heat and mass transfer limitations that contribute to decreased enzyme efficiency. The high concentration of final products, although beneficial, may lead to the inhibition of enzyme activity (Zheng *et al.,* 2009). A combination of these issues ultimately causes a decrease in yield (da Silva *et al.,* 2020).

Table 2.6. XOS production from autohydrolysis residue at low solids loadings

Lignocellulosic material	Pretreatment	Enzyme and solids loading	DP and yield of XOS	Reference
Wheat straw	Auto hydrolysis 160 °C; 15 min	Crude endo-β-(1-4)-xylanase, *Trichoderma viride*; 10% solids loading	DP 3-4 1.23 $g.L^{-1}$	Precup *et al.*, 2022
		Cellic® CTec2; 10% solids loading	DP 3-4 0.59 $g.L^{-1}$	
	Auto hydrolysis 180 °C; 15 min	Cellic® CTec2; 10% solids loading	DP 3-4 0.78 $g.L^{-1}$	
Miscanthus	Steam explosion at 200 °C; 10 min	Novozymes endoxylanase NS22083; 1% solids loading	ca. 52.1% XOS	Bhatia *et al.*, 2020
Wheat straw	Hydrothermal treatment at 190 °C for 10 min	*Aspergillus niger* endo-β-(1,4)-xylanase; 3% solids loading	DP 2-3 9.30 $g.L^{-1}$	Dotsenko *et al.*, 2018
Rye grass			DP 2-3 13.60 $g.L^{-1}$	
Rye grass pulp	Hydrothermal treatment at 140 °C for 60 min	*Cellovibrio mixtus* endo-β-(1,4)-xylanase; 3% solids loading	AXOS DP 2-4 5.50 $g.L^{-1}$	
		Thermotoga maritima endo-β-(1,4)- xylanase; 3% solids loading	AXOS DP 2-4 4.80 $g.L^{-1}$	
Corn powder	Cooked under pressure at 121 °C for 30 min	Endoxylanase Bioxyl P40; 3% solids loading	ca. 77% XOS ca. 3.7 $mg.ml^{-1}$	Aachary and Prapulla, 2009

2.7. Gap in literature

Screw press dewatering as a preliminary step to steam explosion and subsequent enzymatic hydrolysis for XOS production has not been widely explored in valorisation concepts for BSG, but has been shown to be an efficient method to dewater large volumes of BSG (Swart *et al.*, 2020). Enzymatic hydrolysis of the liquid fraction obtained after autohydrolysis for the production of low molecular weight XOS when valorising lignocellulosic biomass has been widely reported (Álvarez *et al*., 2021; Su *et al*., 2021; Bhatia *et al*., 2020; Lian *et al*., 2020). However, only Kabel *et al*., (2002) and Gómez *et al.*, (2015) attempted this in BSG. Furthermore, the enzymatic hydrolysis of the residual solids after hydrothermal treatment at more industrially relevant, high solids conditions, specifically for the production of XOS from BSG has not yet been explored. The use of a screw press is of great significance for dewatering BSG when establishing a continual industrial processes because of its ability to contend with large volumes of wet feedstock which is typically produced by breweries on a daily basis (Swart *et al.*, 2020). In addition, the upcycling of multiple streams acquired after pretreatment is important in creating a sustainable circular bio-economy (Sterling and Karlijn, 2020).

Therefore, the combined process involving screw press dewatering and steam explosion as a pretreatment of BSG, to maximise hemicellulose conversion to XOS using enzymatic hydrolysis will be investigated in this study.

2.8. Aim and objectives

The core aim of the study was to produce low molecular weight xylooligosaccharides (XOS) for potential application in industry from brewers' spent grain, using an integrated process for fractionation and solubilisation of hemicellulose.

This aim was achieved through completing the following objectives:

1. To solubilise hemicellulose in BSG.
    a. Screw press dewatering and steam explosion was employed as a pretreatment for fractionation of BSG and solubilisation of the hemicellulose component. This was done to extract soluble hemicellulose and allow accessibility to XOS prior to depolymerisation through enzymatic hydrolysis of the liquid fraction obtained after steam explosion. In addition, using an endoxylanase, enzymatic hydrolysis of residual solids after steam explosion at a high solids loading was investigated to further solubilise the hemicellulose component in this stream into XOS.
    b. To track the recovery of extracted hemicellulose, all solubilised hemicellulose extracted over the process was to be accounted for at the end of the process of solubilisation in order to identify streams which contained the target product and subsequently quantify the solubilisation yield.
2. To depolymerise long-chained XOS.
    a. To maximise the depolymerisation of solubilised hemicellulose in identified streams into low molecular weight XOS (DP X2 to X6), enzymatic hydrolysis using an endoxylanase was employed. The dosage and time for maximum low molecular weight XOS production was sought during optimisation experiments for enzymatic hydrolysis.
    b. To recover a competitive yield of low molecular weight XOS, the amount of hemicellulose recovered as X2 to X6 and was quantified in each stream to determine the yield of the depolymerisation process.

Chapter 3

Materials and Methods

3.1. Raw material and enzyme

Fresh BSG (approximately 80 kilograms) with a moisture content of approximately 79%, measured using NREL Laboratory Analytical Procedure (LAP), was collected from Stellenbrau microbrewery in Stellenbosch (Sluiter *et al.*, 2012; Sluiter *et al.*, 2008a).

Pentopan mono BG (Novozymes, Johannesburg), a GH-11 commercial xylanase preparation from *Aspergillus oryzae* was used in this study (Vegas *et al.*, 2008). Xylanase activity was measured according to Bailey *et al*., (1992) using the DNS method. Beechwood xylan (1% w/v) was used as a substrate and xylose was used to set a standard curve between 0.5 $g.L^{-1}$ and 10 $g.L^{-1}$. One unit (U) was defined as the amount of enzyme which released 1 µmol of xylose from the xylan substrate at 50 °C. The activity of the enzyme was determined to be 202931.7 $U.g^{-1}$.

3.2. Processing of BSG into different product streams

Four batches of fresh BSG were collected from Stellenbrau over a six-week period. The batches were separately processed (**Figure 3.1.**) to produce a mass balance. Each batch was pressed using a continuous screw press (NEW Eco-tec Verfahrenstechnik GmbH, Mühldorf, Germany) with a screen cage 0.3 m in length and 0.15 m in diameter, with slotted openings, 0.6 mm in size. The single screw was powered by a 2.2 kW 3-phase motor. After pressing, press solids (stream B) and press liquid (stream C) were produced (**Figure 3.2.**). The press liquid was centrifuged using a continuous centrifuge (GEA Westflia Separator group GmbH, Oelde, Germany) which produced two additional streams, the supernatant (stream E) and pellet (stream D).

The press solids were steam exploded in a 19 L capacity “steam gun”. One kilogram (1 kg) of sample with a dry matter content of approximately 27% (w/w) was loaded into the reactor. Saturated steam was rushed into the steam gun to heat up the BSG to the setpoint temperature within 30 seconds. The temperature of the BSG was kept at 180 °C for 10 min before it was explosively expelled from the vessel as previously described (Swart *et al.*, 2020). After collecting the steam exploded slurry (steam G), the solid and liquid was separated by centrifugation using a lab scale spin dryer (Spindel Laundry Dryer). Both residual solids after steam explosion (RSSE-stream J) and steam exploded liquor (SEL-stream H) was stored at -20 °C before enzymatic hydrolysis.

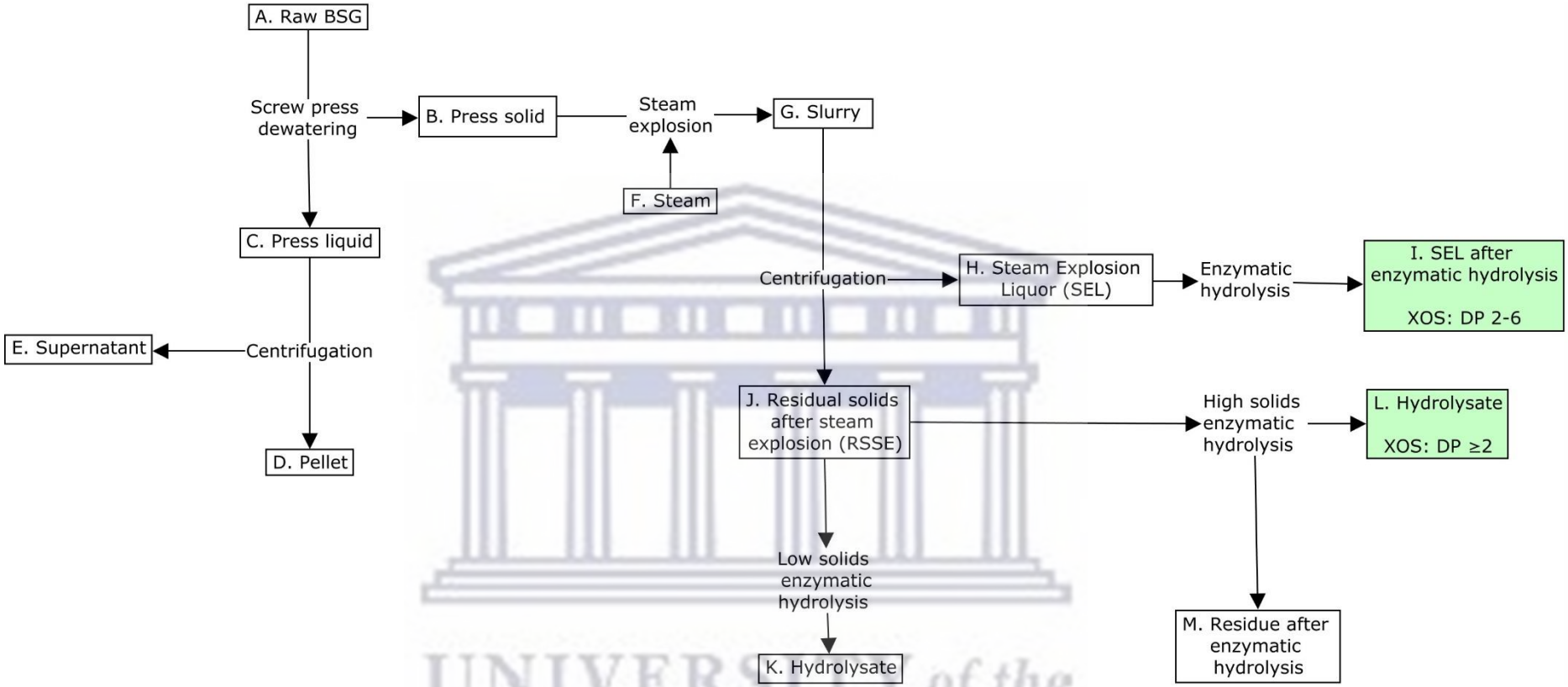

Figure 3.1. Processing of BSG into different product streams. Target products were I. xylooligosaccharides with a degree of polymerisation of 2 to 6 (XOS DP 2-6) in steam explosion liquor (SEL) after enzymatic hydrolysis and L. xylooligosaccharides with a degree of polymerisation of less than 2 (XOS DP ≥2) in the hydrolysate after enzymatic hydrolysis of RSSE.

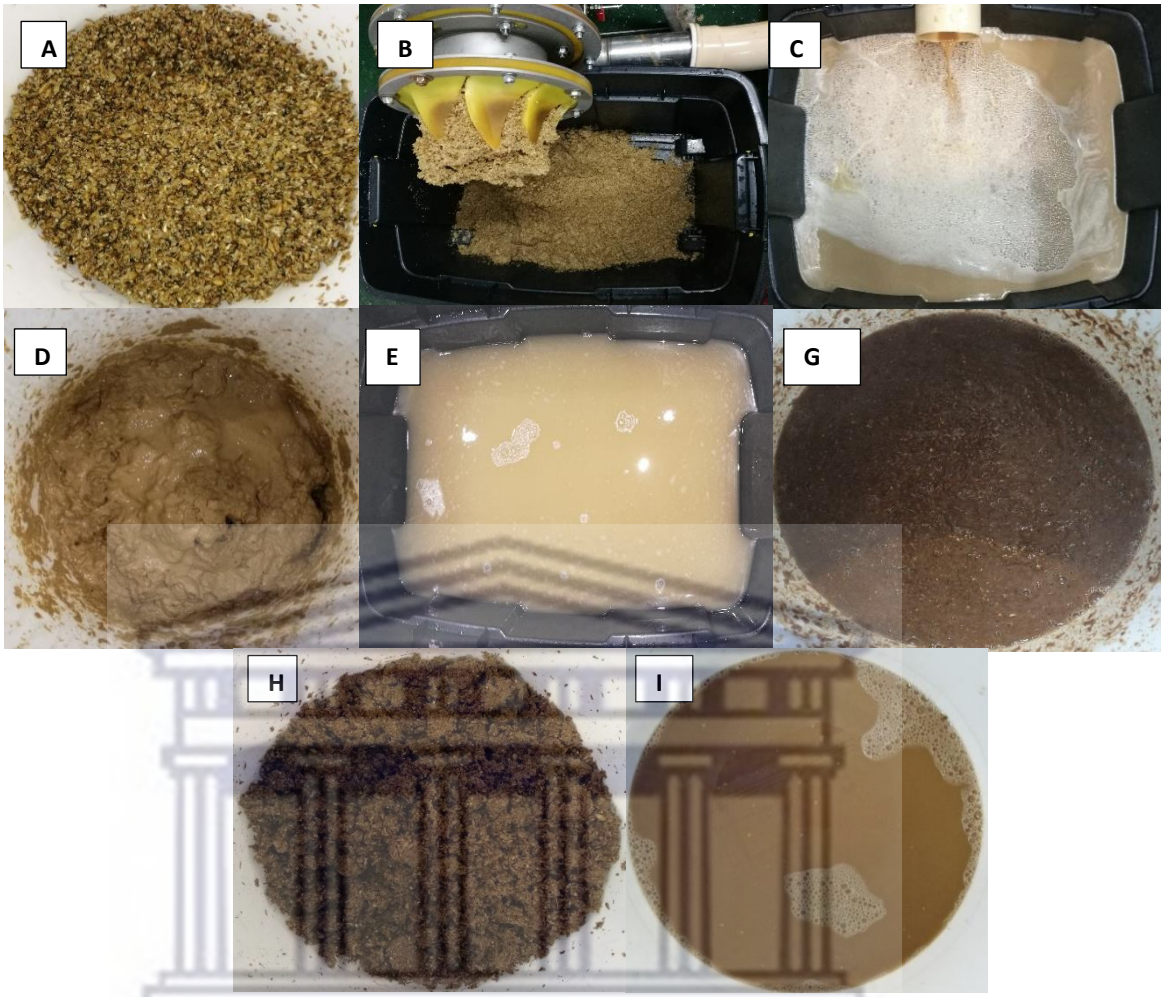

Figure 3.2. Streams produced by fractionation process. A: Raw BSG; B: Press solids; C: Press liquid; D: Pellet after centrifugation of press liquid; E: Supernatant after centrifugation of press liquid; G: Steam explosion slurry; H: Residual solids after steam explosion I: Steam explosion liquor.

3.3. Enzymatic hydrolysis

3.3.1. Enzymatic hydrolysis of Steam Explosion Liquor

Enzymatic hydrolysis reactions of the steam explosion liquor (SEL) took place in 500 ml flasks, with a total volume of 300 ml shaking at 150 rpm, at 50 °C. The pH of the liquor was adjusted to 6 using 5M NaOH prior to enzymatic hydrolysis and monitored throughout the reaction according to Vegas *et al.*, (2008). Enzyme dosages of 1, 5, 25 and 50 $U.ml^{-1}$ liquor was applied and samples were taken at 1, 3, 6, 9 and 24 hrs. The enzymatic reactions were terminated by boiling the liquor samples in a water bath for 10 min before storing at -20 °C.

3.3.2. High solids enzymatic hydrolysis of Residual Solids after Steam Explosion

Prior to high solids enzymatic hydrolysis, Residual Solids after Steam Explosion (RSSE) was washed with RO water to remove any soluble sugars. Horizontal PVC reactors (6 L) with a stainless steel scraper, rotating at 12.6 rpm on a rolling rig were used to carry out high solids enzymatic reactions (**Figure 3.3.**). The reactions took place in at 50 °C with a solids loading of 25% (w/w) made up to a total volume of 300 g with RO water. Enzyme dosages of 25, 100, 500, 1000 and 1500 U.g dried $RSSE^{-1}$ were used and samples were taken at 3, 6, 9 and 24 hrs, while monitoring pH changes throughout the reaction. Enzymes were inactivated by boiling the hydrolysate samples in a water bath for 10 min before storing at -20 °C.

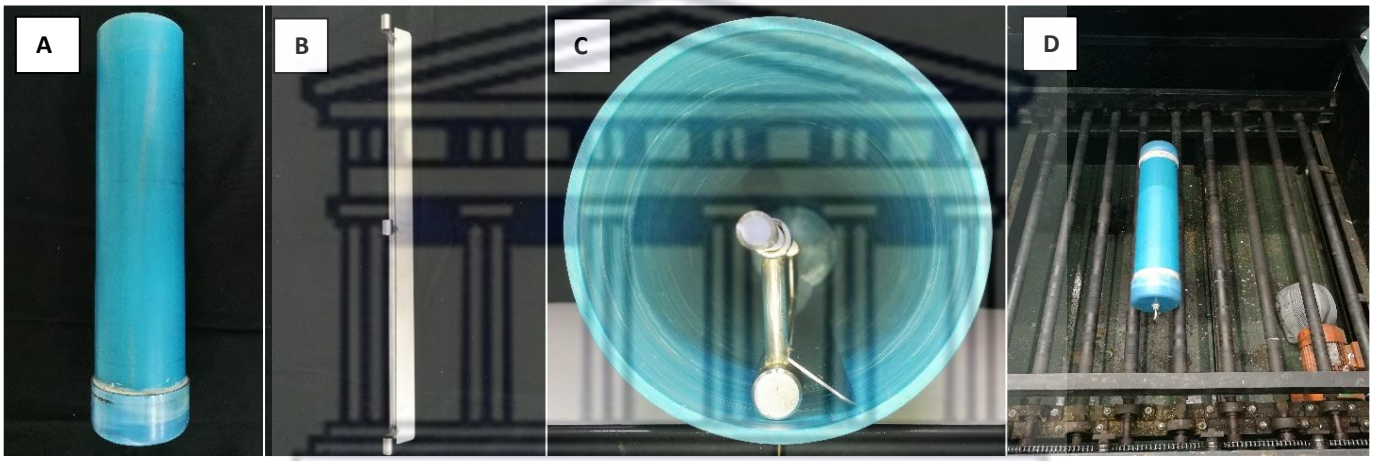

Figure 3.3. Horizontal PVC reactor and components. A: 6 L horizontal PVC reactor; B: Stainless steel scraper; C: Reactor (inside) with stainless steel scraper; D: Reactor on rolling rig.

