Dissecting the structure and inflammatory profile of lignocellulosic waste components for potential applications in sustainable materials

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

With an increasing demand for the development of biomaterials as an alternative to petroleum-based materials there has been great interest in utilising lignocellulosic waste, rich in cellulose and hemicelluloses including ß-glucans. The cell wall composition of plant waste, unprocessed and processed, for their potential use in the extraction of cellulose was investigated using sequential extraction and specific molecular probes including monoclonal antibody-based ELISA and FTIR. Epitope based analysis showed that hemp and tomato wastes were rich in hemicelluloses xylan and glucuronoxylan, whereas sugarbeet waste was richer in pectic polysaccharides. The effect of processing on the cell wall composition from raw hemp and tomato wastes to partially processed pulps and fully processed films was also analysed with hemicellulosic epitope levels reducing in both materials, leaving only traces of xylan, glucuronoxylan and xyloglucan and no ß-1,3-glucan in the final film product. The composition of two spent brewers' grain processing by-products was found to be concentrated in hemicellulose and protein compared to the raw waste. As hemicelluloses have the potential to be used in material applications involving direct contact with mammalian cells/tissue, the effect of these polysaccharides was analysed in an inflammatory cell model. The hemicelluloses xylan and xyloglucan were effective in inducing inflammatory signalling as well as the non-plant derived ß-1,3-glucans laminarin and CM curdlan. In conclusion, the processing of raw wastes to film products reduced the presence of hemicellulosic and ß-1,3-glucan epitopes, however the raw wastes themselves, most notably hemp and tomato, were good sources for extraction of hemicelluloses which have the potential to be reintroduced and utilised as biomaterials in various industries.

Key words: lignocellulosic waste, cell wall, cellulose, hemicellulose, ß-glucan, extraction, glycome profiling, FTIR, inflammation, secreted embryonic alkaline phosphatase (SEAP), sustainability, biomaterials

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Abbreviations

BC – Bacterial cellulose
EC – Ethyl cellulose
MFC – Micro-fibrillated cellulose
NFC – Nano-fibrillated cellulose
ELISA – Enzyme linked immunosorbent assay
mAbs – Monoclonal antibodies
LM – Leeds monoclonal
JIM – John Innes monoclonal

CM – Carboxymethyl
PRR – Pattern recognition receptor
PAMP – Pathogen-associated molecular pattern
TLR – Toll-like receptor
CR – Complement receptor
IL – Interleukin
TNF-α – Tumour necrosis factor α
SEAP – Secreted embryonic alkaline phosphatase
NF- kB – Nuclear factor kappa B
LPS – Bacterial lipopolysaccharide

Chapter 1 - Introduction

At present, petroleum-derived polymers remain widely used as materials with vast applications due to their favourable mechanical properties and low cost. However, as petroleum-derived polymers do not readily biodegrade, there has been increasing concern about the contribution of these materials to global environmental issues such as pollution due to waste disposal in landfills. There has therefore been an increasing demand for renewable and sustainable sources of polymers for the production of biomaterials (Ragauskas et al., 2006).

Biomass, composed primarily of cellulose, hemicellulose, pectins and lignin, is a renewable and carbon neutral source for the production of materials (Zhu et al., 2018). Cellulose, the most abundant and readily available polysaccharide in nature, has advantageous physical and mechanical properties that are exploited in vast applications including as film and packaging materials in the food and pharmaceutical industries (Klemm et al., 2005).

Pulp from wood is currently the main source of cellulose worldwide. However, the increased demand for wood as a source for extraction of cellulose has contributed to increased deforestation, another global environmental issue (Wong et al., 2021). Wood is also highly lignified, and therefore harsh chemical and mechanical treatments are required to isolate and purify cellulose. Agricultural wastes, such as residual hemp biomass leftover from CBD and THC production, are non-woody and contain a lower presence of lignin. Milder treatments are therefore required to extract cellulose fibrils, offering a more environmentally beneficial process than extraction from wood (Ates et al., 2020; Gaidukovs et al., 2021). Plant wastes and by-products are a cheaper, sustainable, and more easily processible source for the extraction of cellulose fibrils, hemicelluloses, and other cell wall polysaccharides (Voicu et al., 2021; Gaidukovs et al., 2021).

1.1. Plant Cell Walls

Cell walls are specialised forms of extracellular matrix that surround plant cells, as well as certain prokaryotes including bacteria and archaea, and eukaryotes fungi and algae (Cooper, 2000). The cell wall can be used to distinguish plant cells from mammalian cells. In plants, cell walls have multiple functions. Firstly, the wall has a structural role, determining the shape of the cell, as well as their organisation into plant tissues which influences the structure of the whole plant. The wall provides a physical barrier to cells, giving them strength and rigidity. The ability of the wall to withstand high stress levels can also help sustain the internal turgor pressure (Bacic et al., 1988; Zhang et al., 2021). The wall also has a role in

plant defence, providing a biological barrier, preventing pathogens entering and damaging the cell (Somerville et al., 2014). Finally, the wall has a metabolic role, containing a significant source of metabolisable energy for the plant, as well as mediating cell communication and transport (Caffall and Mohnen, 2009). This ultimately affects cell differentiation and plant growth, as well as the response of the cells to pathogen infection.

Plant cell walls are composed mainly of cellulose, hemicellulose, and lignin, although pectins and proteins are also present, with the proportions of each constituent depending on the cell type, its specific functions and its stage of development (Burton et al., 2010). The cell wall composition is continuously modified to accommodate cell development and changes in the environmental condition (Caffall and Mohnen, 2009). Plant cell walls have three distinct regions: the middle lamella, the primary cell wall, and the secondary cell wall. The middle lamella is an adhesive region constructed soon after mitosis, where adjacent cells adhere to one another. This region is rich in pectic polysaccharides. Following the deposition of the middle lamella, the primary cell wall is constructed and is continually deposited throughout cell growth and development. The primary cell wall consists of cellulose microfibrils, hemicelluloses, pectins and soluble proteins (Figure 1A). Secondary cell walls are deposited between the primary cell wall and plasma membrane once cell growth has stopped, however secondary cell walls are not developed in all cell types. The secondary cell wall consists of cellulose microfibrils, hemicelluloses and lignin (Figure 1B), with lignin strengthening the wall and protecting the cell from penetration by pathogens (Lack and Evans, 2005; Popper, 2008).

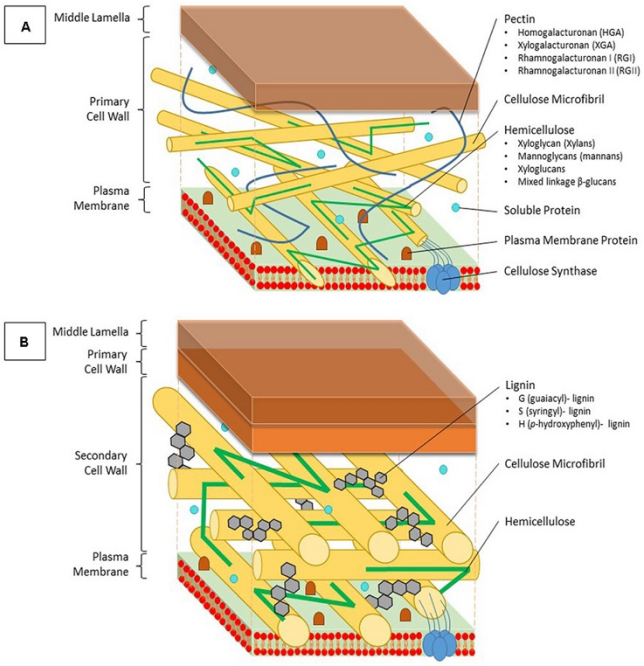


Figure 1. The primary and secondary cell wall structure and composition in plants. A, *the primary cell wall is the outermost wall surrounding the plasma membrane and consists mainly of cellulose microfibrils which can form a bound network with hemicelluloses including xylans, xyloglucans and ß-glucans. The primary cell wall also contains pectins which form a hydrated gel between the cellulose-hemicellulose network and soluble proteins.* ***B,*** *the secondary cell wall is located between the primary cell wall and plasma membrane. In the secondary cell wall the cellulose microfibrils are more orderly structured with lignin and hemicelluloses present in-between microfibrils. From Loix et al. (2017).*

Cell wall structure and composition differs in other organisms. For example, in yeast, the cell wall defines the cell shape during growth, mating, sporulation and pseudohyphae formation. The wall also gives the cell its osmotic integrity and presents glycoproteins on its surface to other yeast cells. The major components of the yeast wall are β-1,3;1,6-glucans, chitin and a variety of proteins, including mannoproteins. The components of the cell wall cross-link in several different ways to form higher-order complexes, with the degree of cross-linking varying during growth and development as well as in response to stress (Orlean, 2012).