3.3.3. Enzymatic digestibility of hemicellulose in Residual Solids after Steam Explosion

Enzymatic digestibility was also conducted at low solids concentration to avoid problems associated with high solid processes. The reactions took place at a solids loading of 3% (w/w) made up to a total volume of 300 ml with RO water. Reactions were conducted in 500 ml flasks shaking at 150 rpm at 50 °C with enzyme dosages of 25, 100, 500, 1000 and 1500 U.g dried $RSSE^{-1}$. Samples were taken at 3, 6, 9 and 24 hrs while monitoring pH changes and the reaction was seized by boiling the samples in a water bath for 10 min before storing at -20 °C.

3.4. Analytical methods

The composition of biomass was determined according to NREL LAP (Sluiter *et al.*, 2012; Sluiter *et al.*, 2008a). Total starch was determined using a starch kit from Megazyme (K-TSTA, Ireland) according to the manufacturers' instructions (Robertson, I'Anson, Treimo, *et al.*, 2010). Crude protein was determined by quantification of nitrogen content using the Dumas method of analysis. Protein content was then calculated by using a conversion factor of 6.25 (Bjerregaard *et al.*, 2019).

The concentration of monosaccharides (xylose, arabinose and glucose), short chain xylooligosaccharides (xylo- biose, -triose, -tetraose, -pentaose and -hexaose) and degradation products (furfural, hydroxymethylfurfural (HMF), acetic acid, formic acid, glycerol and ethanol) was analysed with high performance liquid chromatography (HPLC) using an Aminex HPX-87H Ion Exclusion Column equipped with a Cation-H cartridge (Biorad, Johannesburg, South Africa). All liquid samples were filtered through a 0.2 µm syringe filter before analysis (Swart *et al.*, 2021).

For quantification of oligosaccharides which included xylooligosaccharides (XOS), glucooligosaccharides (Glc-OS) and arabino-oligosaccharides (Ar-OS), liquid samples underwent post hydrolysis according to NREL LAP (Sluiter *et al.*, 2008b). Oligosaccharides were measured as the difference between monosaccharide concentration before and after post hydrolysis (Gómez *et al.*, 2015). Xylooligosaccharide yields after enzymatic hydrolysis of SEL were calculated as the mass percentage of theoretical xylan. Hemicellulose solubilisation yields of monomeric (xylose and arabinose) or oligomeric (XOS or Ar-OS) sugars after enzymatic hydrolysis of RSSE were calculated as the mass percentage of the available hemicellulose in RSSE on a dry weight basis.

3.5. Statistical methods

Enzymatic hydrolysis experiments were done in triplicate and analysed using two-tailed t-tests with unequal variance in Microsoft Excel.

Chapter 4

Results and Discussion

4.1. Composition of raw brewers' spent grain

Fresh BSG samples obtained from a local craft brewery with a moisture content of 79.0% (w/w) were analysed on a dried weight basis to determine their chemical composition. The results (**Table 4.1**) indicated that the hemicellulose content was 19.3% (w/w) with an arabinan/xylan (A/X) ratio of 0.47 indicating that the xylan made up approximately double the amount of arabinan, which is advantageous for the purpose of producing XOS since it is a derivative of xylan. The A/X ratio is consistent with what was reported in literature (Swart *et al.*, 2020; Xiros and Christakopoulos, 2012). As previously reported, BSG has a hemicellulose content of 18.9% to 29.6% (w/w) and although a hemicellulose content 40.0% and above has been obtained, it is extremely rare (Swart *et al.*, 2020; Lynch *et al.*, 2016; Meneses *et al.*, 2013; Waters *et al.*, 2012; Robertson *et al.,* 2010b; Celus *et al.*, 2006; Kanauchi *et al.*, 2001). The BSG used in the current study had a hemicellulose content which was consistent with the lower end of the range reported for BSG and was in line with that of other feedstocks such as, rice straw, wheat straw, barley straw, oat straw and rice husks, which have a hemicellulose content of between 16.1% and 23.4% (Mussatto, 2014). The high hemicellulose content containing a low A/X ratio as observed in this study, favours the production of xylooligosaccharides (XOS) in the context of a biorefinery process where both steam explosion and enzymatic hydrolysis is employed (Álvarez *et al.*, 2021). Furthermore the lower arabinan content renders the solids more susceptible to hemicellulose breakdown (Lynch *et al.*, 2016).

Table 4.1: Chemical composition of fresh BSG used in this study

Component (% w/w dry matter)	Average	± SD
Extractives	**18,5**	**1,7**
NREL water	10,1	1,7
NREL ethanol	8,4	0,3
Cellulose	**17,9**	**2,2**
Hemicellulose	**19,3**	**1,2**
Xylan	13,1	1,1
Arabinan	6,1	0,2
Lignin	**33,7**	**1,7**
Acid-soluble (AS)	7,3	0,4
Acid-insoluble (AI)	26,3	2,1
Ash	**3,3**	**0,2**
Crude Starch	**17,3**	**0,4**
Crude Protein	**22,4**	**1,6**

Data represents the average between four batches of BSG ± standard deviation
NREL: Procedure done according to the National Renewable Energy Laboratory

The various other components of fresh BSG included cellulose (17.9% w/w) and ash (3.3% w/w), which was in agreement with literature (Swart *et al.*, 2020; Lynch *et al.*, 2016). Furthermore, lignin was the largest component in BSG, which constituted up to 33.7% (w/w) of the dried BSG with acid-insoluble lignin making up 78% (w/w) of the lignin component. The lignin content recorded for the BSG used in this study was substantially higher than the 12-28% reported in literature (Swart *et al.*, 2020; Lynch *et al.*, 2016). The high lignin content could disadvantage the process of XOS production by preventing hemicellulose degradation due to the linkage of the two components by various alkyl/aryl-ether bonds which, together with hydrogen bonding, renders a recalcitrant three-dimensional lignocellulosic complex (Huang *et al.*, 2022). The second largest component of fresh BSG was crude protein, making up ca. 22.4% (w/w) dried weight, which was similar to the values reported in literature (Swart *et al.*, 2020; Lynch *et al.*, 2016). Moreover, starch constituted 17.3% (w/w) of the dried BSG, much higher than the 1.0-12.0% reported in literature (Lynch *et al.*, 2016). The high starch content may be attributed to the inefficiency of the mashing regime employed by the craft brewery from which the BSG originated. Differences in composition of BSG from various sources are generally due to inter-brewery variation with respect to the malting and mashing regime, the variety of the grain being used and the time of harvesting of the grain (Lynch *et al.*, 2016).

In the case of valorisation of BSG to produce XOS, the hemicellulose component is of the greatest importance as the source of xylan. Based on the results of the compositional analysis of the fresh BSG, XOS production would be improved by the enrichment of hemicellulose through a process of fractionation. This is done by the removal of components that are not required and thus extracting hemicellulose from the BSG. Furthermore, recyclable streams generated by fractionation may be considered as value added co-products.

4.2. Fractionation of brewers' spent grain by screw press dewatering and steam explosion

Through screw press dewatering of fresh BSG (**Figure 3.1.**), a drier pressed solid with a lower starch and protein content than the fresh BSG should be obtained. This creates an additional starch and protein-rich press liquid stream. Steam explosion of the pressed solids then creates a hemicellulose-rich liquid also containing degraded starch, and a residual solid high in lignin, protein, cellulose and recalcitrant hemicellulose which serves as a substrate for enzymatic hydrolysis. The following section characterises each of the streams generated by screw press dewatering and steam explosion in terms of the change in composition at each step of the process and discusses its origin and significance in terms of XOS production, in order to identify those streams most applicable in the process.

4.2.1. Characterisation of streams generated by screw press dewatering of raw brewers' spent grain

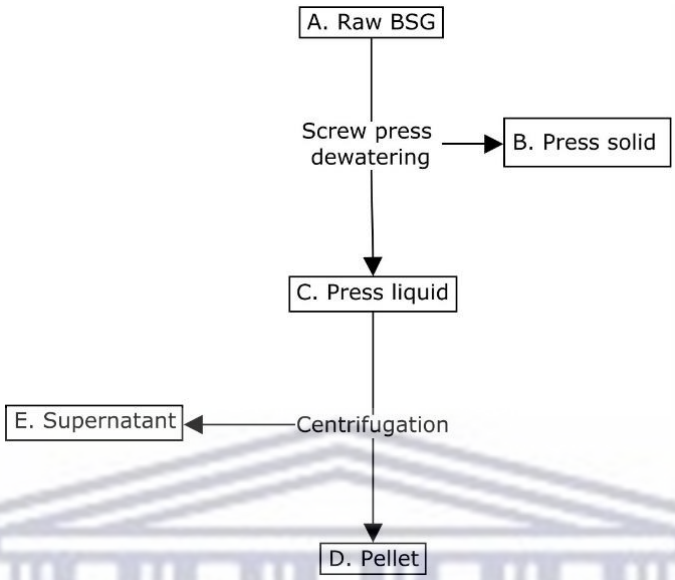

Figure 4.1. Screw press dewatering and centrifugation of raw BSG into various streams (A-E) (excerpt of Figure 3.1.).

Screw press dewatering aids in the fractionation of BSG into a solid and liquid phase. It causes the transfer of insoluble and soluble components such as, starch and protein, from raw BSG (fresh BSG as obtained from brewery in its untreated state) into the press liquid stream in the form of fine particles (smaller than 150 µm) and dissolved solids thereby, enriching the hemicellulose component in the press solids (Finley, 1976; Swart *et al.*, 2020). Using a single-screwed continuous press, the screw press treatment reduced the moisture content from 79.0% to 73.2% (w/w) resulting in pressed solids with a dry matter content (dm) of 24.6% (w/w) (**Figure 4.1.** stream B). The dm obtained after the screw press treatment was near to the optimal for XOS production during steam explosion according to Swart *et al.*, (2020) and therefore, the amount of moisture removal was adequate for the application in this study. However, this amount of moisture removal was mild compared to a previous study, which showed a dm of 37.3% (w/w) could be achieved (Weger *et al*., 2017). In addition, the results indicated that the press liquid (**Figure 4.1.** stream C) had a solids content of 8.4% (w/w), which translated to 13.3% of the dry matter from the raw BSG being recovered in the press liquid (calculated as the mass of solids in the press liquid as a percentage of mass of the solids in the raw BSG, stream A). The remaining dm of the raw BSG 84.7% was recovered in the press solids (stream B). This transfer of mass for dm can be seen in the mass balance (**Table 4.2**), which represents the mass of each measurable component in 1000 kg of raw BSG (stream A **Figure 4.1.**) as it is processed. The mass was subsequently used to calculate the concentration of each component in each stream as a weight percentage.

The transfer of dm from the raw BSG to the press liquid indicated that potentially, a discernible amount of insoluble BSG components had been removed from the starting material, rendering the pressed BSG a more viable substrate for steam explosion and XOS liberation, and allowing the potential use of the press liquid as a stream containing valuable co-products. The starch content of the raw BSG was reduced from 17.3% to 13.3% (w/w) DM during screw pressing (**Table 4.2**), which translates to 35.1% of starch, in terms of mass, being removed from the raw BSG. This was calculated as the difference in the mass of starch in the raw BSG and press solids as a percentage of the mass of starch in the raw BSG. Furthermore, a slight decrease in the protein content of raw BSG from 22.4% to 21.2% (w/w dry BSG) was observed after pressing. According to the mass balance in **Table 4.2**, this translated to 19.6% of the mass of the protein being removed. These results are lower than the 49.1% and 36.0% of starch and protein respectively removed by Swart *et al.*, (2020) during screw press dewatering, yet in line with the fact that the mechanical process of screw press dewatering is inclined to reduce starch. The inefficient removal of starch and protein during screw press dewatering may be attributed to the lack of free water in the raw BSG used in this study (79.0% w/w moisture) as opposed to 85.0% (w/w) moisture reported by Swart *et al.*, (2020), which possibly did not allow for finer particles and more soluble components to be removed, therefore resulting in a mass transfer issue. We hypothesise that during screw pressing, the barley grain and starchy endosperm were compressed, releasing the starch. However, without water, these fine particles were not cleared from the grain and thus, remained entrained in the press solids. Starch removal has been proven to be an essential step for lowering interference by contaminant components during XOS production via autohydrolysis processes such as steam explosion (Swart *et al.*, 2020), liquid hot water treatment (Gómez *et al.*, 2015); and chemical extraction of arabinoxylan (Sajib *et al.*, 2018), through the intensification of the hemicellulose component. Thus, more efficient starch removal was desired in this study. By decreasing the amount of starch prior to autohydrolysis the bulk density is also reduced (Swart *et al.*, 2020). In doing so, more efficient steam penetration of the biomass can occur, which leads to more effective hydrolysis of hemicellulose. The removal of starch and protein also ameliorates downstream issues such as XOS purity of steam explosion liquor which can add to the cost of production of a pure XOS product and furthermore, promote lignin-protein binding in residual solids after steam explosion (Sajib *et al.*, 2018; Kemppainen *et al.*, 2016). It is therefore suggested that future work should seek to address more starch efficient removal techniques through various screw press configurations (Swart *et al.*, 2020), hot water treatments for starch extraction (Gómez *et al.*, 2015) and enzymatic pretreatment with amylases (Sajib *et al.*, 2018).

Table 4.2: Mass balance showing the composition of streams generated by screw press dewatering and steam explosion

Data represents the average mass (kg) and standard deviation (±) of streams generated during quadruplicate experiments

Average and SD (±)	Raw BSG A		Press solid B		Press liquid C		Pellet D		Supernatant E		Steam F		Slurry G		SEL H		RSSE I	
Solid	210,01	6,06	177,70	4,50	27,89	2,00	19,80	1,20	7,23	1,31	0,00	0,00	172,35	3,40	53,15	2,97	119,20	3,11
Cellulose	**37,43**	**3,68**	**29,14**	**1,21**	**n.a.**	**n.a.**	**4,72**	**1,27**	**n.a.**	**n.a.**	**0,00**	**0,00**	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**23,56**	**2,14**
Hemicellulose	**40,52**	**3,17**	**35,00**	**3,75**	**1,51**	**0,10**	**0,06**	**0,10**	**0,22**	**0,04**	**0,00**	**0,00**	**30,81**	**2,02**	**12,08**	**1,48**	**18,73**	**3,11**
Xylan	27,61	2,77	23,64	2,79	1,08	0,08	0,06	0,10	0,16	0,03	0,00	0,00	22,64	1,26	7,93	1,06	14,71	1,18
Arabinan	12,91	0,64	11,36	0,96	0,43	0,03	0,00	0,00	0,05	0,01	0,00	0,00	8,17	1,98	4,15	0,43	4,02	2,35
Lignin	**70,78**	**5,51**	**64,39**	**4,51**	**n.a.**	**n.a.**	**6,92**	**0,82**	**n.a.**	**n.a.**	**0,00**	**0,00**	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**48,80**	**0,73**
Acid-soluble (AS)	15,39	0,43	13,70	1,42	n.a.	n.a.	1,35	0,25	n.a.	n.a.	0,00	0,00	n.a.	n.a.	n.a.	n.a.	8,59	1,92
Acid-insoluble (AI)	55,40	5,86	50,68	3,24	n.a.	n.a.	5,57	0,71	n.a.	n.a.	0,00	0,00	n.a.	n.a.	n.a.	n.a.	40,21	1,52
Ash	**6,90**	**0,45**	**8,26**	**0,83**	**n.a.**	**n.a.**	**0,43**	**0,02**	**n.a.**	**n.a.**	**0,00**	**0,00**	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**4,57**	**1,11**
Monosaccharides	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**0,70**	**0,15**	**n.a.**	**n.a.**	**0,51**	**0,14**	**0,00**	**0,00**	**n.a.**	**n.a.**	**2,65**	**0,31**	**n.a.**	**n.a.**
Xylose	n.a.	n.a.	n.a.	n.a.	0,23	0,04	n.a.	n.a.	0,14	0,04	0,00	0,00	n.a.	n.a.	0,66	0,06	n.a.	n.a.
Arabinose	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,00	0,00	0,00	0,00	n.a.	n.a.	1,55	0,31	n.a.	n.a.
Glucose	n.a.	n.a.	n.a.	n.a.	0,47	0,12	n.a.	n.a.	0,37	0,10	0,00	0,00	n.a.	n.a.	0,45	0,13	n.a.	n.a.
Oligosaccharides	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**13,74**	**1,04**	**n.a.**	**n.a.**	**3,80**	**0,61**	**0,00**	**0,00**	**n.a.**	**n.a.**	**32,17**	**1,29**	**n.a.**	**n.a.**
XOS	n.a.	n.a.	n.a.	n.a.	0,93	0,06	n.a.	n.a.	0,05	0,03	0,00	0,00	n.a.	n.a.	7,62	1,05	n.a.	n.a.
X2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	1,21	0,42	n.a.	n.a.
X3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,00	0,00	n.a.	n.a.
X4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,04	0,07	n.a.	n.a.
X5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	2,78	0,53	n.a.	n.a.
X6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,09	0,16	n.a.	n.a.
> X7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	3,50	1,66	n.a.	n.a.
Ar-OS	n.a.	n.a.	n.a.	n.a.	0,43	0,03	n.a.	n.a.	0,05	0,01	0,00	0,00	n.a.	n.a.	3,03	0,26	n.a.	n.a.
Glc-OS	n.a.	n.a.	n.a.	n.a.	12,38	1,06	n.a.	n.a.	3,70	0,60	0,00	0,00	n.a.	n.a.	21,41	2,45	n.a.	n.a.
Acetal-OS	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,00	0,00	0,00	0,00	n.a.	n.a.	0,10	0,11	n.a.	n.a.
Cellobiose	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,00	0,00	0,00	0,00	n.a.	n.a.	0,00	0,00	n.a.	n.a.
Degradation products	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**0,07**	**0,05**	**n.a.**	**n.a.**	**0,00**	0,00	**0,00**	**0,00**	**n.a.**	**n.a.**	**1,05**	**0,23**	**n.a.**	**n.a.**
Glycerol	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,00	0,00	0,00	0,00	n.a.	n.a.	0,00	0,00	n.a.	n.a.
Ethanol	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,00	0,00	0,00	0,00	n.a.	n.a.	0,00	0,00	n.a.	n.a.
Formic acid	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,00	0,00	0,00	0,00	n.a.	n.a.	0,00	0,00	n.a.	n.a.
Acetic acid	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,00	0,00	0,00	0,00	n.a.	n.a.	0,50	0,13	n.a.	n.a.
Lactic Acid	n.a.	n.a.	n.a.	n.a.	0,07	0,05	n.a.	n.a.	0,00	0,00	0,00	0,00	n.a.	n.a.	0,47	0,11	n.a.	n.a.
HMF	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,00	0,00	0,00	0,00	n.a.	n.a.	0,03	0,01	n.a.	n.a.
Furfural	n.a.	n.a.	n.a.	n.a.	0,00	0,00	n.a.	n.a.	0,00	0,00	0,00	0,00	n.a.	n.a.	0,05	0,02	n.a.	n.a.
Crude Starch	**36,37**	**0,81**	**23,59**	**2,22**	**n.a.**	**n.a.**	**5,81**	**0,87**	**n.a.**	**n.a.**	**0,00**	**0,00**	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**6,78**	**1,21**
Crude Protein	**46,93**	**2,59**	**37,65**	**2,09**	**n.a.**	**n.a.**	**6,42**	**0,43**	**n.a.**	**n.a.**	**0,00**	**0,00**	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**27,38**	**2,10**
Moisture	**789,99**	**6,06**	**485,81**	**29,98**	**308,60**	**32,95**	**114,07**	**8,49**	195,38	29,42	**776,68**	**39,76**	**1267,85**	**60,44**	**973,15**	**62,72**	**294,70**	**24,15**
Total	**1000,00**	**0,00**	**663,51**	**32,99**	**336,49**	**32,99**	**133,88**	**8,48**	**202,62**	**30,38**	**776,68**	**39,76**	**1440,19**	**63,66**	**1026,30**	**65,58**	**413,89**	**27,21**

n.a.: not analysed
HMF: hydroxymethylfurfural
Ar-OS: arabino-oligosaccharides
Glc-OS: gluco-oligosaccharides
Acetal-OS: Acetal-oligosaccharides
SEL: steam explosion liquor
RSSE: residual solids after steam explosion

Due to the selective removal of starch by screw pressing, it was expected that the cellulose and hemicellulose components would become enriched. However, no significant changes in cellulose (17.9 to 16.4% w/w), hemicellulose (19.3 to 19.7% w/w) and lignin (33.7 to 36.3% w/w) took place after screw press treatment as a result of the inefficient removal of starch and protein (**Table 4.2**). Compared to a study by Swart *et al.*, (2020), after pressing, the composition of cellulose (12.6%) and hemicellulose (22.7%) was somewhat lower, whereas lignin (18.5%) and ash (3.6%) was higher. Due to the lack of removal of interfering components and largely unchanged chemical composition of BSG after pressing (**Figure 4.1.** stream B), the anticipated efficiency of hydrothermal treatment may not be observed.

The press liquid fraction was separated into a solid discharge (stream D) and a supernatant (stream E) by centrifugation (**Figure 4.1.**). Stream D and E contained a large amount of suspended solids (water-insoluble components) and dissolved solids (water-soluble components), respectively, originating from stream C. The results (**Table 4.2**) showed that stream D was predominantly comprised of lignin (35.1% w/w dried weight basis), protein (32.5% w/w), starch (29.2% w/w), and minor components such as ash (2.15% w/w) and hemicellulose (0.31% w/w). The composition of protein and starch in this stream was consistent with the 35.5 and 22.0% respectively found by Swart *et al.*, (2020) indicating its potential as a valuable proteinaceous co-product in a biorefinery scenario. The water-soluble components of the press liquid (stream C) were made up of 51.7% (w/w) glucooligosaccharides (Glc-OS), which most likely derived from soluble starch that formed after the mashing process. Furthermore, other soluble components including XOS, arabino-oligosaccharides (Ar-OS), xylose, arabinose and glucose respectively, made up 0.7% (w/w); 0.7%; 1.9%; 0.0%; and 5.1% (w/w) of the stream. Similarly to what was found by Swart *et al.*, 2020., the Glc-OS made up the largest portion of dissolved solids in the press liquid. However, in this study, the stream was composed 51.7% Glc-OS of the dissolved solids, while in the previous study the Glc-OS only made up 39.6% of the dissolved solids in the stream. The fact that more water-soluble starch was found in this fraction in comparison with the previous study, suggests that there was variation between the breweries with respect to the mashing process. This observation also proves that the hydrolysis of starch was less extensive during mashing process employed by the craft brewery. Furthermore, the press liquid fraction is typically treated as an effluent in the process of reducing the moisture content of BSG (Lopez *et al.*, 2012; Finley, 1976). However, waste mitigation strategies for press liquid include valorisation, which is possible due to its high Glc-OS, starch and protein content. For example, without separation of the press liquid, it could be utilised as a substrate for biogas production (Weger *et al.*, 2017). After centrifugation, the solid phase of the press liquid can be applied as a vegetable based protein in food

products (Finley, 1976). Finally, the clarified liquid supernatant can be made into a fermented beverage or can be recycled into the brewing process as wort (Finley, 1976).

4.2.2. Characterisation of streams generated by steam explosion of pressed brewers' spent grain

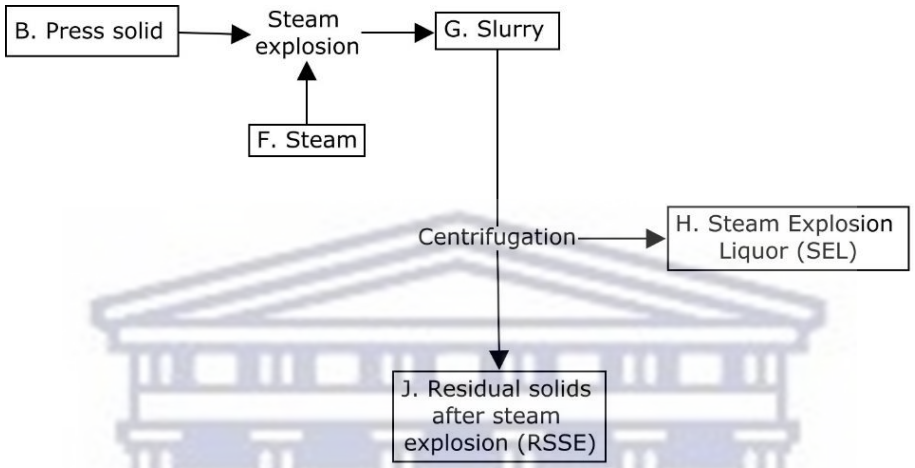

Figure 4.2. Steam explosion of pressed BSG and subsequent centrifugation into various streams (B-J) (excerpt of Figure 3.1.

The tightly bound structure of lignocellulose requires disruption by thermal pretreatment to separate components of the biomass, which enables the release of XOS through the solubilisation of hemicellulose, and provides greater accessibility of enzymes to active sites (Otieno and Ahring, 2012). To achieve efficient fractionation in a biorefinery process, steam explosion (SE) is typically employed as a hydrothermal pretreatment (Álvarez *et al.*, 2021; Cebreiros *et al.*, 2021; Bhatia *et al.*, 2020). The results in **Table 4.2** shows that at a dry matter content (dm) of approximately 25% (w/w) with SE conditions of 180 °C for 10 min, 97% of the dm was recovered in the slurry (stream G **Figure 4.2.**). The high recovery rate indicates that only a small loss of dm occurred over the process. Furthermore, these SE conditions were chosen since it produced the highest XOS yield with a low degradation product yield during SE without an added catalyst according to a previous study by Swart *et al.*, (2020).

The SE process facilitates autohydrolysis, which causes the degradation of hemicellulose (Otieno and Ahring, 2012). At high temperatures, the deacetylation of xylan leads to an increase in the pH, the ultimate breakdown of xylan into XOS and its release from cellulose and lignin resulting in a hemicellulose-rich liquid fraction and a lignin-cellulose-rich solid fraction. According to the mass balance (**Table 4.2**), it therefore, led to a 4.0% decrease in hemicellulose in the press solids (stream B). This translated to 35.3% being removed from the press solids and transferred to SEL (stream H),

which then consisted of 22.9% (w/w dry matter) hemicellulose that included 15.0% (w/w) xylan and 7.9% (w/w) arabinan. Hemicellulose-derived oligosaccharides made up 20.1% (w/w dried mass) of SEL and it included 14.4% (w/w) XOS, 5.7% (w/w) Ar-OS and 0.2% (w/w) acetal-OS. Finally, furfural, a by-product of the steam explosion process which is formed when xylose is degraded, made up 0.1% (w/w) of the dried mass in the stream. It is clear from the composition of SEL that, as expected, XOS was the main product of hemicellulose breakdown at the selected conditions due to the low severity of autohydrolysis as reported in literature (Swart *et al.*, 2020; Gómez *et al.*, 2015). At the steam explosion conditions used in this study, the percentage distribution (**Figure 4.3.**) indicated that XOS with a degree of polymerisation (DP) of 2 made up 16.7%; DP 3, 0%; DP 4, 0.4%; DP 5, 37.9%; DP 6, 1.0% and DP ≥7 44.0% of the total XOS present in SEL. Similar to previous studies, the largest portion of XOS were in the form of longer chains DP ≥4 allowing the opportunity for depolymerisation using enzymatic hydrolysis (Álvarez *et al.*, 2021; Swart *et al.*, 2020; Huang *et al.*, 2017).

Figure 4.3. Degree of polymerisation (DP) distribution as the percentage of total XOS in the steam explosion liquor stream H (X2; X3; X4; X5; X6; X≥7). The percentage distribution represents the average of quadruplicate experiments with standard deviation indicated with error bars.

In the context of optimising XOS production, monosaccharides represent undesired by-products that form during SE, which is largely unavoidable. In this study (**Table 4.2**), monosaccharides derived from hemicellulose made up only 5.0% (w/w) of the dried matter in SEL (stream H **Figure 4.2.**) due to the relatively mild steam explosion conditions employed, which did not allow for extensive degradation of hemicellulose into its monomeric form. This is typical during autohydrolysis where the severity is lower than acid-catalysed reactions and thus, beneficial for a higher XOS yield (Swart *et al.*, 2020). Similarly to what was observed in this study, at 180 °C Kemppainen *et al.*, (2016) found that the liquid fraction contained only ca. 8% (w/w dried mass) monosaccharides. Furthermore, the results in this study indicated that more arabinose (2.9% w/w) was found in SEL compared to xylose (1.3% w/w)

which is consistent with what was previously shown in literature (Swart *et al.*, 2020; Kemppainen *et al.*, 2016; Gómez *et al.*, 2015). This is likely due to the branched nature of arabinan in the hemicellulose matrix, which allows for more extensive hydrolysis than that of the xylan backbone during autohydrolysis. The increased release of arabinose has also been shown to provide more effective hydrolysis of the hemicellulose polymer in the residual solids after steam explosion by the action of xylanases, due to the now increased accessibility to active sites on the xylan chain (Lynch *et al*., 2016).

Furthermore, the results (**Table 4.2**) indicated that along with hemicellulose, ash and starch was also targeted during steam explosion which was consistent with previous reports (Swart *et al.*, 2020; Kemppainen *et al.*, 2016). While 54.7% of ash from the press solids (stream B **Figure 4.2.**) was recovered in RSSE, only 28.7% of starch was recovered in RSSE, which translates to a 7.6% (w/w) decrease after steam explosion. Due to the high amount of starch in the raw BSG and the inefficient removal of starch during pressing, a large amount of starch was transferred to the steam explosion liquor (SEL) in the form of Glc-OS, consistent with what was described in literature (Vegas *et al.*, 2008; Kabel, 2002). Therefore, SEL consisted of 40.3% (w/w dry matter) Glc-OS, while glucose and HMF only made up 0.9 and 0.1% (w/w) respectively. During the production of low molecular weight XOS by enzymatic hydrolysis, the presence of Glc-OS as seen in the results does not hinder the depolymerisation process, but it is not beneficial in terms of purity of a commercial product (Gómez *et al.*, 2015; Vegas *et al.*, 2008). However, though not well researched, the presence of Glc-OS can be advantageous in adding to the prebiotic effect of the hydrolysate as it has been proven to sustain the growth of probiotic bacteria (Karnaouri *et al.*, 2019; Rastall, 2013).

Due to the removal of targeted components, the dry matter of RSSE (stream J **Figure 4.2.**) comprised of 23.0% (w/w) protein, 19.8% (w/w) cellulose, 5.7% (w/w) starch, 3.8% (w/w) ash and 15.7% (w/w) hemicellulose, which was in agreement with a study by Kemppainen *et al.*, (2016). However, lignin, which made up 41.0% (w/w dried RSSE) was approximately 12.1% higher than what was previously reported. Furthermore, while the proportion of acid-soluble (AS) lignin remained the same after steam explosion, the acid-insoluble (AI) lignin increased significantly from 28.6 to 33.7% (w/w of the dried weight) and is similar to what was observed (Kemppainen *et al.*, 2016). In addition, the increase in the concentration of protein, cellulose and lignin, along with the high amounts of the respective components (72.7%; 80.7% and 76.2%) that were recovered in RSSE, suggested that during SE, protein, as well as the lignin-cellulose complex may have become enriched by the removal of starch and hemicellulose (Álvarez *et al.*, 2021; Bhatia *et al.*, 2020; Swart *et al.*, 2020). The high recovery of protein suggested that during SE, protein solubilisation was prevented by aggregation and cross-linking, induced by heat exposure (Kemppainen *et al.*, 2016). Moreover, SE may have allowed lignin to interact

with protein to further increase the capacity of proteins to precipitate. Therefore, the combined effect of enriching these components in the residue and strengthening their interaction, may have led to the remaining hemicellulose becoming more recalcitrant and thus, more resistant to downstream enzymatic hydrolysis which is undesired in the process (Otieno and Ahring, 2012). Overall, the composition of the fractions (**Figure 4.1.** and **Figure 4.2.**) created by screw press dewatering and steam explosion indicated that a starch and protein-rich press liquid (stream C), a hemicellulose-rich liquid containing degraded starch (stream H), and a residual solid high in lignin, protein, cellulose with a substantial amount recalcitrant hemicellulose (stream J) was formed. This suggested that for the valorisation of BSG, specifically to produce high value XOS, streams H and I were most applicable.

4.2.3. Fractionation and recovery of hemicellulose during pretreatment

In a biorefinery process, the recovery of a component plays a major role in tracking reusable and recyclable material in order to maximise the production of the final product (Galbe and Wallberg, 2019). In the context of this study, tracking the losses, recovery and yields of hemicellulose was important in quantifying the efficiency of the fractionation to produce XOS. The tracking and recovery of hemicellulose over the respective screw press and steam explosion process will be discussed in this section.

The mass balance (**Table 4.2**) shows that after pressing, 86.2% hemicellulose was recovered in the press solids due to the removal of fine particles. After steam explosion, 88.9% of hemicellulose was recovered in the slurry (**Table 4.2**), therefore an acceptable loss of only 11.1% took place. This was possibly as a result of arabinan being hydrolysed into arabinose and further being degraded into furfural, which is a volatile compound that evaporates with water (Krzelj *et al.*, 2019). After separating the slurry into RSSE and SEL, 53.6% and 35.3% of hemicellulose was recovered in the respective streams. The hemicellulose yield after steam explosion was thus, 29.8% of the initial hemicellulose in the raw BSG, and 39.2% of the hemicellulose in the slurry. Compared to a study by Swart *et al.*, (2020) who achieved a hemicellulose yield of 76.2% as a percentage of the starting hemicellulose BSG, the yield achieved in this study was much lower. The high amount of lignin and starch present in the pressed BSG may have impacted the efficiency of steam explosion in terms of the release and removal of xylan from the lignocellulosic matrix during autohydrolysis and therefore, subsequent enzymatic hydrolysis for XOS liberation from the residual solids may be negatively affected (Otieno and Ahring, 2012; Berlin *et al.*, 2006). The composition of BSG varies from one brewery to the next and depends on factors which include the cereal variety, time of harvesting and the malting and mashing regime and is thus, expected (Lynch *et al*., 2016). However, the amount of lignin in the raw BSG in this study (**Table 4.1**) was substantially higher than what is described in literature. Furthermore, not only was

there a difference in the chemical composition in the starting material with respect to a higher concentration of lignin and starch, the dry matter content of the slurry after steam explosion in this study (11.1%) was much lower than that obtained by Swart et al., (2020) (20.2%), which is indicative of differences in the steam explosion operation itself. A combination of these factors likely caused the large discrepancy in the hemicellulose yield. The selection of BSG based on its composition is therefore, a crucial consideration for the success of hemicellulose hydrolysis after steam explosion. Thus, it is suggested that a screening process in which the BSG which contains the highest amount of hemicellulose and less lignin, protein and starch is selected. Alternatively, an additional hemicellulose enrichment step could be implemented.

Xylan was almost completely recovered after steam explosion, with 97.6% being recovered in the slurry, and 63.2; and 34.5% of available xylan being recovered in RSSE and SEL, respectively (**Table 4.2**). In contrast, only 71.3% of arabinan was recovered in the slurry, with 34.2 and 37.0% in the RSSE and SEL, respectively, which is indicative of the hemicellulose loss in in the steam explosion process. The xylan yield was 28.7% of the initial xylan from raw BSG and 35.0% of the xylan present in the slurry. Without accounting for the loss, the arabinan yield was 32.1% of the initial arabinan from raw BSG and 50.8% of the arabinan present in the slurry. The respective theoretical solubilisation yields of xylan and arabinan achieved in this study was considerably higher than the 16.1 and 17.8% reported by Kemppainen *et al.*, (2016) who conducted steam explosion of BSG at 200 °C for 10 min. This may be due to the variation in steam explosion operation and starting composition of the BSG. For example, in the previous study, a larger mass (2.4 kg) of feedstock BSG was loaded into the reactor and a higher solid loading of 30.8% (w/w dried BSG) was used. In addition, the hemicellulose component of their starting material only accounted for 9.8% of the dried BSG.

Steam explosion produces XOS through the autohydrolysis of xylan. The theoretical xylan yield of XOS recovered in SEL was 96.1% (**Table 4.2**). However, as a percentage of the initial xylan in raw BSG, the XOS yield was 27.6%, which is lower than the 75.3% reported by Swart *et al.*, (2020). Yet the result obtained is in line with what was reported by Otieno and Ahring, (2012), who described that autohydrolysis in batch systems generally suffers from low oligosaccharide yields of between 15.4-61.2%. Although XOS is the main product of hemicellulose breakdown as a result autohydrolysis, the XOS yield is dependent on optimal conditions for a specific feedstock. Due to the difference in composition of raw BSG used in this study, the optimal conditions reported by Swart *et al.*, 2020 may not have been suitable for obtaining a maximum yield of 75.3%. Furthermore, the Ar-OS yield of the initial arabinan in the raw BSG was 23.5% and 37.1% of the arabinan in the slurry. However, due to the susceptibility of the branched arabinose in the hemicellulose matrix, the arabinose yield after

steam explosion (18.9%- theoretical; 12.0%- initial) was substantially higher than the xylose yield (2.4%- theoretical; 2.8%-initial) similar to what was reported by Kemppainen *et al.*, (2016).