The dynamic plant cell wall not only provides mechanical support, which is essential in the growth and development of plant cells but protect the plant cells from biotic and abiotic stresses. The cell wall structure and composition can also vary depending on the plant species, cell type and developmental stage (Bacic et al., 1988).

1.2. Cellulose

Cellulose, a β-1,4-glucan, is the main structural polysaccharide in plants and the most abundant and readily available carbohydrate in nature, which is traditionally extracted from plants and plant wastes for use in various industrial applications (Klemm et al., 2005). Cellulose often branches with hemicellulose and lignin, which requires harsh alkali and acid treatment to purify the cellulose fibrils (Sun, 2009). Cellulose consists of a long linear chain of glycosidic linked β-D-glucose units (Figure 2). The number of units can be as many as 12,000, but this depends on the origin of the cellulose (Lean, 2006). These chains can aggregate into bundles, forming microfibrils, which can be 10-25nm in diameter in plants (Lack and Evans, 2005).

Cellulose is completely insoluble in water but can be hydrolysed and dissolved by heating with a variety of ionic liquids, including hydrochloric acid, and caustic soda. Humans are unable to digest cellulose as the intestinal enzymes are incapable of hydrolysing the β-1,4 glycosidic bonds. Therefore, cellulose offers no nutritional value to humans, although promotes healthy bowel motility, offering increased protection against bowel cancer. Interestingly, horses and ruminants can digest cellulose, due to their auxiliary stomachs containing enzymes produced by microorganisms that can hydrolyse the glycosidic bonds (Lean, 2006).

Although plants are the major source of cellulose, some aerobic bacteria are able to produce cellulose called bacterial cellulose (BC). BC offers unique physiochemical properties compared to cellulose from plants which gives potential for novel applications in the pharmaceutical, material and food industries (Esa et al., 2014). BC has the same β-1,4-glucan structure as conventional cellulose but the microfibrils are smaller, with a thickness of 3-8nm (Tabuchi, 2007). The degree of polymerisation is also lower in BC at 2000-6000, compared to 13,000 in conventional plant cellulose (Park et al., 2009). BC is a chemically pure form of cellulose without bound hemicelluloses, lignin, and pectin, so acid and alkali treatments are not required. The crystallinity and mechanical strength of BC is higher than that of conventional cellulose, giving potential for use in industrial applications.

1.3. Hemicelluloses

Hemicelluloses are a group of cell wall polysaccharides that consist of glycosidic linked ß-1,4 glucose, xylose or mannose units. Although the monosaccharide units differ between hemicelluloses, the equatorial configuration between C1 and C4 is the same within the backbone, giving the hemicelluloses similar structures (Scheller et al., 2010). Hemicelluloses are branched polysaccharides and have shorter chain lengths than cellulose, with a degree of

polymerisation between 70 and 200 (Ragauskas et al., 2006). The hemicelluloses include xylans, xyloglucans, mannans and ß-1,3-glucans and are components of the cell wall in all plants on earth. Mixed-linkage ß-1,3;1,4-glucans, which are also classed as hemicelluloses, are only present in the cell walls of some plants including cereals and grasses (Lazaridou et al., 2007).

Xylans, the most abundant of the hemicelluloses in land plant cell walls, are a diverse group of polysaccharides. Xylans consist of a ß-1,4-linked xylose backbone however they can exist in multiple structures with short glycosyl side chains in different proportions linking to the O-2 and/or O-3 of the xylose residues (Ebringerova and Heinze, 2000). Glucuronoxylan, part of the group of xylans, contain side branches of 4-O-methyl glucuronosyl, α-1,2-linked to xylose residues in the backbone. Glucuronoxylans are the major non-cellulosic polysaccharide in the secondary cell walls of dicots, whereas in commelinid monocots, xylan is the dominant non-cellulosic polysaccharide. Xylan and glucuronoxylan can also contain residues of arabinose linked to the xylose backbone. These xylans are termed arabinoxylan and glucuronoarabinoxylan (Scheller et al., 2010). Many xylans contain acetyl groups which can affect the physical and mechanical properties of this polysaccharide group (Ebringerová, 2005; Silva et al., 2011).

Xyloglucans consist of a ß-1,4-linked glucose backbone with α-1,6-linked xylosyl side residues. Xyloglucans are formed from repeating units of 4 residues, however the branching on these residues varies. A one-letter code can be used to denote the different branching within the repeating units (Fry et al., 1993). 'G' represents an unbranched glucose residue whereas 'X' represents a branched glucose-xylosyl residue. The two common repeating oligosaccharides are XXXG and XXGG however other repeating oligosaccharides can occur containing different side chains with residues such as galactose and fucose. Xyloglucan is the major hemicellulose present in the primary cell walls of dicots and non-commelinoid monocots. The branching of xyloglucans can affect their solubility with less branched xyloglucans being less soluble (Scheller et al., 2010).

Mannans consist of a ß-1,4-linked backbone containing mannose. In mannans and galactomannans the backbone contains only mannose residues, however in glucomannans and galactoglucomannans the backbone contains both mannose and glucose residues in a non-repeating pattern. Galactomannans and galactoglucomannans contain α-1,6-linked galactosyl side groups (Scheller et al., 2010).

The main biological function of the hemicelluloses is strengthening the primary and secondary cell walls by cross-linking with cellulose, however in some walls they can also cross-link with lignin. The binding strength to cellulose depends on the cell type and type of hemicellulose (Scheller et al., 2010). The role of hemicelluloses in the cell wall also enables the plant cells to change shape during growth until cell maturation, where they help to retain the plant cells final shape (Hayashi and Kaida, 2011).

1.4. Pectins

Pectins are structural polysaccharides consisting mainly of glycosidic linked α-1,4-galacturonic acid units, however variations in structure and composition occur. The exact structure and chemical composition of pectin remains under debate due to the complexity of the polysaccharide (Lara-Espinoza et al., 2018). Pectic polysaccharides include homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RGI) and

rhamnogalacturonan II (RGII). These polysaccharides are not separate molecules within the cell wall region, they exist as covalently linked domains (Harholt et al., 2010). The total pectin content and ratios between the pectic polysaccharides in the domains are variable and depend on the species, tissue type and environmental conditions (Zablackis et al., 1995), however HG is typically the most abundant of the polysaccharides (Mohnen, 2008). Pectins are commonly located in the primary cell wall and middle lamella of plants and can compose one third of the dry substances of dicots and some monocots (Willats et al., 2001).

HG consists of a linear chain of α-1,4-galacturonic acid residues. These residues can be methylated at C-6 and acetylated at O-2 or O-3 at various degrees (Mohnen, 2008). XGAs are HGs with ß-1,3-linked xylosyl side residues at O-3, and occasionally O-4, on the galacturonan backbone (Zandleven et al., 2006). The degree of xylosidation in xylogalacturonan can vary between 40 and 90%, depending on the plant source (Mohnen, 2008). The galacturonan backbone can be methylated at various degrees independently of the xylosyl side residues (Schols and Voragen, 1996). RGI is the only pectin type not to contain a pure galacturonan backbone. RGI contains repeating units of α-1,2-galacturnoic acid and α-1,4-rhamnose in the backbone (Harholt et al., 2010). Branches containing neutral sugars such as arabinose and galactose occur predominantly at O-4 on rhamnose residues, forming arabinan, galactan and arabinogalactan side chains (Maxwell et al., 2012). RGII consists of a linear α-1,4-galacturonic acid residue backbone with side residues of rhamnose and galactose, alongside rarer sugar residues at O-2 or O-3 on the galacturonan backbone. RGII is structurally the most complex pectic domain and is conserved across many plants (Lara-Espinoza et al., 2018).