4.3. Low molecular weight xylooligosaccharide production from steam explosion liquor (SEL) using an endoxylanase (*refer to* Figure 4.4.)

The composition of the liquor after steam explosion, specifically the large portion of longer chained XOS (DP ≥7), makes it a suitable substrate for the production of low molecular weight XOS (Álvarez *et al.*, 2021; Su *et al.*, 2021; Bhatia *et al.*, 2020; Lian *et al.*, 2020; Huang *et al.*, 2017). Preliminary experiments showed that after 24 hours of incubation at 50 °C, the maximum amount of sugar was obtained (appendix **Figure A 1.**). Hereafter, sugar concentrations decreased, possibly due to microbial contamination. The dosages used in this set of experiments were 1; 5; 25 and 50 U.ml SEL^{-1}.

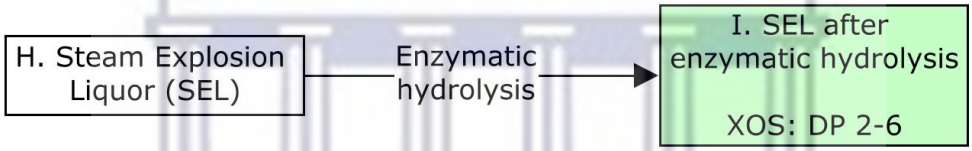

Figure 4.4. Enzymatic hydrolysis of Steam Explosion Liquor (SEL) in the production of low molecular weight XOS (streams H & I) (excerpt of **Figure 3.1.**).

4.3.1. Effect of enzymatic hydrolysis on monosaccharide and oligosaccharide formation

Figure 4.5. A and B indicates that minimal monomer formation of xylose and arabinose had taken place in the treated SEL. This was in agreement with literature and beneficial to the production of XOS as it indicates that the reducing ends of the xylan chain were not cleaved off (Bhatia *et al.*, 2020; Huang *et al.*, 2017; Vegas *et al.*, 2008). The monomers that were present in SEL was not due to the addition of enzyme, but a product of steam explosion since no major increases in xylose or arabinose occurred as a result of increasing the enzyme dosage or reaction time. Throughout the reaction, the xylose and arabinose concentration remained below 1.0 $g.L^{-1}$ and 2.5 $g.L^{-1}$ respectively. The minimal formation of monomers was attributed to the fact that Pentopan Mono BG, the commercial enzyme used, is a GH-11 enzyme classified as a 1-4, endoxylanase, which lacks β-xylosidase activity, which is beneficial for low molecular weight (DP 2-6) XOS production (Vegas *et al.*, 2008). The prevention of further breakdown of XOS chains observed here was attributed to the mechanism of action by which the endoxylanase binds to active sites which are located within the xylan backbone or long XOS chain and cleaves it into shorter XOS chains, without the ability to bind to at the end of the xylan chain cleaving

off one xylose monomer as with a β-xylosidase (Carvalho *et al.*, 2013). Moreover, **Figure 4.5. C and D** shows that there was no correlation between oligosaccharide (XOS and Ar-OS) production and enzyme dosage over time. Since minimal xylose or arabinose was formed, no decrease in XOS and Ar-OS was observed; and because no additional polymeric hemicellulose was available to be hydrolysed into oligosaccharides, no increase was observed.

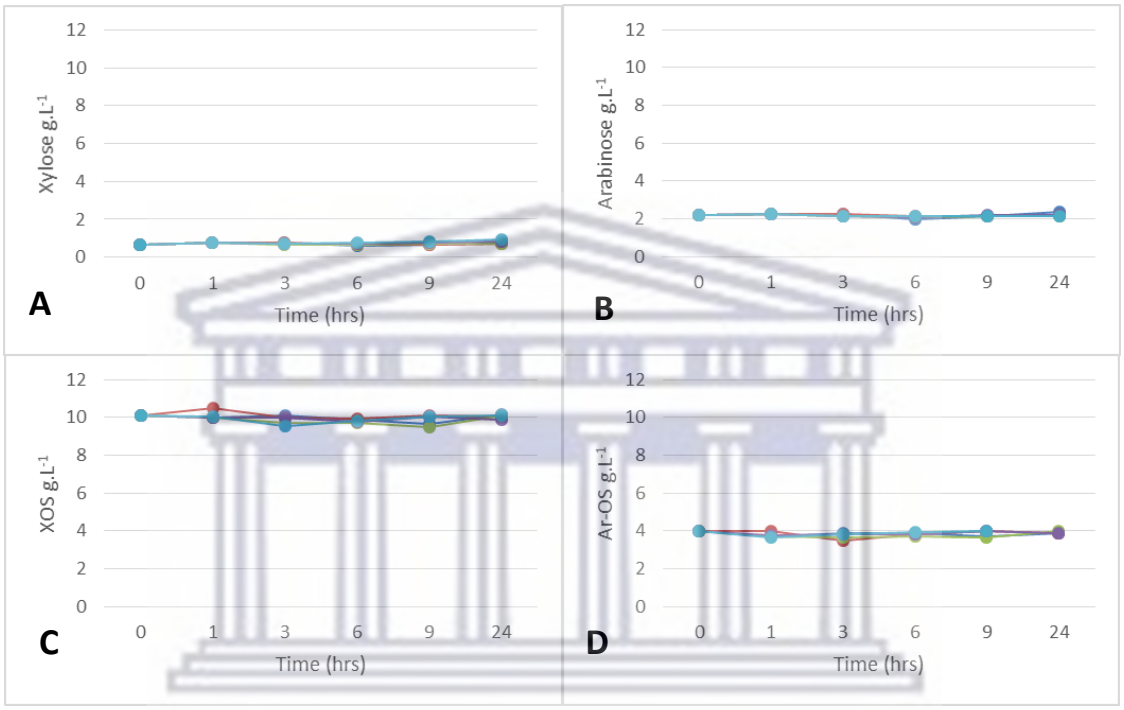

Figure 4.5. Time course of monosaccharide and oligosaccharide concentrations (g.L^{-1}) during enzymatic hydrolysis of SEL (stream I) with Pentopan Mono BG at various dosages (● 0 U.ml^{-1}; ● 1 U.ml^{-1}; ● 5 U.ml^{-1}; ● 25 U.ml^{-1}; ● 50 U.ml^{-1}). A: Xylose concentration. B: Arabinose concentration. C: XOS concentration. D: Ar-OS concentration. Data represents the average of triplicate experiments.

4.3.2. Effect of enzymatic hydrolysis on depolymerisation of XOS

As established in section **4.2.2.** at the beginning of the enzymatic hydrolysis of the steam explosion liquor (SEL), a large amount of XOS with a DP of ≥7 was present. **Figure 4.6. A** shows that the component yield at the start of the reaction was 76.3%. With the addition of the endoxylanase (1 U.ml^{-1}), the yield decreased significantly, by 19% after 3 hrs. Conversely, the yield of xylobiose to xylohexaose (X2-6) increased by 19% after 3 hrs when the endoxylanase was added. This observation suggested that the addition of an endoxylanase resulted in the formation of X2-6, which was dependent on the degradation of X≥7, and was in agreement with a previous report (Huang *et al.*,

2017). Furthermore, due to the lack of polymeric xylan in this stream, no further solubilisation of xylan and therefore, no increase in X≥7 was observed with the passage of time during the reaction.

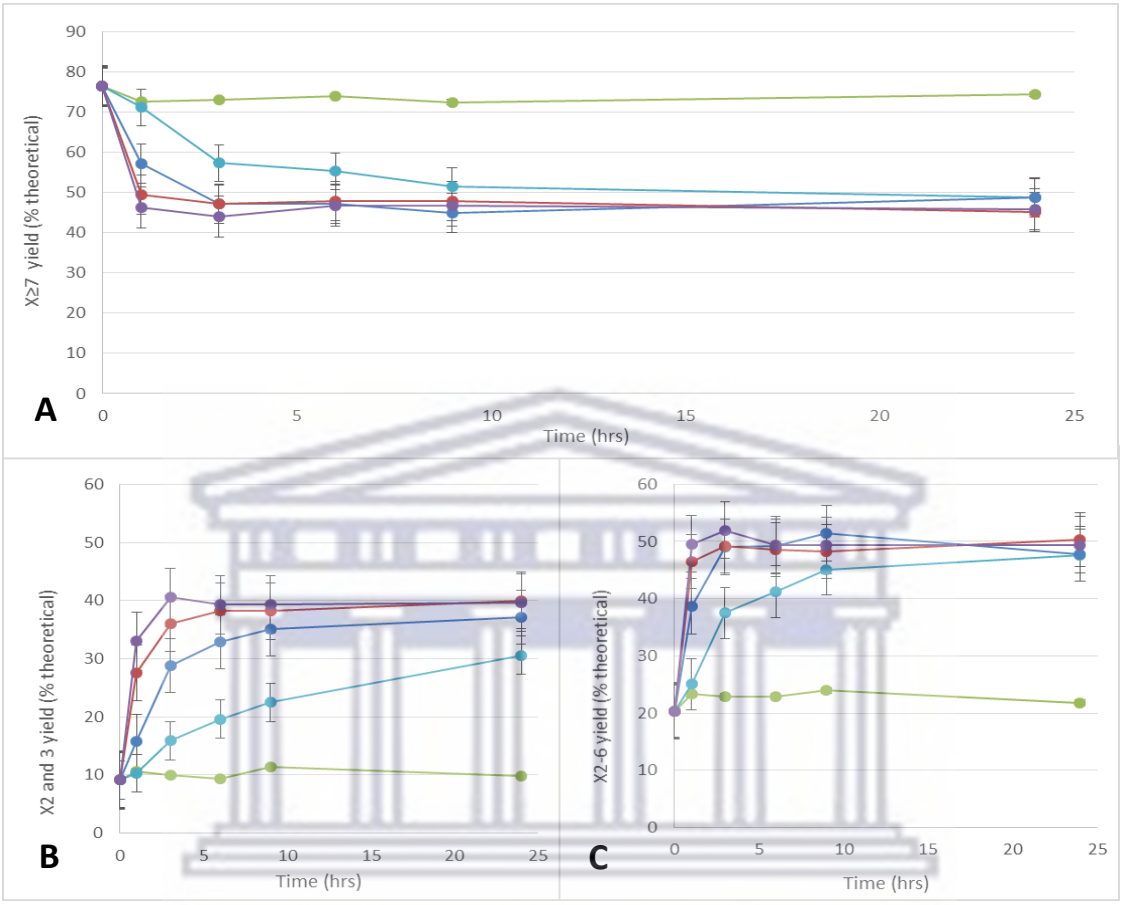

Figure 4.6. XOS yield calculated as a percentage of theoretical xylan released as XOS during enzymatic hydrolysis of SEL (stream I) with Pentopan Mono BG at various dosages (● 0 U.ml^{-1}; ● 1 U.ml^{-1}; ● 5 U.ml^{-1}; ● 25 U.ml^{-1}; ● 50 U.ml^{-1}). A: component yield of XOS DP ≥7. B: component yield of XOS DP 2-6. C: component yield of XOS DP 2 and 3. Results indicate experiments done in triplicate with standard error bars.

According to **Figure 4.6. C,** at 1 hr the respective enzyme dosages of 1, 5, 25 and 50 U.ml $^{-1}$ yielded 25.0, 38.7, 46.5 and 49.6% X2-6. This indicated that at 50 U.ml^{-1} at 1hr, approximately 98% of the maximum yield was achieved, showing that the production of X2-6 was near-complete. Moreover, at 24 hrs, there was no difference between the X2-6 yields produced by the various dosages except for when 1 U.ml^{-1} was compared with 25 and 50 U.ml^{-1}, respectively. This observation suggested that higher enzyme dosages favoured a shorter reaction time, indicating that there may be a trade-off between the cost of using a higher enzyme dosage and a smaller volume reactor since time is a function of reactor volume (Li *et al*., 2017). Furthermore, the results indicated that the maximum X2-6 yields of 51.9%, found at 50 U.ml SEL $^{-1}$ at 3 hrs; and 50.3% at 25 U.ml^{-1} at 24 hrs was attained. These yields are lower compared to other studies which reported an X2-6 yield of 63.4-97.5% (Álvarez *et al.*, 2021; Su *et al.*, 2021; Lian *et al.*, 2020).

When examining the more bioactive and commercially desirable XOS (DP 2 and 3), a stronger trend between enzyme dosage and yield was visible (**Figure 4.6. B**). As the enzyme dosage increased, the X2 and X3 yield increased. For example, after 24 hours at all dosages except for 25 and 50 $U.ml^{-1}$, the yields were significantly different ($p < 0.05$) to one another. Compared to the X2-6 yields, increasing the enzyme dosage therefore, had greater effect on X2 and X3, which meant that all dosages were inclined to produce similar X2-6 yields, but only higher dosages produced X2-3, suggesting that a higher enzyme activity was required for X2-3 production. However, similarly to the X2-6 yields, high dosages also favoured shorter reaction times. Thus, the best conditions for X2 and X3 yields were also found to be at 50 $U.ml^{-1}$ at 3 hrs; and 25 $U.ml^{-1}$ at 24 hrs, producing a maximum yield of 40.5 and 40.0%, respectively. These maximum yields were lower than the 62.8-74.7% reported in literature (Su *et al.*, 2021; Bhatia *et al.*, 2020; Lian *et al.*, 2020; Huang *et al.*, 2017). Furthermore, when comparing the condition of 50 $U.ml^{-1}$ at 3 hrs to 5 $U.ml^{-1}$ at 24 hrs (37.1% yield), though the difference in yield is significant ($p < 0.05$), it makes up 92% of the maximum yield. In industry, it may be more feasible to use 10 times less enzyme, but the energy and volume requirements to run the reaction for an additional 21 hrs to achieve a slightly lower yield may negate the efforts to save on expenses relating to the enzyme. On the contrary, when comparing the maximum yields achieved after 24 hours at 25 $U.ml^{-1}$ and 5 $U.ml^{-1}$, it may be more beneficial to use 5 time less enzyme.

It is important to note that during preliminary testing (results in appendix **Figure A 2.**) it was found that with 50 $U.ml^{-1}$ at 3 hrs the yield of X2-6 and X2 and X3 was 80.7 and 61.4% respectively. Long term storage of the liquor at -20° C may have caused compositional changes to take place with regards to the DP distribution before enzyme treatment, as seen in the hemicellulose mass balance (**Figure 4.17.**), which negatively impacted the X2 and X3 yield. Moreover, the autohydrolysis process associated with steam explosion produced a low pH environment (pH 4.24) that may have stimulated these compositional changes during storage. In addition, XOS is known to exhibit cryoprotective effects on starch during freezing by attaching to the surface of starch granules and moving towards its interior (Su *et al.*, 2020). It is proposed that this aggregation during freezing may have contributed to inhibited access of the enzyme to active sites on XOS, thus decreasing enzyme activity after storage.

The percentage distribution (**Figure 4.7.**) of the low molecular weight XOS shows that the various optimal conditions (5 $U.ml^{-1}$ at 24 hrs; 25 $U.ml^{-1}$ at 24 hrs; 50 $U.ml^{-1}$ at 3 hrs) produced a similar distribution pattern of XOS chain lengths. After enzymatic hydrolysis, the percentage distribution of X2 and X3 increased from 9.5% at 0 $U.ml^{-1}$ at 0 hrs to 38.4; 42.0; and 41.6% at the respective conditions. Other studies have reported varying results on percentage distribution, for example, Vegas *et al.*, (2008) obtained 20-25% of X2 and X3, Su *et al.*, (2021) obtained 65.7%. Huang *et al.*, (2017) obtained

74.7% and Bhatia *et al.*, (2020) obtained 80.8%. Furthermore, the percentage distribution of X≥6 decreased significantly from 80.4% at 0 U.ml^{-1} and 0 hrs to 38.4; 42.0; and 41.6% at the respective conditions, indicating that although the extent of depolymerisation was mild, a fair amount of longer XOS chains were hydrolysed. Therefore, the xylanase had a net positive effect on the production of short-chained XOS. In addition, during the preliminary test at 50 U.ml^{-1} at 3 hrs (**Figure 4.17.**), the XOS consisted of 64.1% X2 and X3 and 35.9% X≥4. Compared to percentage distribution of X2 and X3 obtained in final triplicate experiments at the same condition, the preliminary test was ~20% higher. Since after storage at -20 °C X≥7 still made up 47.3-50.5% it may have been possible that those longer chains were involved the aggregation and thus, could not be hydrolysed by the enzyme.

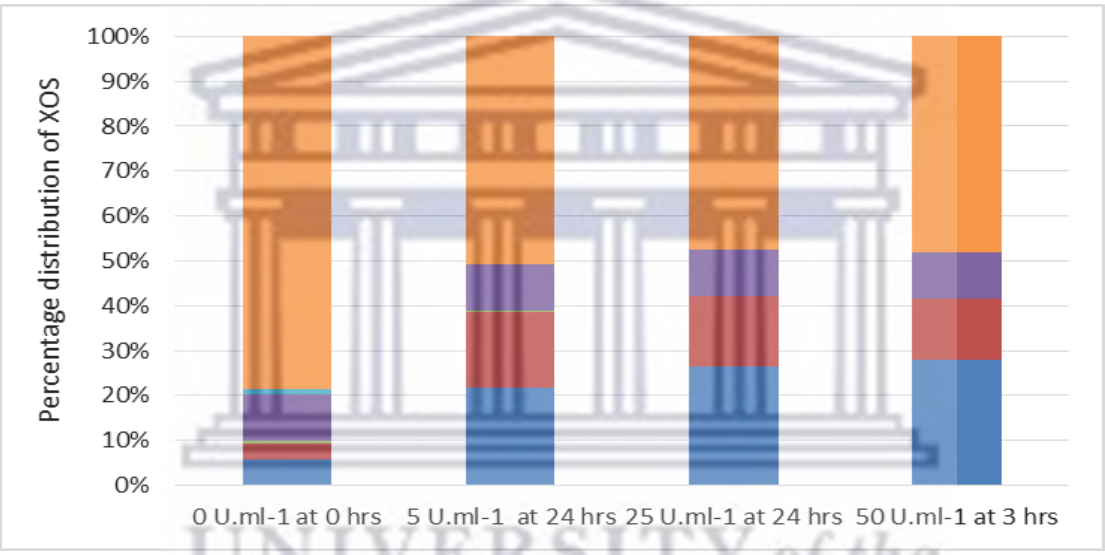

Figure 4.7. Degree of polymerisation (DP) distribution at various enzymatic hydrolysis conditions using Pentopan Mono BG on SEL (stream I). Percentage distribution was calculated as the percentage DP of XOS after enzymatic hydrolysis (■ X2; ■ X3; ■ X4; ■ X5; ■ X6; ■ X≥7). Data represents experiment performed in triplicate.

Altogether, higher enzyme dosages led to a higher yield of X2 and X3 with shorter reaction times. Therefore the condition of 50 U.ml^{-1} at 3 hrs was most suitable. The low yield and percentage distribution after the final experiment in triplicate indicated that the longer XOS chains became resistant to enzymatic hydrolysis after a long storage period. Depolymerisation was thus, relatively unsuccessful. However, value has been added to SEL by the production of a significant amount of X2 and X3. By conducting enzymatic hydrolysis on a freshly a generated SEL stream as would be done in a typical biorefinery operation, larger yields of low molecular weight XOS can likely be achieved.

4.4. Xylooligosaccharide (XOS) production from Residual Solids after Steam Explosion (RSSE) using high solids enzymatic hydrolysis

After steam explosion of pressed BSG, 53.6% of the hemicellulose was recovered in RSSE (see section **4.2.3.**), indicating that potentially a substantial amount of XOS could be liberated from this feedstock during solubilisation. Upcycling secondary streams in the extraction of high-value compounds such as XOS from a low value feedstock such as BSG, is required in an efficient biorefinery process (Galbe and Wallberg, 2019). In industry, economically viable strategies for extraction include high solids enzymatic hydrolysis due to the reduced reaction volume, which leads to a reduction in equipment volume. In addition, when compared to low solids reactions, less free water is available leading to an increase in the final product concentration and less water to be removed in downstream processes (da Silva *et al.*, 2020). During preliminary high solids enzymatic hydrolysis tests, results showed that at 24 hours of incubation at 50 °C, the maximum amount of oligosaccharides with varying degrees of polymerisation were obtained (appendix **Figure A 3.**). Hereafter, either a decrease in concentration or no notable changes were observed. Furthermore, higher dosages of 25; 100; 500; 1000 and 1500 U.g $RSSE^{-1}$ were used to overcome the recalcitrant nature of the hemicellulose in RSSE, and possible constraints associated with high solids reactions, which include mass and heat transfer problems brought about by the ratio of solid to moisture content of the mixture.

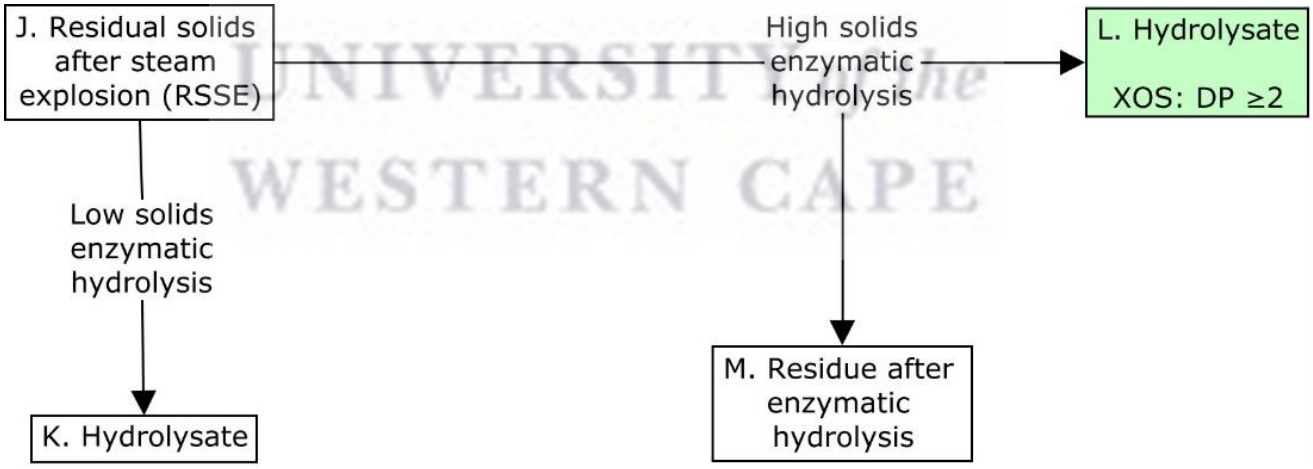

Figure 4.8. Enzymatic hydrolysis of Residual Solids after Steam Explosion (RSSE) in the production of XOS (streams J-M) (excerpt of Figure 3.1.).

4.4.1. Effect of high solids enzymatic hydrolysis on the solubilisation of hemicellulose into xylooligosaccharides

4.4.1.1. Monomer formation

The enzyme employed in this study was a GH 11 xylanase, reported to have an inclination for the production of XOS DP 2-5 with minimal monomer formation (Sajib *et al.*, 2018; Vegas *et al.*, 2008). **Figure 4.9**. **A** shows that although the hemicellulose conversion yield of xylose remained below 2.5%, as the enzyme dosage increased, xylose yield did increase. In addition, as the reaction time increased, the xylose yield increased partially because of the "high solids effect", which occurs in reactions with higher solids loadings. For instance, due to the accumulation of XOS, it is possible that the enzyme may have started to attack its own reaction products in which the low β-xylosidase activity present resulted in the increase in xylose (da Silva *et al.*, 2020; Vegas *et al.*, 2008). Furthermore, as shown in **Figure 4.9**. **B**, no arabinose was formed, likely due to the lack of specificity the enzyme has for arabinan hydrolysis.

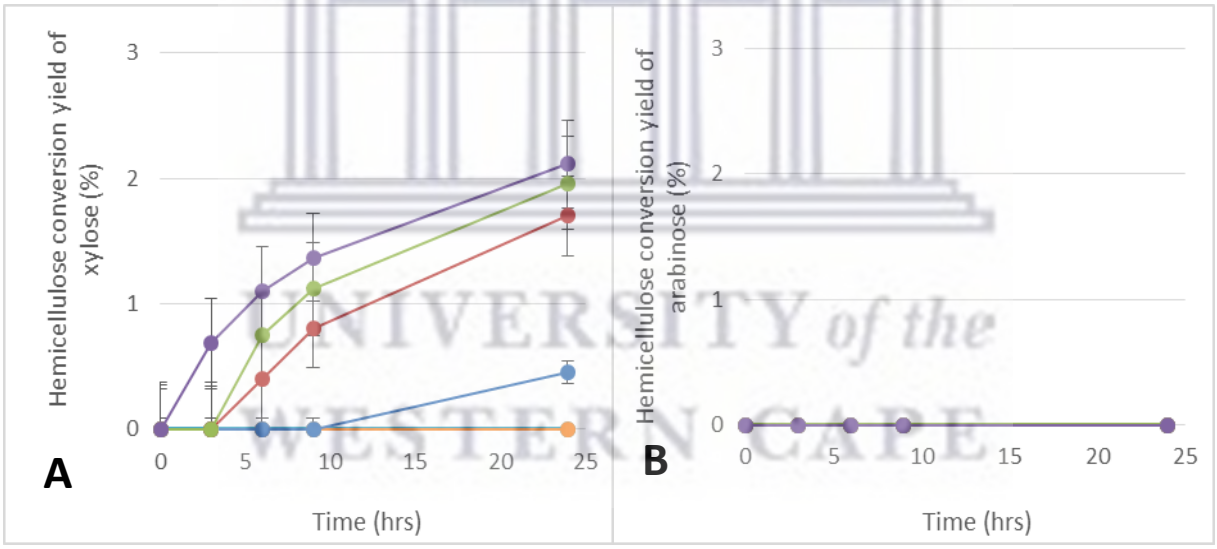

Figure 4.9. Hemicellulose conversion yield (%) of monomers formed during high solids enzymatic hydrolysis of RSSE (stream J) (● 0 U.g^{-1}; ● 25 U.g^{-1}; ● 100 U.g^{-1}; ● 500 U.g^{-1}; ● 1000 U.g^{-1}; ● 1500 U.g^{-1}). A: Hemicellulose conversion to xylose. B Hemicellulose conversion to arabinose. Yield calculated as the mass of xylose released as a percentage of hemicellulose in the residual solids. Data shown as the average of triplicate experiments with standard error bars.

4.4.1.2. Oligosaccharide formation

Figure 4.10. A, shows that the hemicellulose conversion yield of XOS increased as the enzyme dosage increased. Treatments at 1000 and 1500 U.g^{-1} produced XOS levels that were significantly ($p < 0.05$) higher than that of 100 U.g^{-1}. This result also indicated that the dosage had to increase exponentially,

so 10 times more enzyme was needed to produce a significant amount of XOS (yield increased by 3.5% from 29.8% at 100 U.g^{-1} 24 hrs to 33.3% at 1000 U.g^{-1}), which would dramatically increase the cost involved in producing XOS. Furthermore, 3hr increments of time did not have a significant effect on the hemicellulose conversion into XOS. However, the difference in the yield was significant ($p < 0.05$) at all dosages (except the control sample 0 U.g^{-1}) when 3 hrs was compared with 24 hrs. Since there was no significant difference between 1000 and 1500 U.g^{-1}, the maximum yield (33.3%) of XOS achieved was at a dosage of 1000 U.g^{-1} at 24 hrs. However, due to the higher costs associated with increasing the enzyme dosage by 10-fold it would likely be more feasible in industry to use the condition of 100 U.g^{-1} at 24 hrs as it produces 89.5% of the maximum yield. Moreover, due to the fact that the reaction volume was reduced by means of high solids loading, the trade-off between reaction time and reactor volume decreases (Li *et al*., 2017). The low XOS yield was not unexpected since mass transfer problems become prevalent at higher solids loadings (25% w/w) due to the lack of free water. This gives rise to poor mixing and heat transfer limitations, contributing to a decrease in enzyme efficiency, which ultimately causes a decrease in yield (da Silva *et al.,* 2020; Zheng *et al.,* 2009).

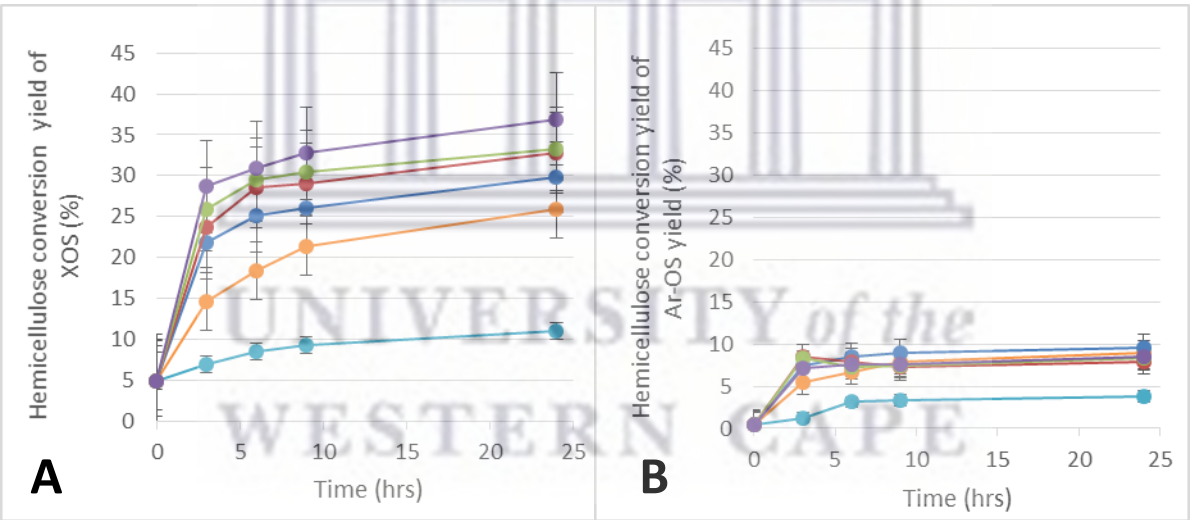

Figure 4.10. Hemicellulose conversion yield (%) of oligosaccharides formed during high solids enzymatic hydrolysis of RSSE (stream J). (● 0 U.g^{-1}; ● 25 U.g^{-1}; ● 100 U.g^{-1}; ● 500 U.g^{-1}; ● 1000 U.g^{-1}; ● 1500 U.g^{-1}). A: Hemicellulose conversion yield of XOS. B Hemicellulose conversion yield of Ar-OS. Yield calculated as the mass of oligosaccharide released as a percentage of hemicellulose in the residual solids. Data shown as the average of triplicate experiments with standard error bars.

At 100 U.g^{-1} at 24 hrs, according to **Figure 4.10. B** the Ar-OS yield was 9.5%. Although there was no correlation between increasing enzyme dosages and the resultant hemicellulose conversion yield to Ar-OS, it is clear from the results that Ar-OS production was affected by the addition of the xylanase. There was no difference between the yield at the lowest (25 U.g^{-1}) and highest (1500 U.g^{-1}) dosage; and after 3 hrs no further Ar-OS was produced. As confirmed by NREL analysis (appendix **Figure A 5.**), this may be an indication that all of the available arabinan was solubilised. This observation is notable

because, due to the lack of specificity of Pentopan Mono BG for arabinan hydrolysis into arabinose monomers (seen in the previous section), it may have been possible that the Ar-OS was formed as a consequence of xylan being solubilised by the xylanase, thereby cleaving off XOS or xylose that was branched with a certain degree of arabinose monomers. Although the degree of polymerisation of these arabino-xylooligosaccharides were unknown in this study because HPLC standards where specific to X2-6 molecules and not their branched form, they are also generally sought after for their ability to provide a prebiotic effect and therefore, stimulate a range of health benefits (Lynch *et al.,* 2016).

4.4.1.3. Hydrolysate composition after high solids enzymatic hydrolysis

Overall, the results in this section indicated that there was potential for XOS production by means of high solids enzymatic hydrolysis of steam exploded BSG residue. Due to an increase in XOS and Ar-OS because of enzymatic hydrolysis, the composition of the hydrolysate was altered. For example, **Figure 4.11.** shows that the control sample (0 $U.g^{-1}$) was made up of 34.1% XOS and Ar-OS, whereas the treated sample (100 $U.g^{-1}$) was made up of approximately 61.1% of these components. The proportion of Glc-OS also dramatically decreased from 64.3% in the control to around 27.6% in the treated samples. Furthermore, as the enzyme dosage increased, the concentration of the Glc-OS decreased slightly by being hydrolysed into glucose monomers. In a similar sense, more xylose was formed as the enzyme dosage increased possibly due to XOS that were broken down. Additionally, the hydrolysate of the treated samples contained small amounts of acetic acid, which may have derived from the cleaving of branched acetyl groups in the hemicellulose chain (Otieno and Ahring, 2012). The results demonstrate that the crude process employed to produce XOS generated a hydrolysate which contained a large portion of the target product. However, a downstream refining process may be necessary to increase the purity of the hydrolysate in a commercial setting. The removal of Glc-OS would contribute greatly to a purer final product. Since it is likely that the origin of Glc-OS is starch, an amylase could be employed to hydrolyse the Glc-OS into their monomeric form, followed by size exclusion chromatography or anionic and cationic exchange and activated charcoal chromatography to separate the XOS from the monomers as reported in literature (Precup *et al.*, 2022; Gomez *et al.*, 2015; Vegas *et al.*, 2008; Yang *et al*., 2007).

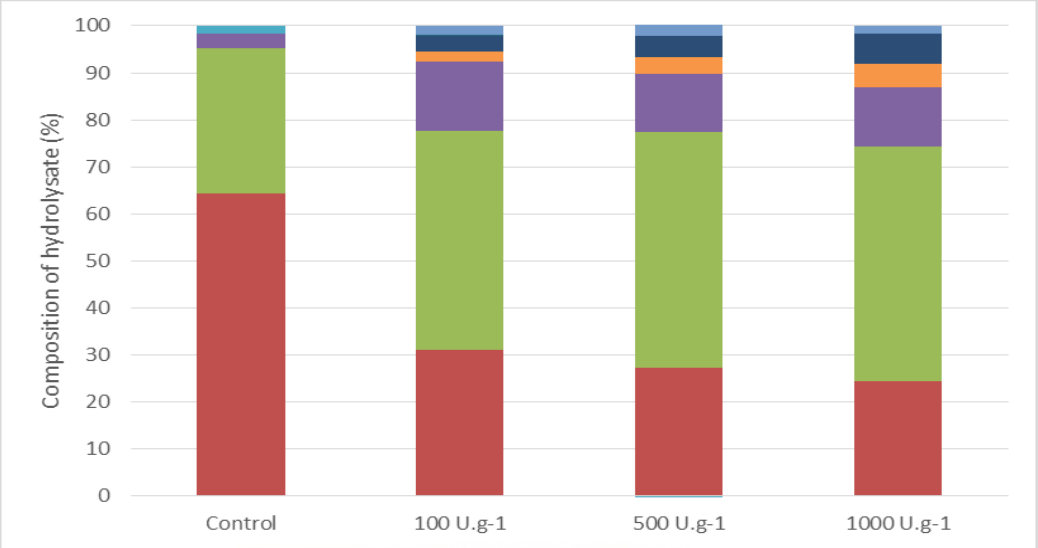

Figure 4.11. Composition of hydrolysate (stream L) after enzymatic hydrolysis at 0 (control); 100; 500 and 1000 U.g^{-1} at 24 hrs (■ Glc-OS; ■ XOS; ■ Ar-OS; ■ acetal-OS; ■ glucose; ■ xylose; ■ ethanol; ■ acetic acid). Data represents averages between triplicate experiments.