Pectins can be classified into two groups based on their degree of esterification with methyl groups. High methoxyl (HM) pectins have over 50% of their carboxyl groups esterified and low methoxyl (LM) pectins have less than 50% of their carboxyl groups esterified. This classification affects the gelling mechanism of pectins and differs between HM and LM pectins (Kuuva et al., 2003). Pectins can also be ferulated, with ferulic acid ester linkages forming at O-2 of arabinose residues and O-6 of galactose residues in RGI (Ralet et al., 2005). The presence of ferulic acid residues allow the cross-linkage of polysaccharide chains which can impact on the physiology of the cell wall (Ishii, 1997).

Pectins are present mainly in the primary cell wall and middle lamella of plants and are often associated with cellulose, hemicellulose, and lignin (Harholt et al., 2010). Pectins are often deposited in the initial stages of primary cell wall growth with involvement in the development of plant cells (Parre and Geitmann, 2005). They provide turgidity to cells and aid resistance to harsh environmental conditions such as drought and low temperatures. Pectins have a role in the structure and firmness of plant tissue, contributing to the mechanical resistance of the cell wall and intercellular adhesion (Northcote, 1972).

1.5. ß-glucans

β-glucans are a type of polymer characterised by the presence of glycosidic linked β-D-glucose. These polysaccharides naturally occur in the cell walls of plants, bacteria, fungi, yeasts, algae and lichens, with distinct differences in their molecular structure which can also influence their physiological functions (Volman et al., 2008). Examples of β-glucans are cellulose, callose, mixed linkage β-glucans and xyloglucan (Cosgrove, 2005).

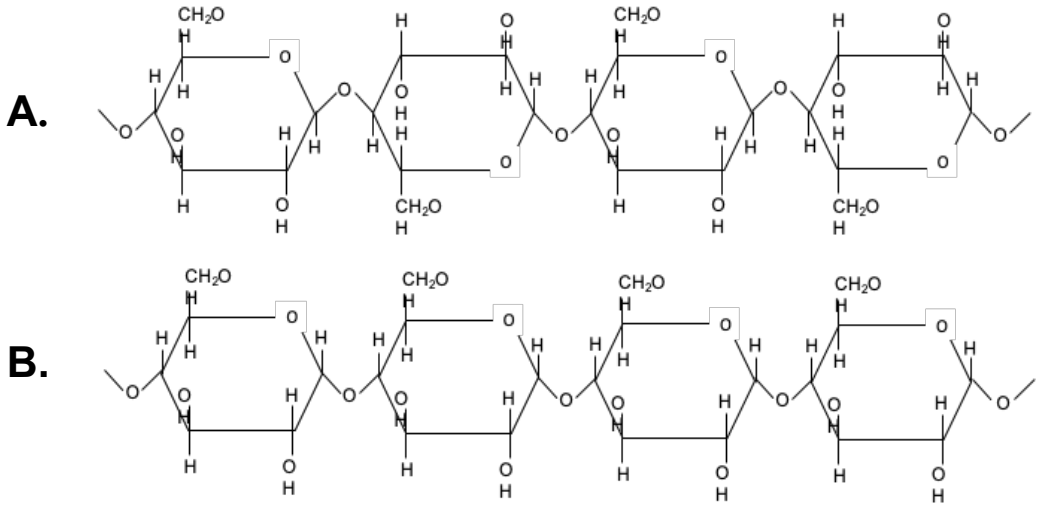


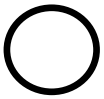
Figure 2. Backbone chain structures of two plant β-glucans*. **A,** part of a cellulose chain, showing the ß-1,4-glycosidic linkages between each ß-D-glucose residue. **B,** part of a callose chain, showing the ß-1,3-glycosidic linkages between each ß-D-glucose residue.*

β-glucans are also present in human and animal diets as part of the endosperm cell walls in cereals such as oats and barley. These β-glucans exist as mixed linkage β-1,3;1,4-glucans (Lazaridou et al., 2007). Dietary β-glucans have been shown to modulate the immune response which can be beneficial and increase the resistance against invading pathogens (Volman et al., 2008). Dietary β-glucans can also reduce blood glucose and cholesterol concentrations attenuating the risk for diabetes and cardiovascular disease. β-glucans have displayed antioxidant properties and scavenge reactive oxygen species, thereby reducing the risk of multiple diseases including cancer. The abundance of beneficial bacteria and metabolic activity of the gut microbiome can be stimulated by dietary β-glucans, which not only enhances the functioning of the gastrointestinal tract but reduces inflammation and therefore the risk of colon cancer (Ciecierska et al., 2019).

β-1,3-glucans are a type of ß-glucan with linkages between the hemiacetal oxygen at C-1 and C-3 on the next glucose residue (Figure 2). They assume different names depending on their origin and structure (Table 1). The simplest β-1,3-glucans are linear and unbranched, such as curdlan, which is extracted from *Alcaligenes faecalis* (Stone, 2009). β-1,3-glucans can also occur as branched β-1,3;1,6-glucans. These branches can be branch-on-branch, such as in yeast and fungal cell walls (Bacic et al., 2009) or cyclic such as in the bacteria, *Rhizobium loti* (Estrella et al., 2000). Most β-1,3-glucans, including linear β-1,3-glucans and branched β-1,3;1,6-glucans, tend to form helical chain shapes due to the orientation and repetition of the glycosidic linkages between residues giving a twist (Gidley and Nishinari, 2009).

Table 1. The structure of β-1,3-glucans from various natural sources*. Adapted from Seo et al. (2019).*

Origin/Common names	Structure	Description
Bacterial: Curdlan	———	Linear β-1,3-glucan backbone (unbranched)

Bacterial: From the *Rhizobiaceae* family		Cyclic β-1,3;1,6-glucan
Fungal and algae: Pachyman and Laminarin		Linear β-1,3-glucan backbone with short β-1,6-linked branches
Yeast: *Saccharomyces cerevisiae*		Linear β-1,3-glucan backbone with long β-1,6-linked branches
Plant: Callose		Linear β-1-3-glucan backbone with relatively few β-1,6-linked branches

The β-1,3-glucans exhibit a range of solution and gel properties. These properties depend on the chemical structure and molecular size of the β-glucan, their biological origin, and the extraction method of the β-glucans (Gidley and Nishinari, 2009). Many β-1,3-glucans are water soluble, particularly those with a low degree of polymerisation such as the branched β-1,3;1,6-glucans laminarin, however some can only be dissolved in aprotic solvents such as formic acid. Linear β-1,3-glucans are the least soluble in water (Gidley and Nishinari, 2009), but can be dissolved in dilute bases such as NaOH (Yotsuzuka, 2001). Dilute solutions of β-1,3-glucans have a high viscosity which is due to the stiffness of the polysaccharide chain (Gidley and Nishinari, 2009).

β-1,3-glucans have various biological functions in nature. In the fungal cell wall, β-1,3;1,6-glucans have a structural role, aiding defence against invading pathogens. They also help prevent dehydration and provide osmotic stability. When β-1,3;1,6-glucans are stored inside the fungal cell they can act as a source of metabolisable energy during development (Dalonso et al., 2015). The fungal β-1,3;1,6-glucans also contribute to fixing cell wall proteins and can be associated with chitin. This association has been shown to protect the fungal cell wall from penetration by glucanases (Teparić and Mrša, 2013).