4.4.1.4. Hemicellulose solubilisation yield

When quantifying the sugars present in the hydrolysates, **Figure 4.12.** (treatment at 1500 U.g^{-1} for 24 hrs), showed that the hemicellulose in RSSE was solubilised by 47.6% via the endoxylanase. This is consistent with the solubilisation yield of xylan (42.3%) and arabinan (48.5%) obtained by Kemppainen *et al.*, (2016) at a solids loading of 20% (w/w). However, the mass balance (**Figure 4.17.**) showed that 129.2% of hemicellulose was recovered after enzymatic hydrolysis meaning that the residue showed no change in hemicellulose component after enzymatic hydrolysis. This discrepancy may have resulted from the inability of the NREL process to accurately measure small compositional changes especially if those components were present in small quantities. Furthermore, although there was a significant difference between the hemicellulose conversion yield at 100 U.g^{-1} (41.4%) and 1500 U.g^{-1}, with respect to hemicellulose solubilisation, using 100 U.g^{-1} would be the more feasible as 15 times less enzyme would be required to obtain 87.2% of the maximum yield. The low hemicellulose solubilisation yield was expected due to the nature of the solid-state reaction in which no free water was available to aid the mitigation of mass transfer limitations. It is possible that enzyme activity was inhibited due to product inhibition, which restricts access to the substrate. Additionally, the steam explosion pretreatment enriched the protein component, as well as the lignin-cellulose complex through the removal of starch and hemicellulose (section **4.2.2**), causing the recalcitrant hemicellulose left behind after steam explosion to become even more resistant to enzymatic hydrolysis by adding to the inability of the enzyme to gain access to active sites in the substrate and by promoting possible the unproductive binding of xylanase to acid-insoluble lignin (Li *et al.*, 2015; Otieno and Ahring, 2012).

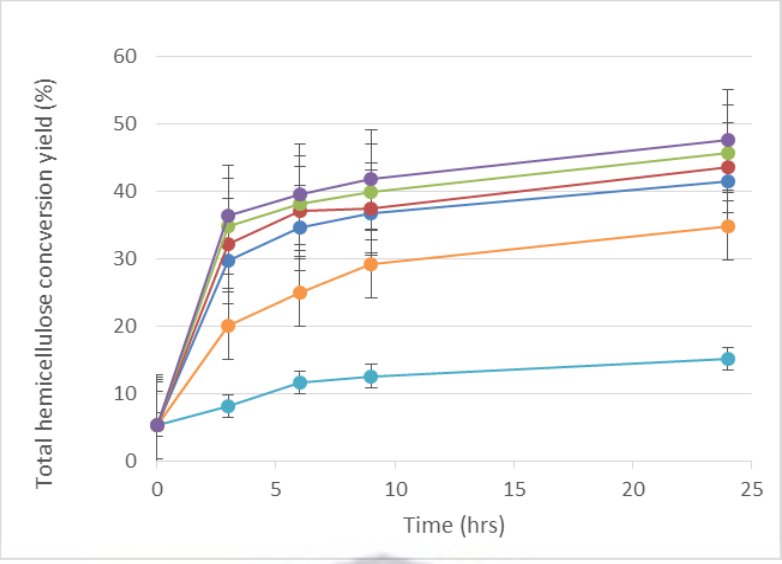

Figure 4.12. Total hemicellulose conversion yield (%) calculated as a mass percentage of monosaccharides and oligosaccharides (stream L) of hemicellulose in residual solids (stream J) (● 0 U.g^{-1}; ● 25 U.g^{-1}; ● 100 U.g^{-1}; ● 500 U.g^{-1}; ● 1000 U.g^{-1}; ● 1500 U.g^{-1}). Data represents average of triplicate experiments with standard deviation shown as error bars.

4.4.2. Effect of high solids enzymatic hydrolysis on depolymerisation of xylooligosaccharides

Xylobiose and xylotriose (X2 and X3) are sought after due to their prebiotic activity (Sajib *et al.*, 2018; Gómez *et al.*, 2015; Adamberg *et al.*, 2014; Gullón *et al.*, 2008; Moura *et al.*, 2007). Processing conditions should, therefore, select for and concentrate these short chain XOS. It is clear from **Figure 4.13. A.** that as the enzyme dosage increased, the X2 and X3 yield increased. While the dosage of 1000 U.g^{-1} at 24 hrs produced the maximum amount of X2 and X3 (13.9%), which was significantly more than at 500 U.g^{-1} at 24 hrs (8.9%). Here, the exchange between the doubled cost of the enzyme and hemicellulose conversion yield may result in a profitable outcome since 36.0% more hemicellulose is being converted to X2 and X3 at the higher yield.

The formation of low molecular weight XOS is brought about by the depolymerisation of longer chained XOS (Huang *et al.*, 2017). The results (**Figure 4.13. B and C**) reiterated this fact by indicating that for most dosages, the maximum yield of X≥7 was achieved within 3 hours, yet for X2-6 the maximum yield was achieved after 9 hours. In addition, for dosages of 100, 500, 1000 and 1500 U.g^{-1} about 75-96% of the X≥7 was formed in the first 3 hours (appendix **Figure A 6.**), whereas approximately 53-67% of the X2-6 was formed in the same time. This suggests that polymeric hemicellulose is being hydrolysed into X≥7 prior to the further depolymerisation of these longer chains. To add to this, the percentage distribution (**Figure 4.14.**) showed that at 1000 U.g^{-1} at 24 hrs, X2 and X3 made up 42.0% of the total XOS in the hydrolysate, while the rest were in the form of X≥7.

It is postulated that due to a build-up of product at a relatively high concentration (5.4 g.L $^{-1}$), the enzyme was not able to access the larger chains with X≥7. This was made clear by the fact that 99.4% of the X2-6 was made up of X2 and X3. In other words, almost all available X4-6 was hydrolysed. Product build up is common in high solids reactions due to the lack of free water, which dilutes and shifts the product allowing for contact with active sites on target molecules, which in this case would be X≥7 (da Silva *et al.*, 2020). Furthermore, due to the dependency of the formation of X2-6 on the availability of X≥7, it was expected that X≥7 would decrease as X2-6 was formed. However, the results **Figure 4.13. C** showed that the hemicellulose conversion yield either increased or remained stable as the reaction time increased. This phenomenon may indicate that due to the availability of polymeric hemicellulose in the RSSE, the process of solubilising the hemicellulose into longer chained XOS was occurring continually, likely until feedback inhibition of the enzyme by X2 and X3 occurred.

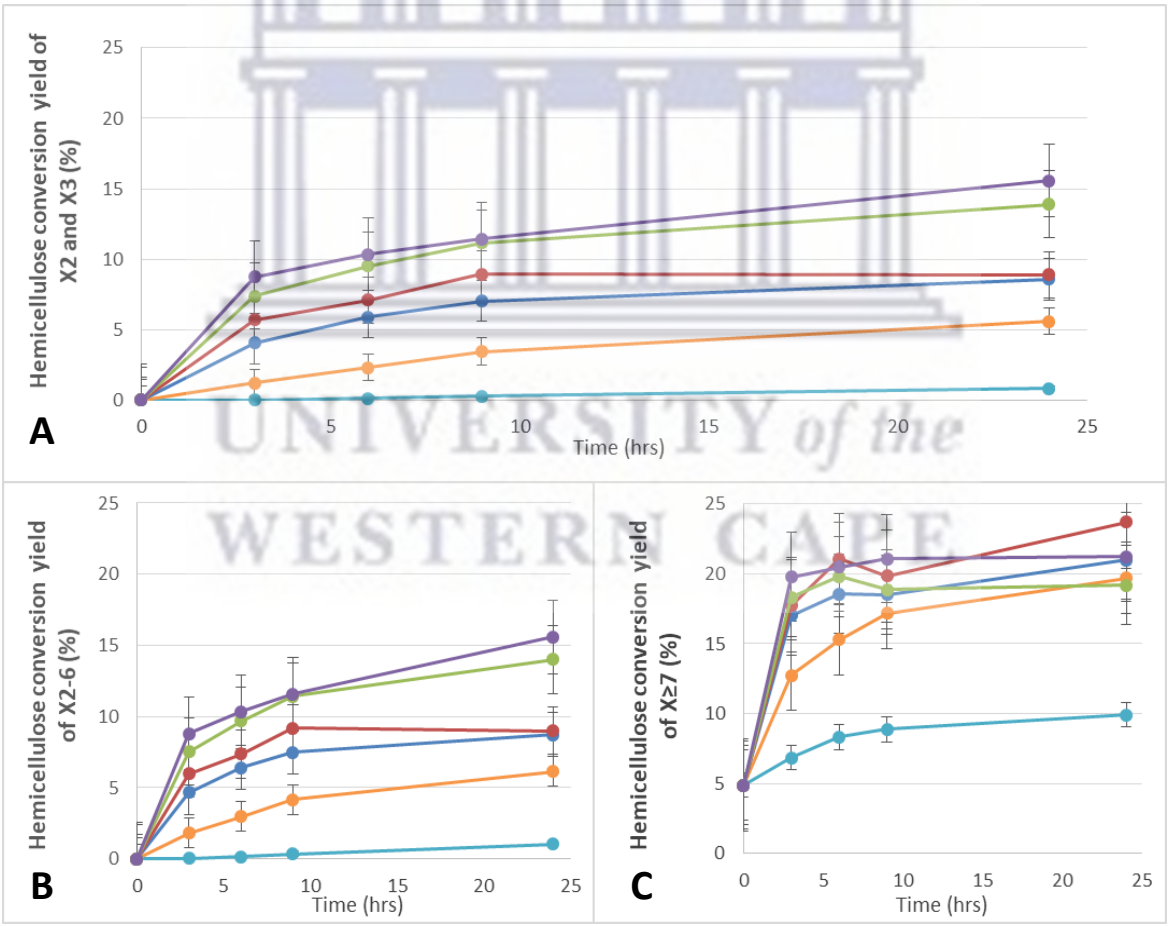

Figure 4.13. Hemicellulose conversion yield (%) of XOS with various degrees of polymerisation (DP) calculated as a mass percentage of XOS of hemicellulose in RSSE (● 0 U.g^{-1}; ● 25 U.g^{-1}; ● 100 U.g^{-1}; ● 500 U.g^{-1}; ● 1000 U.g^{-1}; ● 1500 U.g^{-1}). A: DP 2-6. B: DP ≥7. C: DP 2 and 3. Data represents the average of triplicate experiments with standard deviation shown as error bars.

Overall, the solubilisation of hemicellulose into XOS at a high solid loading of 25% (w/w) was more successful than depolymerisation, due to restricted access to X≥7 which resulted in lower X2 and X3 yields. In addition, different dosages were favoured for producing different XOS derivatives. In the production of XOS and Ar-OS, 100 $U.g^{-1}$ for 24 hrs was adequate, whereas a higher dosage of 1000 $U.g^{-1}$ at 24 hrs was required in the production of X2 and X3. However, irrespective of the low yields achieved, value was added to the stream by enzymatic hydrolysis of hemicellulose. Furthermore, the balance between solids loading and enzyme dosage must be optimised to combat product inhibition and achieve improved depolymerisation where 25% (w/w) solids loading can be regarded as the point of reference for the production of XOS from steam exploded BSG residue. In addition, the solubilisation of hemicellulose resulted in a residue high in cellulose (30.0% w/w dried BSG) and lignin (35.0%) at dosages of 500-1500 $U.g^{-1}$ (appendix **Figure A 5.**).

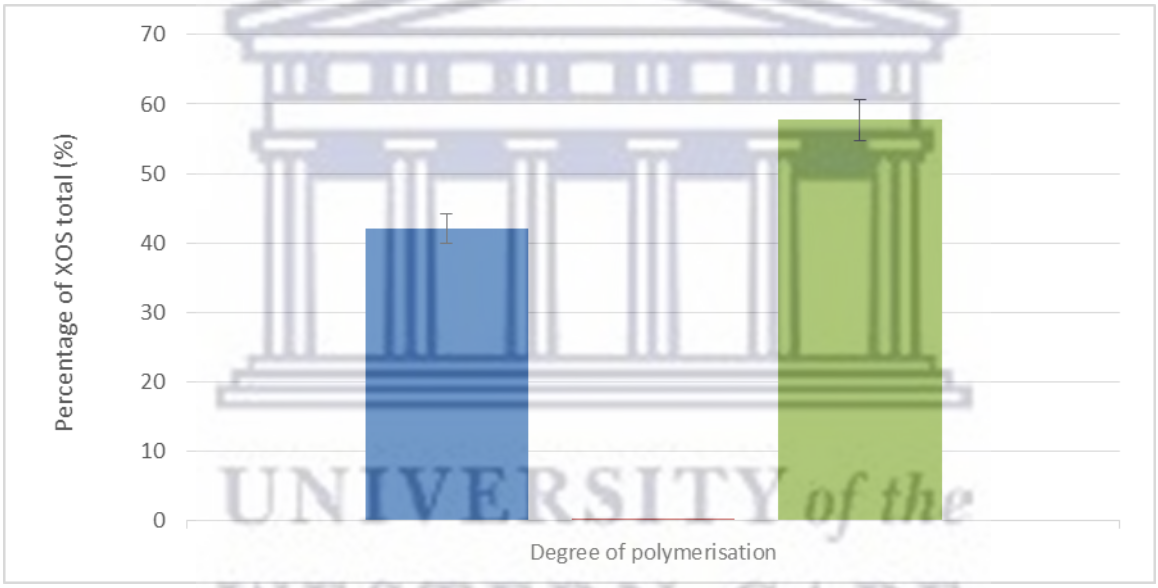

Figure 4.14. Percentage distribution of degree of polymerisation (DP) of XOS in hydrolysate at 1000 $U.g^{-1}$ at 24 hrs (■ DP 2 and 3; ■ DP 4-6; ■ DP ≥7). Data represents average between triplicate experiments.

4.4.3. Xylooligosaccharide production from RSSE at low solids concentration

At a low solids concentration (defined as less than 15% w/w solids) sugar yields are typically higher than at high solids loadings, while the opposite is true for sugar concentration (Weiss *et al*., 2019). To demonstrate the potential digestibility of the residual solids after steam explosion (RSSE) and to serve as a benchmark for whether maximum solubilisation of hemicellulose could be achieved at the conditions used, the experiment was repeated at 3% solids.

4.4.3.1. Effect of low solids enzymatic hydrolysis on hemicellulose solubilisation and oligosaccharide production

Figure 4.15. A indicated that no monomers were formed during enzymatic hydrolysis at a low solids concentration. Furthermore, it was also shown that as the enzyme dosage increased, the XOS yield increased. For instance, the yield for the respective dosages of 25; 100 and 500 $U.g^{-1}$ at 24 hrs was 14.9; 17.9 and 24.4% with the latter corresponding to a maximum concentration of 0.9 $g.L^{-1}$. A similar trend was observed with the hemicellulose conversion yield of Ar-OS (**Figure 4.15. B**). Here, the yield at 500 $U.g^{-1}$ at 24 hrs was 8.7% which was equivalent to a concentration of 0.3 $g.L^{-1}$. The total hemicellulose conversion yield (**Figure 4.15. C**) was thus, 33.1%. However, compared to the high solids reaction, in section

4.4.1.4., the hemicellulose yield was 10.4% less at the same enzyme dosage and time. Moreover, the concentration of XOS and Ar-OS was higher at 12.7 and 3.1 $g.L^{-1}$, respectively (appendix **Figure A 4.**). The high solids effect occurs when high substrate concentrations used in enzymatic hydrolysis results in low component yields due to mass transfer issues, which result in high product concentrations caused by an accumulation of product therefore, causing product inhibition (da Silva *et al.*, 2020). Whereas, for low solids concentrations, a higher yield and a low product concentration was expected, but a low yield and concentration was obtained. This indicated that the higher substrate concentration may have enabled the enzyme to have more access to the hemicellulose component, thereby producing a higher solubilisation yield. This suggested that solids concentration must be increased from 3% (w/w) in order to allow better enzyme-substrate binding.

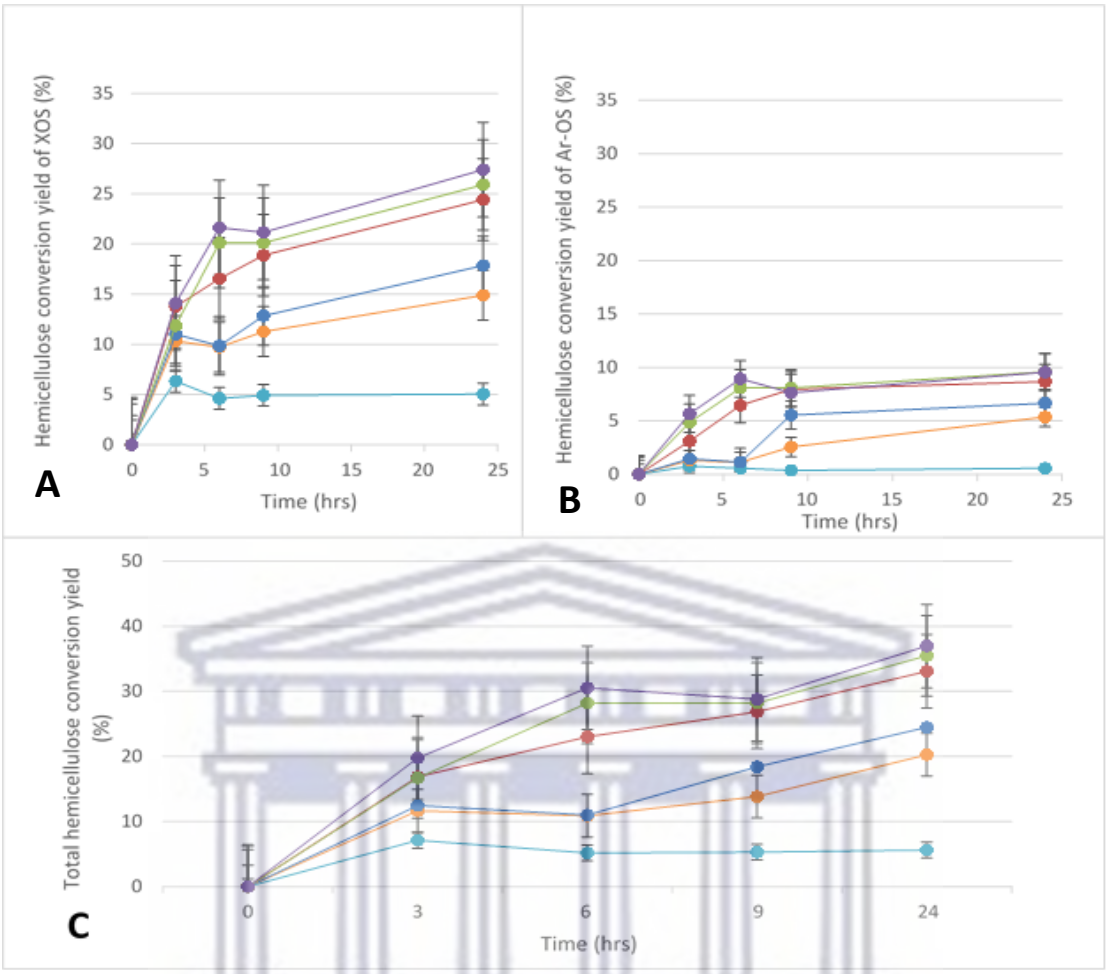

Figure 4.15. Hemicellulose yields of oligosaccharides produced at 3% solids loading calculated as the mass percentage of oligosaccharides of hemicellulose in RSSE (● 0 U.g^{-1}; ● 25 U.g^{-1}; ● 100 U.g^{-1}; ● 500 U.g^{-1}; ● 1000 U.g^{-1}; ● 1500 U.g^{-1}). A: Hemicellulose conversion yield of XOS. B: Hemicellulose conversion yield of Ar-OS. C: Hemicellulose conversion yield of oligosaccharides including XOS and Ar-OS. Data represents the average between triplicate experiments with standard deviation shown as error bars.