In plants, β-1,3-glucans have a structural role in the cell wall and act as a matrix for the deposition and fixing of various other cell wall components but also have important regulatory functions (Parre and Geitmann, 2005). Callose is a β-1,3-glucan cell wall polymer that can be found in higher plants and multicellular green algae (Scherp et al., 2001). Callose consists of a linear chain of glycosidic linked β-D-glucose units (Figure 2). Callose is involved in multiple fundamental biological processes which are highlighted in Figure 3. In plants, callose is localised mainly around plasmodesmata which are intercellular networks responsible for intercellular communication and transport (Maule et al., 2011). The capacity of callose to complex with aniline blue fluorochrome can be used to identify and quantify the β-1,3-glucan at various locations in plant tissues (Zavaliev and Epel, 2015).

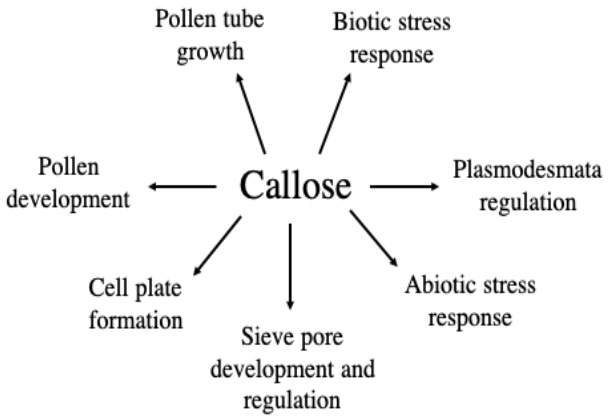


Figure 3. The roles of callose in higher plants*. Callose is involved in various aspects of plant growth and development and response to stresses. Adapted from Chen and Kim (2009).*

Callose has a minor role in developing cell walls but has important regulatory functions in plant defence, intercellular signalling, and organogenesis (Ellinger and Voigt, 2014; Amsbury et al., 2018). Reversible accumulation of callose occurs at the neck region of the plasmodesmata in response to wounding and exposure to various biotic and abiotic stresses (Stone, 2009), with established correlations between increased callose accumulation and decreased symplastic transport (Vaten et al., 2011). Increased accumulation of callose reduces the aperture of the plasmodesmata which restricts the size of macromolecules that can pass cell-to-cell, inhibiting intercellular signalling and transport. Degradation of callose increases the aperture of the plasmodesmata, allowing intercellular signalling and transport to resume (Amsbury et al., 2018).

Callose is also involved in cell plate formation during cell division and in the development and regulation of sieve pores which connect the phloem vascular system (Barratt et al., 2011). Under normal unstressed conditions, callose is present in the sieve plate at a basal level, however when the plant is subjected to stress, callose accumulates rapidly, plugging the sieve pores (Xie and Hong, 2011). Callose is present in large amounts in pollen and is involved in cell wall resistance to circumferential tension stress in pollen tubes. Reduced amounts of callose in pollen tubes reduces cellular stiffness and increases viscoelasticity highlighting the load bearing capacities of the polymer. Callose in pollen also controls cell turgor-equilibrium (Parre and Geitmann, 2005).

1.6. Extraction of cellulose and hemicelluloses from biomass

Agricultural wastes containing lignocellulosic biomass are in plentiful supply. The cell walls of lignocellulosic biomass from agricultural wastes contain cellulose as the major component (35-65%) but also contain hemicelluloses (20-45%) and lignin (10-25%) (Sarkar et al., 2012). These wastes therefore offer a low-cost, renewable, and biodegradable source of cellulose and other valuable hemicelluloses and ß-glucans (Abdeshahian et al., 2020).

The major issue is the lack of established protocols for the purification of the different polysaccharides from the lignocellulose mixtures as the hemicelluloses and lignin can be strongly bound to the cellulose fraction in the biomass. The extraction and purification method for cellulose, hemicelluloses and lignin from agricultural wastes is also important as different methods can affect the yield, purity, structure, rheological properties, molecular weight, and the functional properties of the resulting fractions (Ahmad et al., 2012).

Single cellulose microfibrils can aggregate into cellulose macrofibrils which have varying degrees of crystallinity with crystalline, sub crystalline and non-crystalline chains. Therefore, different types of native cellulose fibrils can be obtained by processing using various mechanical and chemical treatments. Individual microfibrils obtained from extraction are termed either "micro-fibrillated cellulose" (MFC) or "nano-fibrillated cellulose" (NFC), and have a diameter of between 10 and 100 nm. MFCs can be modified following treatments to enhance their physical and mechanical properties, further improving their versatility (Ten and Vermerris, 2013).

Several approaches are now in place to isolate cellulose from lignocellulosic waste, however many of the extraction methods require a pre-treatment of the waste to separate the cellulose fraction from the hemicellulose and lignin (Mulyaningtyas and Sediawan, 2019). Enzymatic or chemical pre-treatments can be used. The pre-treatment not only separates the cellulose fraction but also modifies the structure, crystallinity, and polymorphism of the cellulose (Shi et al., 2011). Enzymatic pre-treatment with cellohydrolase targets the hydrogen bonds that link the cellulose microfibrils (Osong et al., 2016), reducing the degree of crystallinity (Abdeshahian et al., 2020). This treatment increases the glucose released from hydrolysis which can be beneficial with applications in bioethanol production (Maurya et al., 2015). The pre-treatment process also reduces the amount of energy required in the extraction process (Alvira et al., 2010), enhancing the economic viability of the process (Li and Chen, 2020).

There has also been interest in developing effective methods to obtain hemicellulose of high purity and yield. Current methods include alkaline extraction, liquid hot water extraction, ultrasonication-based extraction, and steam explosion-based extraction. As the hemicelluloses contain various groups of polysaccharides with different chemical and physical structures, fractionation and purification are required to acquire more homogeneous hemicellulosic fractions. Fractionation and purification methods include ethanol precipitation, ammonium sulfate precipitation, iodine complex precipitation and column chromatography (Peng et al., 2012). Various membrane fractionation technologies including ultrafiltration have received great interest as sustainable fractionation and purification methods for lignocellulosic products, as most other extraction methods of hemicelluloses use more severe pre-treatment and extraction conditions (Huang et al., 2008).

The extraction and utilisation of β-glucans from lignocellulosic and agricultural-food waste not only offers a low-cost source of β-glucans but offers a potential sustainable and biodegradable source for novel materials with application potentials in various industries including those described in previous sections.

1.7. Industrial applications of cellulose and hemicelluloses

Following the extraction of cellulose and other cell wall polysaccharides from lignocellulosic biomass, the production of functional materials with properties similar to petroleum-derived materials are required. Cellulose has been widely used in industry, maybe most notably in paper and packaging applications (Elmira et al., 2010). The applications of cellulose are vast, with utilisation in the food and biomedical industries alongside others, thus demand has increased (Zhang and Chang, 2011). Aside from cellulose, other cell wall components such as hemicelluloses and ß-glucans are also valuable biopolymers for utilisation in industry (Zhu et al., 2020). ß-1,3-glucans have received particular interest as they have shown to have multiple

beneficial effects on aspects of human health, including immunological effects. Although the health-related effects of the plant ß-1,3-glucan, callose, in isolation, is unknown, some research has been done in wall ß-1,3-glucans from other natural sources including curdlan and pachyman extracted from bacterial and fungal walls respectively (Vetvicka and Sima, 2017).

1.7.1. Applications of cellulose and hemicelluloses as functional food ingredients

Cellulose from wood-pulp can be used as a stabiliser in the food industry. An investigation carried out by Murray et al. (2011a), created complex mixtures of cellulose derived from wood-pulp with ethyl cellulose (EC). These complexes were shown to act as surface active particles offering stability to foams and emulsions. Murray et al. (2011b) highlighted the importance of the EC: cellulose ratio in these mixtures. If the ratio of EC: cellulose is too high then particle aggregation occurs, forming a gel, which is unfavourable for use as a stabiliser in this case. If the ratio is too low, then the particles are unable to adsorb onto the bubble surface. Although the cellulose complexes were shown to act as successful stabilisers, it was suggested that combining these particles with proteins would increase the stabilisation potential further (Murray et al., 2011a).

As a dietary fibre, BC is "generally recognised as safe" by the Food and Drug Administration (FDA) so can be utilised in the food industry (Park et al., 2009; Shi et al., 2014). BC has great potential as a food ingredient due to its high purity, with no bound impurities as in untreated conventional cellulose. BC also can form a variety of textures shapes including particles, filaments and films and has the capability to acquire colour and flavour changes (Shi et al., 2014).

The applications of BC as a functional food ingredient are diverse. BC can improve the rheology of food products by thickening, gelling, and water-binding. The properties and structure of BC enable stabilisation of food in different temperatures, pH's, and freeze-thaw conditions, even at low concentrations. Other applications involve BC producing low calorie food products and low-cholesterol products (Ullah et al., 2016).

Thammakiti et al. (2004) investigated the potential of β-1,3;1,6-glucans from *Saccharomyces cerevisiae* (yeast) as a functional food ingredient. Samples that were homogenised prior to analysis had a greater β-glucan content than non-homogenised samples. The homogenisation also increased the viscosity, water-holding capacity, and emulsion stabilisation capacity of the resulting β-glucan preparations. These properties were also shown to be higher in the β-glucan preparations from homogenised brewers' yeast than in preparations from commercial baker's yeast, although the oil-binding capacity was very similar. It was therefore concluded that the brewers' yeast β-glucans have the potential to be utilised as stabilisers in the food industry (Thammakiti et al., 2004).

1.7.2. Effects and opportunities for using ß-glucans to improve human health

There has been great interest into the effect of biopolymers on human health over the last decade, with particular focus on the immunological effects of the β-glucans. Various other aspects including their effects on respiratory and cardiovascular health have also been investigated, and in some cases adverse effects have been observed.

1.7.2.1. Immunomodulatory effects of ß-glucans

The ability to detect pathogens is crucial for the defence and survival of multicellular organisms including vertebrates and invertebrates. The presence of pattern recognition receptors (PRRs) allows infectious foreign structures to be recognised. The structures that bind and are recognised by these PRRs are termed pathogen-associated molecular patterns (PAMPs) (Muthuramalingam et al., 2019). Upon recognition and subsequent binding of PAMPs such as β-1,3-glucan, an innate immune response is triggered, which eliminates foreign molecules via phagocytosis, protecting the host from invasion (Brown and Gordon, 2005).

β-1,3-glucans can act as immunostimulants, inducing trained immunity in the host immune cells (Brown et al., 2003). β-glucans are not produced by mammalian cells which means they can be recognised as PAMPs by PRRs on the surface of immune cells such as monocytes, macrophages, neutrophils, and dendritic cells. When the β-1,3-glucan binds to the PRRs, a rapid immune response is initiated with immune cells secreting cytokines, B cells and T cells, components of adaptive immune function (Figure 4), which aids the production of a more effective immune response when the host is exposed to a certain pathogen a second time (Castro et al., 2020). Brown et al. (2003), Chan et al. (2009) and Mueller et al. (2000) have reported Dectin-1, complement receptor 3 (CR3) and toll-like receptors (TLR) as being the most important PRRs for β-1,3-glucans.

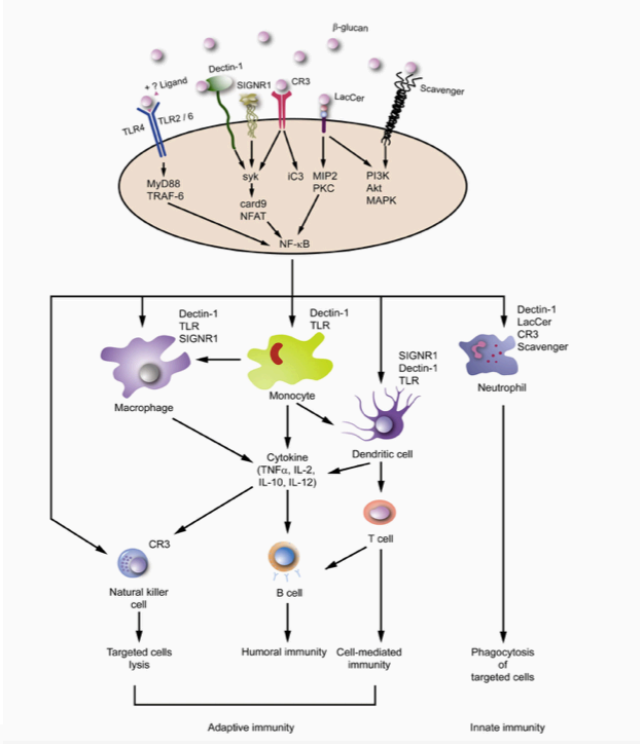


Figure 4. Overview on β-glucan induced immune activation. *β-glucans can bind to various receptors on immune cells either individually or in combination with other ligands. The recognition and subsequent binding of the β-glucans to the receptors activates signalling via different immune cells including macrophages, monocytes, dendritic cells, and neutrophils. β-glucans can induce both the innate and adaptive immune responses with the corresponding receptors, immune cells and signalling cascades listed. From Chan et al. (2009).*

Various physicochemical parameters including molecular weight, solubility and biochemical composition influence the biological activity and immunological properties of the β-1,3-

glucans (Soltanian et al., 2009). The characteristics of the immune response also depend on the cell types and the receptors involved. (Camilli et al., 2018) reported that soluble and particulate (insoluble) yeast β-glucans stimulated the immune system via different receptors and subsequent signalling cascades. The particulate β-1,3;1,6-glucans were recognised by Dectin-1 which subsequently led to phagocytosis by dendritic cells and macrophages via the Dectin-1 receptor pathway. This induced T-cell differentiation into Th1-cells and cytokine release which are components of the adaptive immune system. Soluble β-glucans were able to bind to and activate the CR3 receptor, which initiates a complement system mediated immune response involving specific antibodies.

Multiple studies investigating the effect of yeast β-1,3-glucans on the immune system have been carried out. Huang et al. (2012) reported that yeast β-1,3-glucans were recognised by the PRR, Dectin-1, which are expressed on phagocytes. The subsequent binding of the β-1,3-glucan particles activated the complement cascade, which facilitated the phagocytosis of the glucans both in vitro and in vivo in mice. Kankkunen et al. (2010) also identified that particulate yeast β-1,3-glucans initiated an interleukin-1β (IL-1β) mediated cellular response via Dectin-1 signalling in human macrophages. Upon oral administration, the yeast β-1,3-glucans have been shown to be taken up by macrophages found in the upper intestinal lymphatic tissue via the Dectin-1 receptor, and transported to the spleen, lymph nodes and bone marrow. Macrophages within the bone marrow degraded the large β-1,3-glucans into smaller soluble β-glucan fragments which were released. Upon pathogenic challenge the antimicrobial and inflammatory response was more efficient compared to a control (Castro et al., 2020).

Zymosan, a cell wall particulate β-1,3-glucan from yeast, has been the focus for multiple studies. Zymosan can activate phagocytes which leads to the destruction of invading pathogens by phagocytosis (Brown et al., 2002). Zymosan can also stimulate macrophages to secrete cytokines including interleukin (IL)-2, IL-10 and IL-12 and immunomodulators, which activate adaptive immunity (Brown et al., 2002; Sato et al., 2003; Young et al., 2004; Brown, 2006; Du et al., 2015). Other fungal β-glucans including β-1,3;1,6-glucans derived from *Candida albicans* and *Trametes versicolor* have been reported to also induce trained immunity in monocytes through TLRs including TLR-2 and TLR-4 (Saeed et al., 2014; Novakovic et al., 2016; Mitroulis et al., 2018).

Not only β-1,3;1,6-glucans from fungi and yeast are inducers of trained immunity but β-1,3;1,4-glucans from oat (Pan et al., 2020). Oat-derived β-1,3;1,4-glucans induced trained immunity via metabolic reprogramming. The oat β-glucans bound to TLR-2 and TLR-4 ligands on monocyte-derived macrophages, which increased the production of Tumour Necrosis Factor-α (TNF-α) and IL-6, pro-inflammatory cytokines that are involved in the immune response. An upregulation of mRNA expression was also found upon activation of the macrophages. It has previously been reported that β-1,3;1,6-glucan induced trained immunity is mediated by metabolic processes including glycolysis and the tricarboxylic acid cycle (TCA cycle) (Saeed et al., 2014; Novakovic et al., 2016). Upon the oat-derived β-1,3;1,4-glucan stimulating the TLR 2 and 4 ligands, multiple key enzymes in the glycolytic pathway and TCA cycle were significantly upregulated. When these enzymes were inhibited, the production of TNF-α and IL-6 decreased. This highlighted that oat-derived β-glucans can induce trained immunity (Pan et al., 2020).