4.4.3.2. Effect of low solids enzymatic hydrolysis on the depolymerisation of xylooligosaccharides

Figure 4.16. A shows that a significant increase ($p < 0.05$) in the hemicellulose conversion yield of both X2 and X3 took place as the enzyme dosage increased except for between 1000 and 1500 U.g^{-1}. Additionally, in contrast with the high solids, increasing the reaction time had a significant effect on the yield especially at the higher dosages. Therefore, the highest hemicellulose conversion yield for X2 and X3 (5.0%) was achieved at 1000 U.g^{-1} at 24 hrs with a concentration of 0.2 g.L^{-1}. Furthermore, the results indicated that throughout the reaction, no X4-6 could be measured. At a solids loading of 3%, Dotsenko *et al.*, (2018) was able to obtain X2 and X3 concentrations of 9.3 and 13.2 g.L^{-1} in wheat straw and rye grass, respectively, after hydrothermal pretreatment at 190 °C for 10 min. The concentration obtained by those authors was considerably higher than in this study due to

concentration by evaporation of 96% of the moisture prior to analysis. Moreover, the yield reported by Dotsenko *et al.*, (2018) was 7% and 5% as a percentage of dried wheat straw and rye grass, respectively. In another study, after autohydrolysis conditions of 160 °C for 15 min, in a reaction containing 10 % (w/w) dried wheat straw, Precup *et al.*, (2022) obtained a concentration of only 1.23 $g.L^{-1}$ and 0.59 $g.L^{-1}$ using an endoxylanase and Cellic® CTec2 respectively. At autohydrolysis conditions of 180 °C for 15 min, using Cellic® CTec2, the authors obtained 0.78 $g.L^{-1}$, which was more in line with what was achieved in this study.

Furthermore, the percentage distribution of X2 and X3 was 19.6% at 1000 $U.g^{-1}$ indicating that a large portion of XOS was made up of X≥7. In addition, similarly to what was observed in section **4.4.2.** , X≥7 increased or remained stable across the reaction (**Figure 4.16. B**) due to the availability of polymeric hemicellulose which the enzyme had access to. Furthermore, a higher X2 and X3 yield, concentration and percentage distribution was obtained at 25% (w/w) solids loading which again suggested that because of the dilute nature of the reaction in a larger volume flask (300 ml), the enzyme had difficulty gaining access to or making contact with the longer XOS chains as their concentration in the mixture was small.

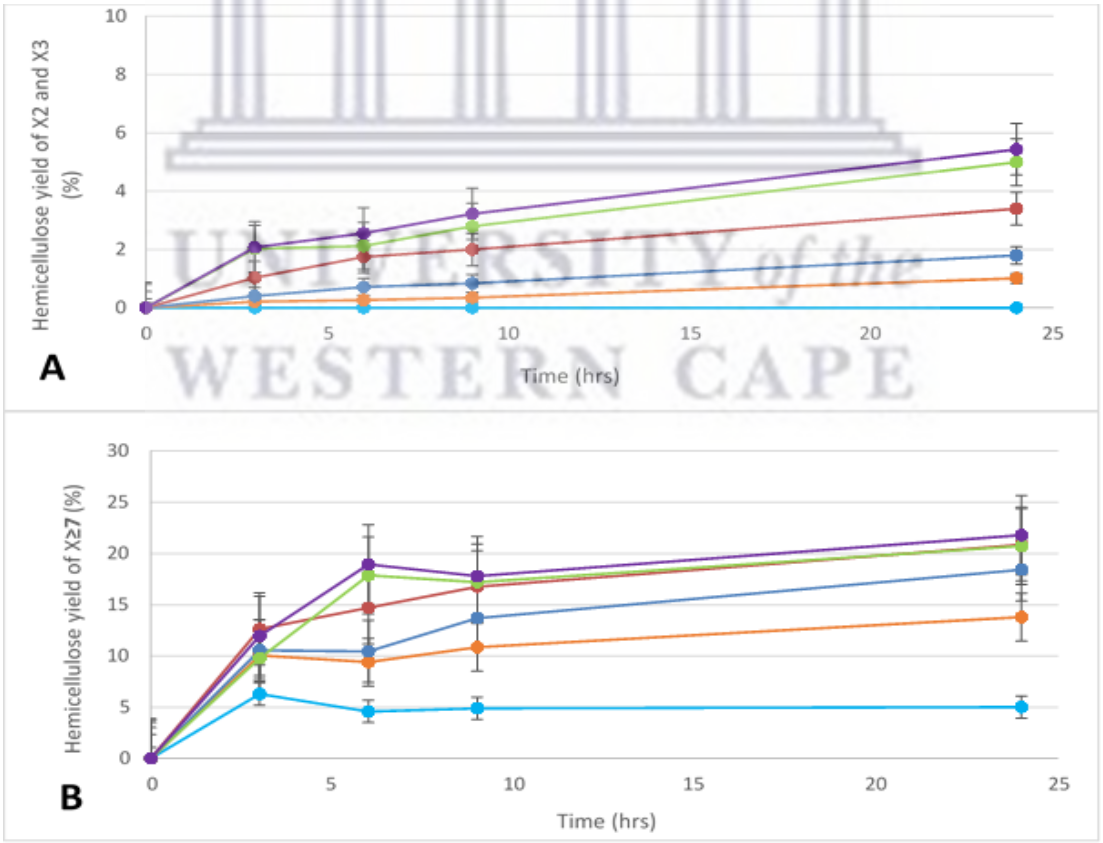

Figure 4.16. Hemicellulose conversion yield of XOS with various degrees of polymerisation (DP) calculated as the mass percentage of XOS release from hemicellulose in RSSE (stream J) at a low solids loading (● 0 $U.g^{-1}$; ● 25 $U.g^{-1}$; ● 100 $U.g^{-1}$; ● 500 $U.g^{-1}$; ● 1000 $U.g^{-1}$; ● 1500 $U.g^{-1}$). A: DP 2 and X3; B: DP ≥7. Data represents the average between triplicate experiments with standard deviation shown as error bars.

The larger reaction volumes and requirement for downstream water removal associated with the production of target products at low solids loadings, in addition to the unsuccessful solubilisation and depolymerisation evident in this section, shows that this is not a viable option as a biorefinery process. Although the high solids enzymatic hydrolysis (section **4.4.2.**) presented product inhibition issues which resulted in a low yield (13.9%) for X2 and X3, increasing solids loading to 25% (w/w) resulted in better hemicellulose solubilisation and XOS depolymerisation compared to the low solids reactions and is thus, more feasible in a biorefinery scenario. Improvements can still be made to the high solids process by ameliorating product accumulation to aid depolymerisation while still working at a high substrate concentration, which would allow for sufficient enzyme-substrate binding.

4.5. Hemicellulose mass balance after fractionation and enzymatic hydrolysis of brewers' spent grain

The hemicellulose recovery was vital to the valorisation of BSG as the source of XOS. The processing of hemicellulose from BSG into different product streams using various unit operations is depicted in **Figure 4.17.** , as a mass balance. Hemicellulose entered the process as part of BSG where it was fractionated into different streams followed by a process of solubilisation and depolymerisation using screw press dewatering, steam explosion and enzymatic hydrolysis. One tonne of wet BSG contained 36.2 kg of hemicellulose. After pressing, 609.3 kg of wet, pressed BSG and 30.0 kg of hemicellulose remained, which showed that even though 6 kg of hemicellulose was lost during the process, the concentration of hemicellulose in the wet BSG significantly increased from 3.6% to 4.9%. This indicated that the removal of moisture, water-soluble and water-insoluble solids during screw press treatment (section **4.2.1.**) resulted in the enrichment of the hemicellulose component. After steam explosion of the pressed BSG, 93.3% of the hemicellulose was recovered in the slurry, which was made up of steam explosion liquor (SEL, stream I) and residual solids after steam explosion (RSSE-stream H). The respective streams contained 14.4 kg of hemicellulose solubilised by steam explosion and 13.7 kg of polymeric hemicellulose, which indicated that these streams could be upcycled for XOS production.

Stream I and J were disconnected through a waiting period in which the substrate was stored at -20 °C for two months, while preliminary testing (stream L and M) was being completed. During storage the steam explosion liquor underwent a compositional change with respect to the low molecular weight XOS (DP 2-6). For example, directly after steam explosion, X2-6 made up 27.1% of the hemicellulose in SEL, whereas during preliminary testing and the final experiment, the control sample showed that the X2-6 fraction made up 22.1 and 13.3% of the hemicellulose in the respective streams. Although it is reported that XOS is stable over a wide range of pH's and temperatures little to no

research has been done on its stability during long term storage in the form of autohydrolysis liquor (Contesini *et al.*, 2019; Carvalho *et al.*, 2013). It is also possible that XOS aggregation with contaminant components during freezing may have inhibited accessibility to enzyme binding sites (Su *et al.*, 2020). Due to these possible changes, depolymerisation of XOS was affected which resulted in a smaller X2 and X3 yields. During preliminary testing, at 50 $U.ml^{-1}$ at 3 hrs the theoretical hemicellulose yield indicated that 55.0% of the hemicellulose in the stream was recovered as X2-6 and 46.3% was recovered as X2 and X3 while at the same condition after an additional 1.5 months in storage, 34.0 and 25.3% of hemicellulose was recovered for the respective degrees of polymerisation.

The RSSE (stream H) was made up of 48.9% of the remaining recalcitrant hemicellulose (13.7 kg polymeric hemicellulose with 0.06 kg of soluble hemicellulose still present after washing). High solids enzymatic hydrolysis enabled the solubilisation of 5.9 kg of this hemicellulose into XOS, which translates to a theoretical hemicellulose yield of 41.3% and resulted in a yield for X2 and X3 of 8.4%. Though no research has previously been done on high solids enzymatic hydrolysis of steam exploded BSG residue for the production of XOS, Bhatia *et al.*, (2020) achieved a yield of 52.1% of XOS at 1% solids loading. Considering that a higher solids loading was used in this process, the yields compare well with one another. In addition, compared to the enzymatic hydrolysis at 3% (section **4.4.3.**) the yield achieved by high solids enzymatic hydrolysis was higher due to the increased substrate concentration. This, along with high costs associated with low solids reactions, indicates that the high solids scenario is a viable option in a biorefinery scenario. Altogether as a percentage of the hemicellulose present in the slurry (28 kg), the process of solubilisation and depolymerisation by steam explosion pretreatment and enzymatic hydrolysis yielded 47.9% XOS, 16.1% Ar-OS, 23.9% X2-6, 20.4% X2 and X3. However, the process could potentially yield 51.4% XOS, 15.0% Ar-OS, 36.4% X2-6, 29.3% X2 and X3 if, freshly generated SEL is used as the case would be in typical biorefinery operation. Nevertheless, by producing satisfactory yields of XOS at various degrees of polymerisation, value was added to BSG which is indicative of a viable strategy for the valorisation of BSG.

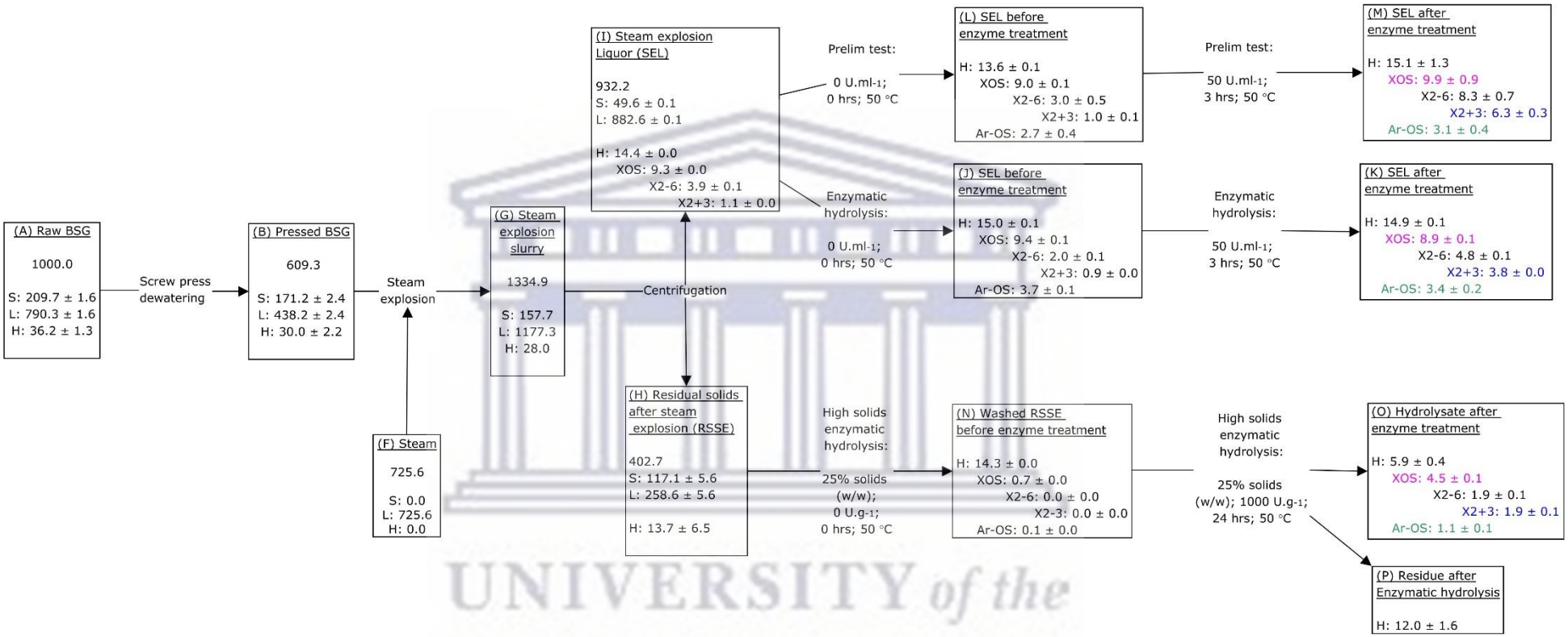

Figure 4.17. Hemicellulose mass balance from 1000 kg of BSG. S: solid; L: liquid; H: hemicellulose. Preliminary enzymatic hydrolysis test (L and M) performed in duplicate, data represents average and ± standard deviation in kilograms (kg). Enzymatic hydrolysis data represents the average and standard deviation (±) of triplicate experiments.

Chapter 5

Conclusions and Recommendations

The main aim of this study was to establish a crude process to produce xylooligosaccharides (XOS) in the context of valorisation of BSG. This aim was achieved by fractionating BSG into various streams to gain access to the hemicellulose component, after which enzymatic hydrolysis was employed for both depolymerisation and solubilisation of the hemicellulose in the selected streams. Time course experiments were then carried out at various enzyme dosages to determine the most efficient conditions for XOS production.

5.1. Fractionation of brewers' spent grain

Screw press treatment aided in the moisture reduction from 79.0% to 73.2% (w/w) thereby removing water soluble and insoluble components such as, Glc-OS, lignin, protein, and starch from the raw BSG and transferring it to the press liquid fraction. After centrifugation of this press liquid, the supernatant was composed of 50.1% (w/w) Glc-OS and the pellet was comprised of 35.1% (w/w) lignin, 32.5% protein and 29.2% starch. Due to its composition, the clarified press liquid after centrifugation could potentially exploited as a fermented beverage, or recycled back into the wort (Finley *et al.*, 1976). An acceptable amount of hemicellulose (86.2%) that was recovered in the screw pressed solids indicated that hemicellulose remained relatively unchanged. For further enrichment of hemicellulose, enzyme pretreatment with an amylase prior to screw press dewatering may be beneficial. Steam explosion of screw pressed BSG further fractionated the remaining hemicellulose component, of which 53.6% and 35.3% was recovered in RSSE and SEL, respectively. The steam explosion process produced a satisfactory XOS yield of 27.6%, which was mostly comprised of X5 and X≥7 making it a suitable substrate for depolymerisation to obtain X2 and X3. However, due to compositional differences such as, higher amounts of lignin and starch in the raw BSG and inefficient starch and protein removal, combined with possible operational differences compared to a previous study (Swart *et al.*, 2020), steam explosion was not as efficient in producing XOS as expected. Since the barley grain sourced in breweries, and beer brewing processes varies from one brewery to the next, BSG with lower lignin, starch and protein could be targeted as they would be desirable to enable larger XOS yields.

5.2. Xylooligosaccharide production from streams generated by fractionation

After a xylanase treatment of 3 hrs at 50 $U.ml^{-1}$ on SEL, a maximum yield as a percentage of the theoretically available xylan of 51.9% and 40.5% was obtained for X2-6 and X2-3, respectively as a

result of the depolymerisation of X≥7, therefore adding value to the SEL stream. However, using freshly produced liquors immediately after hydrothermal pretreatment could significantly improve low molecular weight XOS yields by up to 29% for X2-6 and by 21% for X2-3.

At a xylanase treatment of 100 $U.g^{-1}$ on RSSE for 24 hrs, 41.1% of the available hemicellulose was solubilised mainly into XOS and Ar-OS with minimal monomer formation at a high solids loading (25% w/w). However, a much higher dosage of 1000 $U.g^{-1}$ was necessary to achieve a depolymerisation yield for X2-3 of 13.9% likely due to product inhibition. At a low solids loading (3% w/w) lower solubilisation and depolymerisation yields were achieved as a result of inefficient enzyme-substrate binding. Further investigation into optimisation of solids loading in the enzymatic hydrolysis of RSSE is required to balance the trade-off between product inhibition and enzyme-substrate binding.

5.3. Summary and recommendations

The fractionation and enzymatic hydrolysis process described in this study could serve as an additional strategy for BSG valorisation in a biorefinery process by the production of high-value XOS from two streams which contain significant amounts of hemicellulose. However, the process can be improved with further optimisation. Firstly, BSG with lower lignin, starch and protein should be sourced to obtain higher XOS yields after steam explosion. Secondly, freshly produced liquor should be made use of as the substrate for enzymatic hydrolysis of SEL to enable higher low molecular weight XOS yields. Finally, further investigation into solids loadings of RSSE is required for the purpose of mitigating mass transfer issues associated with high solids loading enzymatic hydrolysis.

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Appendix

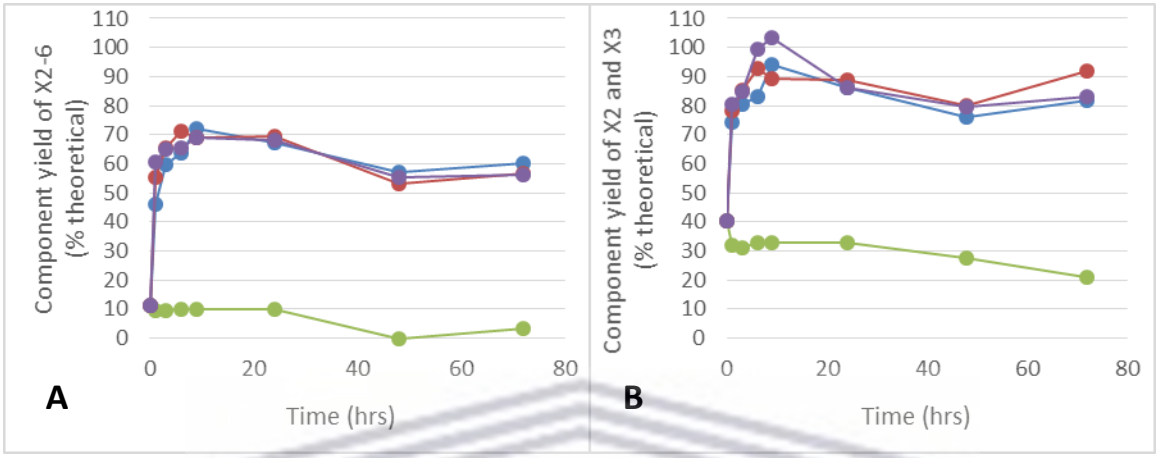

Figure A 1. XOS yield in preliminary experiments for enzymatic hydrolysis of SEL (● 0 U.g^{-1}; ● 50 U.g^{-1}; ● 100 U.g^{-1}; ● 200 U.g^{-1}). A: X2 and X3 yield. B: X2-6 yield. Yield calculated as a percentage of xylan available in SEL.

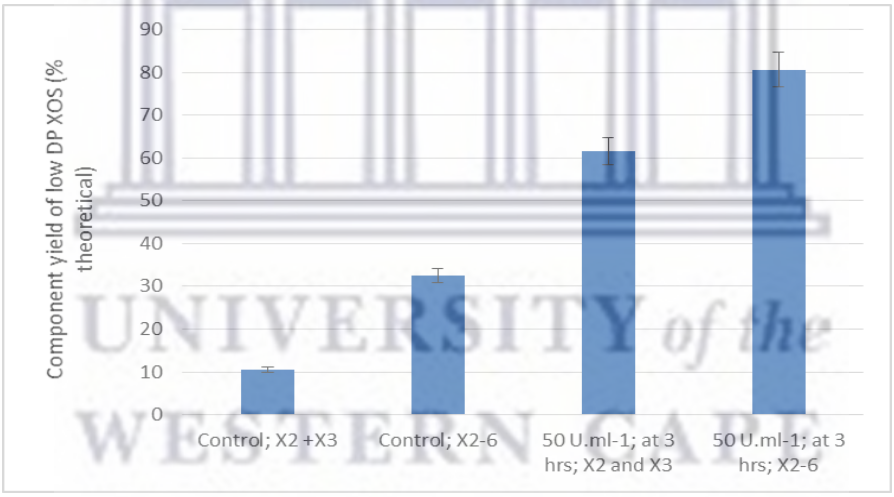

Figure A 2. XOS (DP 2 and 3; and 2-6) yield obtained during preliminary testing. Data represents the average of duplicate experiments with standard error bars.

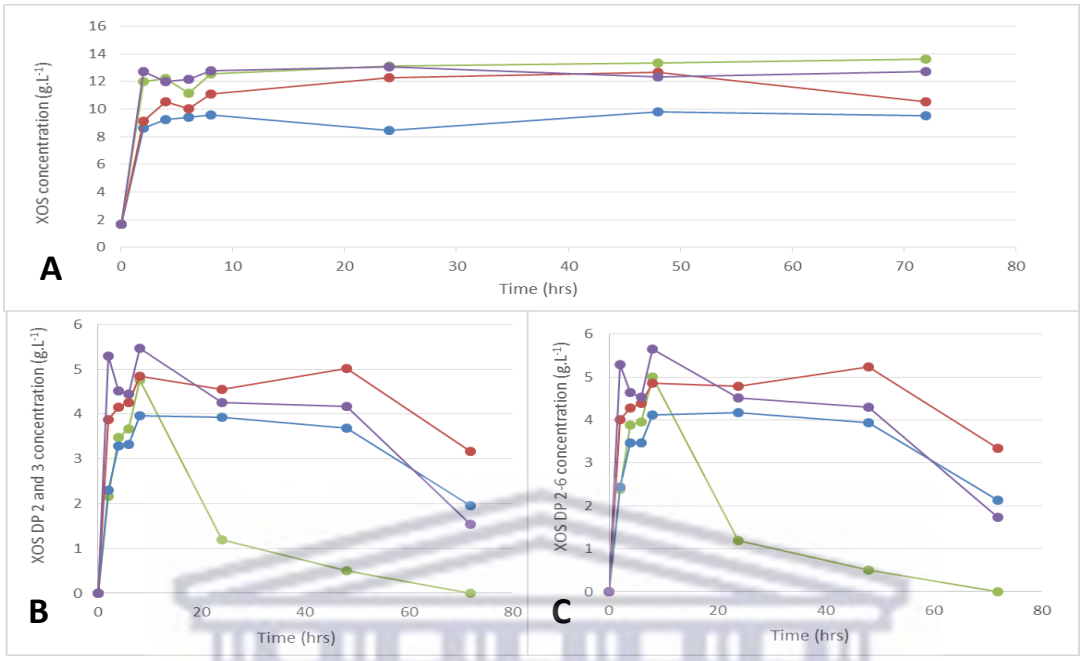

Figure A 3. Concentrations (g.L^{-1}) of XOS with varying degrees of polymerisation (DP) obtained during preliminary testing of high solids enzymatic hydrolysis (● 100 U.g^{-1}; ● 500 U.g^{-1}; ● 1000 U.g^{-1}; ● 1500 U.g^{-1}).

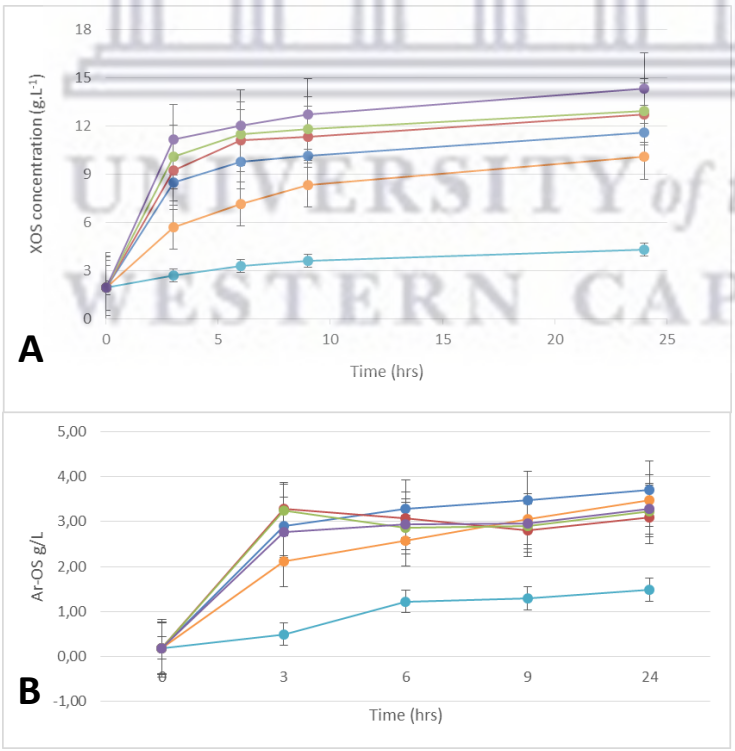

Figure A 4. Oligosaccharide concentration (g.L^{-1}) obtained during high solids enzymatic hydrolysis (● 0 U.g^{-1}; ● 25 U.g^{-1}; ● 100 U.g^{-1}; ● 500 U.g^{-1}; ● 1000 U.g^{-1}; ● 1500 U.g^{-1}). A: XOS concentration. B: Ar-OS concentration. Data represents average of triplicate experiments with standard error bars.

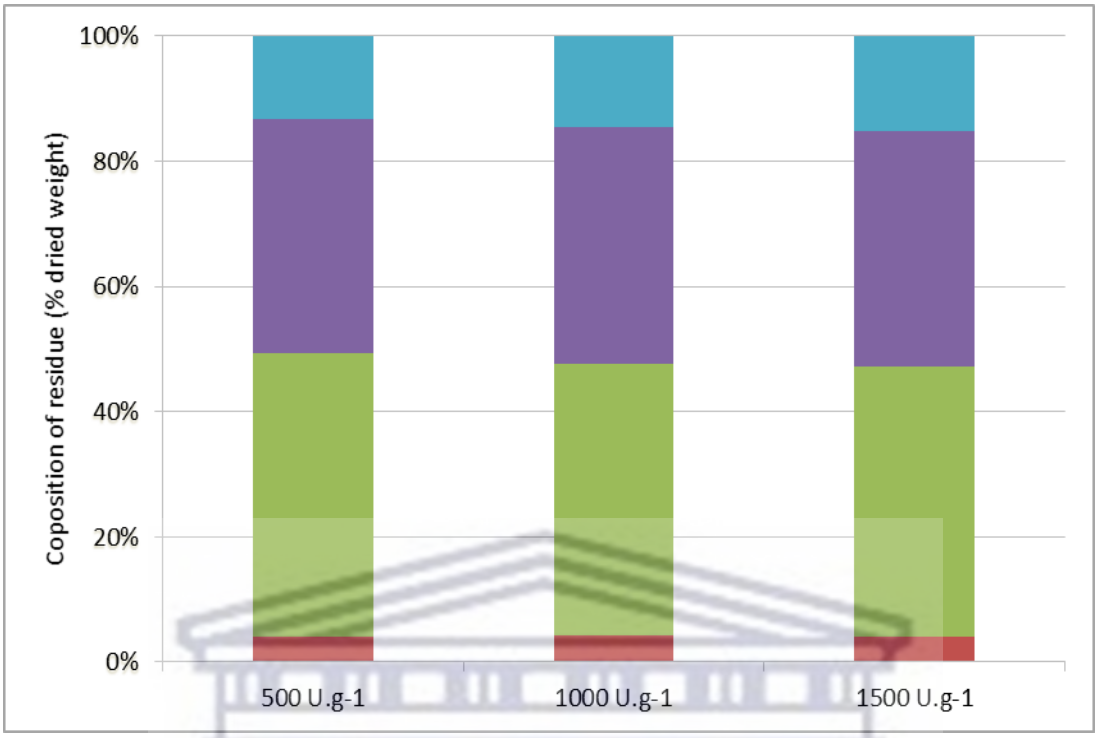

Figure A 5. Composition (% w/w) of residue after high solids enzymatic hydrolysis at various dosages after 24 hrs (▪ ash; ▪ lignin; ▪ cellulose; ▪ hemicellulose made up of xylan). Data showing average of triplicate experiments.

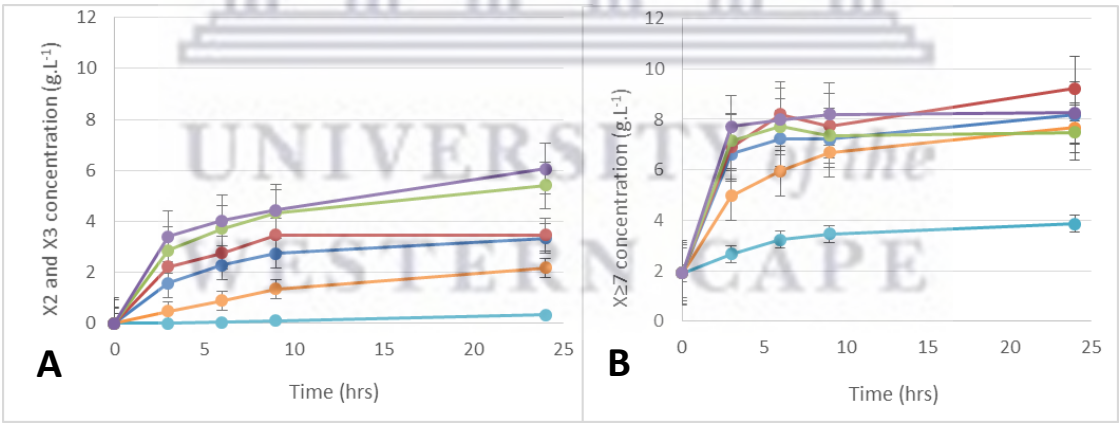

Figure A 6. XOS with varying degrees of polymerisation (DP) concentration (g.L^{-1}) obtained during high solids enzymatic hydrolysis (● 0 U.g^{-1}; ● 25 U.g^{-1}; ● 100 U.g^{-1}; ● 500 U.g^{-1}; ● 1000 U.g^{-1}; ● 1500 U.g^{-1}). A: X2 and X3. B: X≥7. Data represents average of triplicate experiments with standard error bars.

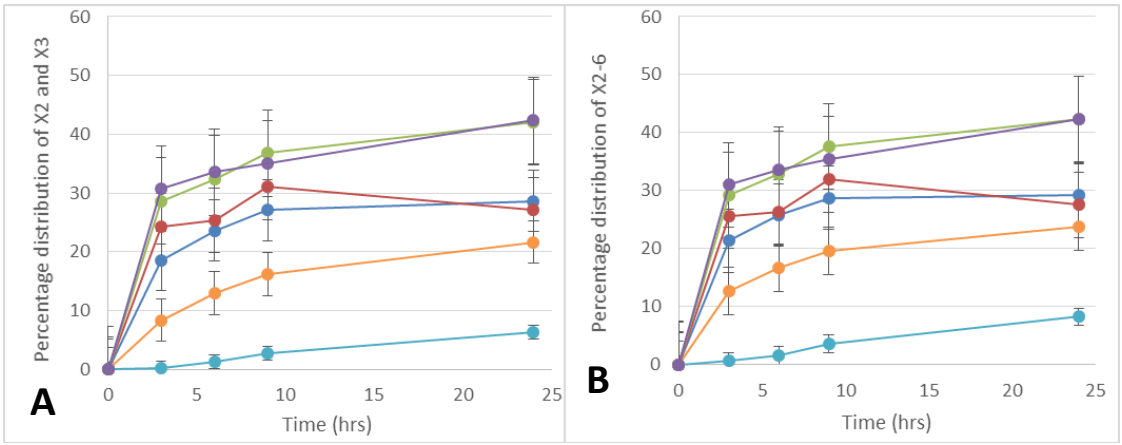

Figure A 7. Percentage distribution (%) of XOS with various degrees of polymerisation (DP) obtained during high solids enzymatic hydrolysis (● 0 U.g^{-1}; ● 25 U.g^{-1}; ● 100 U.g^{-1}; ● 500 U.g^{-1}; ● 1000 U.g^{-1}; ● 1500 U.g^{-1}). Calculated as a fraction of the total XOS. A: Percentage distribution of X2 and 3. B: Percentage distribution of X2-6. Data represents average of triplicate experiments with standard error bars.

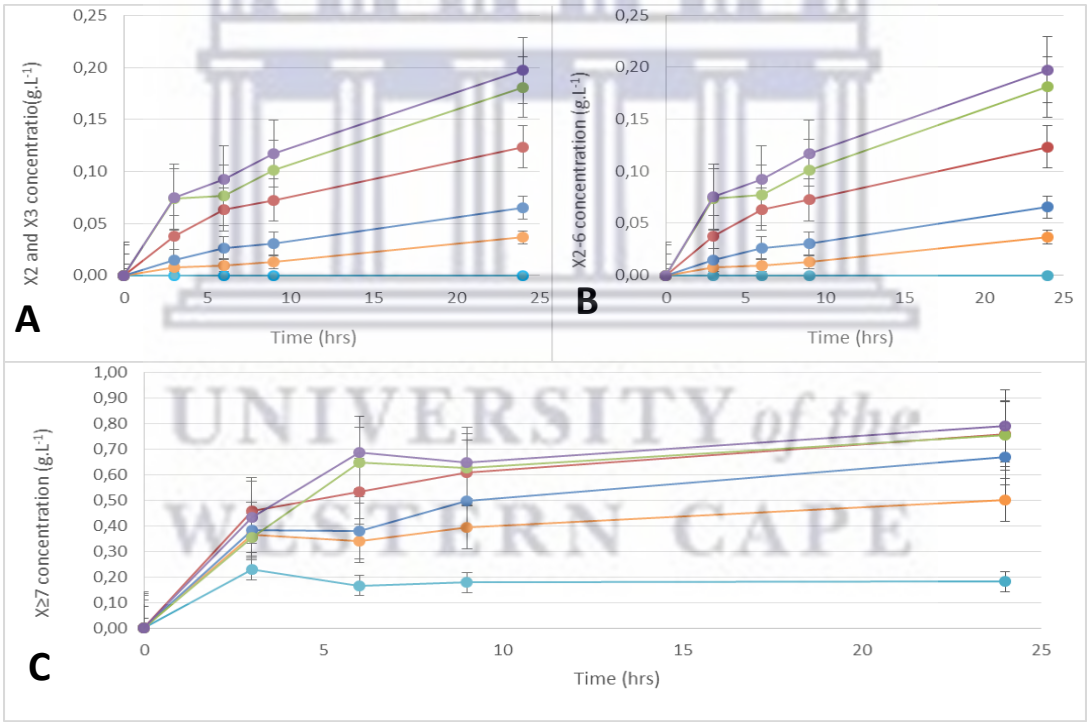

Figure A 8. XOS concentration (g.L^{-1}) at various degrees of polymerisation (DP) obtained by enzymatic hydrolysis in a 3% solids reaction (● 0 U.g^{-1}; ● 25 U.g^{-1}; ● 100 U.g^{-1}; ● 500 U.g^{-1}; ● 1000 U.g^{-1}; ● 1500 U.g^{-1}). Data represents the average of experiments in triplicate with standard error bars.