1.7.2.2. Other applications of ß-glucans in human health

β-1,3-glucans have also shown beneficial effects on general human health. With Covid-19 causing a global pandemic, there has been recent interest in the role of β-glucans in reducing the severity of SARS-Coronavirus symptoms. The induction of trained immunity can be targeted to the respiratory system to reduce the severity of respiratory infections. A study conducted by Dharsono et al. (2019), found that yeast β-1,3;1,6-glucans decreased the severity of the physical symptoms of upper respiratory tract infections compared to a placebo, as well as successfully reducing the systolic and diastolic blood pressure of the participants. It was later suggested by Geller and Yan (2020), that the use of β-glucans could be implemented in Covid-19 patients, as those requiring intensive care treatment with the most severe symptoms have been shown to have significantly higher blood pressures than those not requiring intensive care treatment (Huang et al., 2020).

Dietary β-glucans have been shown to reduce the concentration of cholesterol in the blood. Reyna-Villasmil et al. (2007) reported that oat β-1,3;1,4-glucans improved the lipid profile in overweight males with diagnosed mild to moderate hypercholesterolemia. The high-density lipoprotein (HDL) plasma concentration significantly increased and low-density lipoprotein (LDL) plasma significantly decreased.

Dietary oat and barley β-1,3;1,4-glucans have also been shown to modulate the glucose and insulin responses in humans. An investigation conducted by Behall et al. (2006), found that a treatment involving the consumption of 5.8 g β-1,3;1,4-glucans /tolerance significantly lowered the glucose and insulin responses compared a control in men with mild insulin-resistance. The β-1,3;1,4-glucans have been found to alter the properties of chyme in the upper gastrointestinal tract which effects nutrient absorption, gut motility and gastric emptying, resulting in a lower GI and insulin response. β-1,3;1,4-glucan intake can therefore aid the control of diabetes (Daou and Zhang, 2012).

Antioxidant activity of β-1,3-glucans have been reported. Kao et al. (2012) isolated a low molecular weight β-1,3-glucan from the fruiting bodies of *Ganoderma lucidum*. The fraction was shown to reduce H_2O_2-induced cell death and decreased the production of H_2O_2-induced intracellular reactive oxygen species (ROS). It was concluded that isolated β-1,3-glucans from fruiting bodies could be used as a food supplement source of antioxidants for health maintenance.

1.7.2.3. Adverse effects of ß-glucans

β-glucans have displayed many positive health effects, however previous studies have also identified adverse effects of β-1,3-glucans including contribution to asthma (Rylander and Lin, 2000; Maheswaran et al., 2014), and other allergenic diseases such as Japanese cedar pollinosis (Kanno et al., 2021). Toxic effects on the respiratory system (Rand et al., 2010), and lethal toxicity when combined with certain non-steroidal anti-inflammatory drugs (NSAIDs) have also been reported (Yoshioka et al., 1998; Takahashi et al., 2001).

An early study conducted by Yoshioka et al. (1998), reported that the fungal β-1,3-glucans sonifilan, grifolan and zymosan induced lethal toxicity in mice when orally administered alongside the NSAID, Indomethacin. Lethal toxicity occurred when mice were orally administered with 5 mg/kg^{-1} once a day for two weeks. A later study confirmed these observations and reported that the lethal toxicity was due to the onset of peritonitis by enteric bacteria (Takahashi et al., 2001).

A recent investigation conducted by Kanno et al. (2021), studied the effect of latent β-1,3-glucan extracted from Japanese cedar pollen (JCP) on the production of Japanese cedar pollinosis-specific antibody production in mice. The isolated β-1,3-glucan increased the production of TNF-α and IL-6 via a Dectin-1 receptor pathway. When JCP grains were administed intranasally, JCP-specific IgE and IgG were also detected in the blood serum of the mice. Interestingly, the mice also showed increased sneezing behaviours. It was concluded that inhibiting the interaction of JCP β-1,3-glucan and Dectin-1 could act as an effective therapeutic prevention of pollinosis, however, the study was carried out in mice so further study in humans is required to confirm the results.

Rylander and Lin (2000) reported that the inhalation of mould-derived β-1,3-glucans caused symptoms from the upper respiratory tract in humans with children being a group at particular risk. In review of the evidence, it was suggested that these respiratory symptoms could be fully or partly due to the β-1,3-glucan content in moulds. Maheswaran et al. (2014) associated exposure to β-glucan dust levels in the home in children aged 7-10 to atopic asthma at age 11-14. The likelihood of bronchial hyper-responsiveness (BHR) almost doubled with unit increases of β-glucan in asthmatic children. The exposure to high β-glucan levels at 7-10 years old increased the risk of BHR in adolescence to children without pre-existing asthma. These findings suggest that dust β-glucan exposure at school age is a risk factor to the onset of asthma and BHR.

Multiple β-1,3-glucan sources have been shown to induce trained immunity via different mechanisms, however the effect of the higher plant β-1,3-glucan, callose, on the immune system in isolation, is not yet known. As some sources of β-glucans have been shown to host a negative effect on the immune system and health, research should be carried out to find out whether structural differences in β-1,3-glucans (Table 1) influence the immune response positively or negatively.

1.6.3.1. Exploitation of cell wall biopolymers in the development of biologically active materials

1.6.3.2. Wound dressings and tissue engineering

An important aspect in successful wound treatment is to provide a suitable base for the healing process while removing exudates from the surface of the wound. A successful wound dressing should also prevent dehydration at the wound (El-Kased et al., 2017). Hydrogels have been used in biomedical applications due to their biocompatibility, non-toxicity, and biodegradability. They also exhibit superabsorbent properties and have a soft consistency which can resemble the environment of living tissue (Van Vlierberghe et al., 2011).

Muthuramalingam et al. (2019) developed a hydrogel from a fungal β-1,3;1,6-glucan and poly-vinyl alcohol (PVA) as a wet dressing material. The β-glucan/PVA hydrogel dressing significantly accelerated the wound healing process both *in vitro* and *in vivo* in mice. The hydrogel also showed a high water-holding capability which enabled the dressing to provide sufficient moisture to the wound, preventing dehydration. The hydrogel was also a successful flavonoid carrier which increased the growth of skin appendages and the rate of the wound closure. A similar study using a β-glucan/PVA film was carried out *in vivo* in rats by Huang and Yang (2008). Wound healing time was found to reduce by 48% when the β-glucan/PVA film was used compared to a cotton gauze dressing.

Nair et al. (2016) investigated the effects of a cyclic β-1,3;1,6-glucan on wound healing. Cyclic β-1,3;1,6-glucan/carrageenan hydrogels were prepared and tested both *in vitro* and *in vivo* in rats. Both studies showed increased wound healing rates compared to the control, which supports the potential use of β-1,3;1,6-glucans in novel dressing materials. Medeiros et al. (2012) developed a cream containing β-1,3;1,6-glucan isolated from baker's yeast to investigate the effects on ulcer healing. The β-1,3;1,6-glucan cream enhanced ulcer healing and increased fibroblast proliferation which also aided the healing process.

The process of wound healing is complex, involving inflammation, proliferation and maturation and development of cells and tissues (Singh et al., 2013). Reviewing the evidence from various studies suggests that β-1,3-glucans have a positive effect on wound healing and give potential for the development of novel wound dressings.

1.6.3.3. Drug encapsulation and modulation

The encapsulation of drugs can aid drug stabilisation since the capsule material provides a physical barrier and a controlled release at the target site in biological systems (Paramera et al., 2011). The encapsulation or complex with certain materials can also enhance targeted delivery with a higher affinity to the cell membrane.

β-1,3-glucans derived from yeast can act as an effective platform for targeted delivery of payload drugs to immune cells such as macrophages, as they present the receptor specific to Dectin-1 on their membranes (Brown and Gordon, 2001; Huang et al., 2012). Volpato et al. (2018) investigated the effect of a drug, T6, encapsulated with β-1,3;1,6-glucan particles from yeast, on the progression of Leishmaniasis, a tropical disease affecting humans. The β-glucan particles successfully encapsulated the T6 with a maximum encapsulation efficacy (EE) of 60.95%. The T6 encapsulated with β-glucan particles resulted in a significant reduction in the specific macrophage toxicity compared to just the T6 in isolation suggesting that the β-glucan particles decreased the cytotoxicity of the T6 compound.

Subedi et al. (2009) used curdlan to encapsulate an anti-cancer drug, doxorubicin, in a lipid core of glyceryl caprate. The curdlan successfully encapsulated the drug with an EE of 67.5%. The release of doxorubicin was shown to be higher at pH 5, than pH 7.4 *in vitro.* It was previously reported by Kim et al. (2005), that aggregation of particles, resulting in a decreased EE, could occur if insufficient curdlan is available to adsorb to the surface of the lipid particles. It is therefore important to investigate curdlan: lipid ratios for the optimum EE.

Another study by Li et al. (2010), developed nanoparticles of cholesterol-conjugated carboxymethyl curdlan (CCMC) to encapsulate an anti-cancer agent, epirubicin, which is derived from doxorubicin. A study was carried out *in vitro* and *in vivo* in rats to assess the potential of the CCMC nanoparticles as anti-cancer drug carriers. *In vitro* the EPB-loaded CCMC nanoparticles were more cytotoxic than the free EPB and had a broader distribution within cells. *In vivo* the EPB-loaded CCMC nanoparticles effectively suppressed tumour growth in rats, compared to when EPB was sued in isolation. Compared to free EPB, the CCMC increased the retention time in the blood plasma enhancing the therapeutic efficacy and aiding the distribution within tissues. It was concluded that the β-1,3-glucan, curdlan derivative, conjugated with cholesterol has the potential to act as an effective anti-cancer drug carrier.

1.6.3.4. Potential applications by exploiting mechanical properties of hemicellulose biopolymers

The interactions and complexes formed between cell wall biopolymers offer potential for the development of new applications in industry and in plant modification for enhanced biological functions (Abou-Saleh et al., 2018). It is however important to understand how the components interact and following this, how the physical and mechanical properties change in various applications.

The hemicellulose xylan has previously been used to form poly (2-hydroxyethyl methacrylate) (pHEMA)-based hydrogels with the effects of the presence of acetyl groups on the physical properties of the hydrogels being investigated. Silva et al. (2011) reported that the presence of acetyl groups from xylan increased the stiffness of hydrogels compared to the presence of unacetylated xylan. This increased stiffness also reduced the water swelling capacity of the gels which was ultimately reported to improve the drug release properties of the hydrogels. Another study conducted by Karaaslan et al. (2011), investigated the potential use of modified xylan and cellulose pHEMA hydrogels in replacement articular cartilage. It was reported that the hydrogels had similar mechanical properties, viscoelastic and water swelling capacities to load bearing tissues.

Abou-Saleh et al. (2018) investigated the interactions of callose and cellulose using a simplified model of composite hydrogels to represent a plant cell wall environment. Callose was shown to alter the mechanical properties of cellulose hydrogels, reducing the stiffness of the gels to a greater extent than that predicted by the ideal mixing rule. The elasticity of the hydrogels was also increased with the addition of callose, where there was a sudden failure in 100% cellulose hydrogels. It was therefore proposed that callose enhances the cell wall resilience to high strain conditions by increasing the elasticity of the wall, resulting in a gradual deformation rather than a sudden failure and tearing of the cell wall. These results suggest that the interactions of callose and cellulose not only offer the potential to regulate various biological processes but offer a potential source of novel biodegradable materials.

A study carried out by Mikkelsen et al. (2015), investigated the interactions of mixed-linkage β-1,3;1,4-glucans and arabinoxylan with bacterial cellulose. The effect of β-1,3;1,4-glucans and arabinoxylan on the mechanical properties of bacterial cellulose hydrogels were studied. Both the β-1,3;1,4-glucans, at concentrations between 27% and 29%, and arabinoxylan, at concentrations between 8.5% and 50%, altered the mechanical properties of the hydrogels, reducing the tensile stress, strain and resulting Young's modulus of the hydrogels compared to the 100% bacterial cellulose hydrogel.

The mechanical properties of cell wall biopolymers and interactions with cellulose and other polysaccharides offer the opportunities for the design of strategies to regulate various plant biological processes and a potential source of novel biodegradable components for the material and food industries.

Increasing demand for utilising lignocellulosic wastes for the extraction of cellulose has led to interest in finding alternative non-woody waste sources for the extraction of cellulose, hemicelluloses alongside other cell wall biopolymers for the production of biomaterials with various industrial applications.

Chapter 2 - Objectives of work

The work proposed had three major objectives:

1. To dissect the molecular composition of lignocellulosic cell walls from different waste plant sources.

2. To determine compositional changes during processing of raw plant waste to pulp, film and their by-products.

3. To assess the effect of plant waste biopolymers towards induction of inflammation in mammalian cells.

2.1. Dissecting the molecular structure of cell walls in lignocellulosic wastes

Lignocellulosic wastes not only offer a sustainable source of cellulose but offer a potential source of other cell wall polysaccharides such as xylans and ß-glucans for extraction and applications in industry. To investigate the cell wall molecular structure of these agricultural wastes, different compositional techniques were used to explore the polysaccharide profile of cell walls from various waste sources. Sequential weak to strong cell wall extractions were carried out to extract epitopes free in the cell wall domain up to those strongly bound to cellulose fibrils. Specific molecular probes were used to firstly qualitatively screen waste materials in different extracts for a range of pectic, hemicellulosic and glycoprotein epitopes. Secondly, quantification of polysaccharide epitopes rich in initial screening, alongside hemicelluloses of interest including the ß-1,3-glucan, callose, which unlike other ß-1,3-glucans, has not been extensively studied.

2.2. Compositional comparison of the cell walls from raw wastes to partially and fully processed waste pulps and films alongside by-products

It is important to investigate the effect of processing on the composition of the waste materials to identify whether any impurities or components heavily bound to cellulose remain. To investigate the compositional differences in raw wastes, partially processed pulps and fully processed films, specific molecular probes will be employed for quantification of polysaccharide epitopes rich in the raw wastes alongside the ß-1,3-glucan, callose. The data will allow visualisation of the processing effects from raw waste into pulps and film products. These probes alongside other techniques such as protein determination will be useful in determining whether any polysaccharide epitope has been concentrated in by-products of processing, with different potential applications in industry, further improving the sustainability of the process. To compliment this analysis, the use of FT-IR will also be employed for structural characterisation by highlighting the presence of bond structures and functional groups allowing comparison between different waste materials.

2.3. Assessment on the effect of cell wall biopolymers towards induction of inflammation in mammalian cells

As cell wall polysaccharides have the potential to be used in industrial applications involving direct contact with mammalian cells, it is important to investigate the effect of these

components on aspects of the immune response in mammalian cells. The induction of inflammation is one such response which also has a role in adaptive immunity.

The effect of hemicelluloses rich in waste materials and ß-1,3-glucans on the induction of inflammation will be investigated using a murine cell reporter model in a dose dependant manner. As the ß-1,3-glucan, callose, is difficult to isolate, other ß-1,3-glucans from various natural sources will be used in the analysis as an indicator to the effect callose may have.

At the end of the project the aim is to assess the potential for utilisation of these agricultural waste materials for the sustainable production of biocompatible films for a variety of applications.

Chapter 3 - Materials and Methods

3.1. Materials

3.1.1. Compositional analysis materials

Agricultural waste, pulp and film materials were provided by Futamura, UK. The commercial standards of xylan (beechwood), xyloglucan (tamarind seed) and glucuronoxylan were purchased from Megazyme. Laminarin, microcrystalline cellulose (Avicel), 3,3', 5,5; -tetramethylbenzidine (TMB) and Nunc flat bottomed 96 well Maxisorp immuno-plates. were purchased from Sigma-Aldrich (Dorset, UK) alongside all other chemicals and buffers used in the analysis. The non-fat milk powder was purchased from the local supermarket. Primary rat monoclonal antibodies (mAbs) were obtained from PlantProbes (Leeds, UK) and secondary antibody, anti-rat horseradish peroxidase was purchased from Invitrogen. For the protein assay, Bovine Serum Albumin (BSA) solids were purchased from Sigma-Aldrich and Coomassie brilliant blue G-250 dye was obtained from Bio-Rad Laboratories, Inc.

3.1.2. Cell culture materials

RAW-Blue™ Cells, derived from murine RAW 264.7 macrophages, were purchased from InvivoGen. The culture medium containing Dulbecco's Modified Eagle Medium (DMEM) was purchased from ThermoFisher Scientific alongside Foetal Bovine Serum (FBS) and Penicillin-Streptomycin. Zeocin was purchased from InvivoGen as well as the QUANTI-Blue™ Reagent.

The commercial ß-glucan standards, CM curdlan from Megazyme, CM pachyman from Biosupplies Australia Pty Ltd., and Laminarin and CM cellulose from Sigma-Aldrich were purchased. Hemicellulose standards in 3.1.1. were used alongside arabinoxylan (wheat) from Megazyme. Bacterial lipopolysaccharide (LPS from *E. coli*) was purchased from InvivoGen. Sterile flat bottomed 24 well and 96 well immuno-plates were purchased from Sigma-Aldrich.

3.2. Methods

3.2.1. Cell Wall Fractionation

Cell wall fractionation for each waste material was carried out sequentially according to Santiago-Doménech et al. (2008), with some modifications. 50 mM cyclohexylenediaminetetra-acetic acid (CDTA) pH 6, 4 M KOH and 1 μg/mL cellulase 5a were used in the extractions. Sequential mild to strong extractions were required due to different binding strengths of polysaccharides to cellulose fibres, with some being free within the cell wall domain and others being heavily bound to cellulose. Raw wastes underwent the full three sequential extractions, but waste pulps and films were only extracted in KOH and cellulase.

Samples were initially grinded to reduce the particle size. Depending on the experiments, 5-100 mg of samples (sample mass was accounted for in calculations) were placed in 2 mL tubes and 2 ball bearings were added into the tubes before grinding in a Tissue Lyser for 5 minutes at 50 Hz. 50 mM CDTA was added to the tube and mixed in the Thermomixer for 20 minutes at 1000 rpm before rocking for 40 minutes and centrifuging for 15 minutes at 14,000 rpm. The supernatant (CDTA extract) was transferred to a separate tube and kept with the sample pellet being subjected to the next stage of extraction. 4 M KOH with 10% $NaBH_4$ was added on top of the pellet and mixed in the Thermomixer for 40 minutes at 1000 rpm before centrifuging for 15 minutes at 14,000 rpm. The supernatant (KOH extract) was transferred to a separate tube and kept with the sample pellet being subjected to the final stage of extraction. 1 μg/mL cellulase 5a in 20 mM Tris buffer pH 8.8 was added on top of the pellet and incubated at 37 °C in the Thermomixer for 3 hours at 500 rpm before centrifuging for 15 minutes at 14,000 rpm. The supernatant (cellulase extract) was transferred to a separate tube and kept. All extracts were refrigerated at 4 °C and used within 7 days.

3.2.2. Cell Wall Glycome Profiling

Glycome profiling is an ELISA based technique that can be used for analysis of polysaccharide epitopes within extracted cell wall extracts (Figure 5) (Pattathil et al., 2010). The cell wall extracts were diluted 1:10 with PBS with the KOH extract also being neutralised using 80% acetic acid before coating on 96 well immuno-plates overnight at 4 °C. The plates were washed with tap water 6 times, patted dry and then blocked with 5% (w/v) non-fat milk powder in PBS (MP/PBS) for 2 hours at room temperature. The plates were then washed 9 more times, patted dry and monoclonal antibodies (mAbs) (

Table ***2***), diluted 1:10 with 3% MP/PBS, were added in triplicate wells and incubated for 1.5 hours at room temperature. The plates were washed 9 more times, patted dry and a secondary antibody (Anti-rat IgG HRP), diluted 1:1000 with 5% MP/PBS, was added and incubated for 1 hour at room temperature. The plates were washed 9 times before the addition of a TMB substrate, diluted 1:5 in de-ionised water, to generate the signal over 5 minutes. The reaction was stopped using 2.5 M sulphuric acid, producing a yellow colour. The absorbance was read at 450 nm using a MultiSkan plate reader, determining the binding strength of mAbs. PBS negative controls were included on the plate using primary mAbs and secondary HRP. Due to the varying affinities of mAbs, comparisons between antibodies are qualitative, however the data can be used for quantitative comparisons between wastes, pulps and films and extracts (Cornuault et al., 2018).

Table 2. Targeted cell wall polysaccharide epitopes and corresponding antibodies*. LM (Leeds Monoclonal), JIM (John Innes Monoclonal) (PlantProbes, Leeds UK).*

	Antibody	*Polysaccharide epitope*
Pectins	***LM5***	*ß-1,4-D-galactan*
	LM6	α-1,5-L-arabinan
	LM13	α-1,5-L-arabinan (linear)
	LM19	*Homogalacturonan (Unesterified)*
	JIM7	*Homogalacturonan (Esterified)*
Hemicelluloses	***LM10***	*Xylan (Unsubstituted)*
	LM11	*Xylan/Arabinoxylan*
	LM21	*Mannan*
	LM23	*Xylosyl residues*
	LM25	*Xyloglucan*
	LM28	*Glucuronoxylan*
	10H2	*Callose*
Glycoproteins	***LM1***	*Extensin*
	JIM13	*Arabinogalactan*

3.2.3. Quantification of Cell Wall Polysaccharide Epitopes

Commercial standards of xylan, xyloglucan, glucuronoxylan and laminarin were used to quantify the amount of certain polysaccharide epitopes present in sample extracts. Standard curves with linear regression equations were produced using the ELISA based technique as described in 2.2 with commercial standards of increasing concentrations and mAbs specific to the polysaccharide epitopes (Figure 6). Standard stock solutions of 1 mg/mL were produced by adding PBS to sample powder and vortexing for 20 seconds until a clear solution containing no un-hydrated powder remained. Stock solutions were diluted sequentially using PBS, vortexing for 5 seconds between each subsequent dilution.

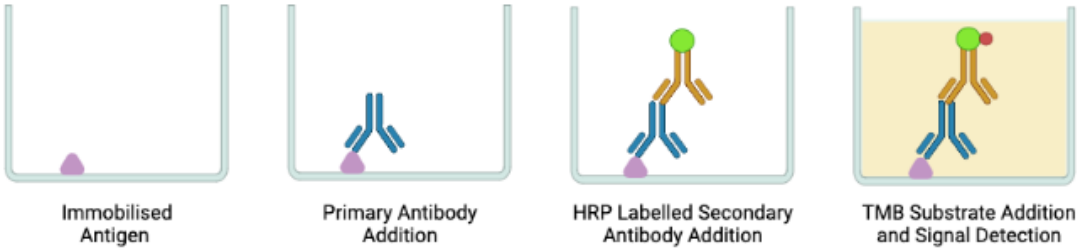


Figure 5. ELISA workflow to determine the polysaccharide profile of agricultural waste products. *Antigens from the cell wall extracts were immobilized and detected using monoclonal antibodies specific to epitopes of polysaccharides. HRP labelled anti-rat antibody was used to detect primary mAbs and TMB substrate added after washing the excess of non-bound antibody. The reaction was stopped with sulfuric acid and the yellow signal colour development was detected and measured at 450 nm. (Created with BioRender.com).*

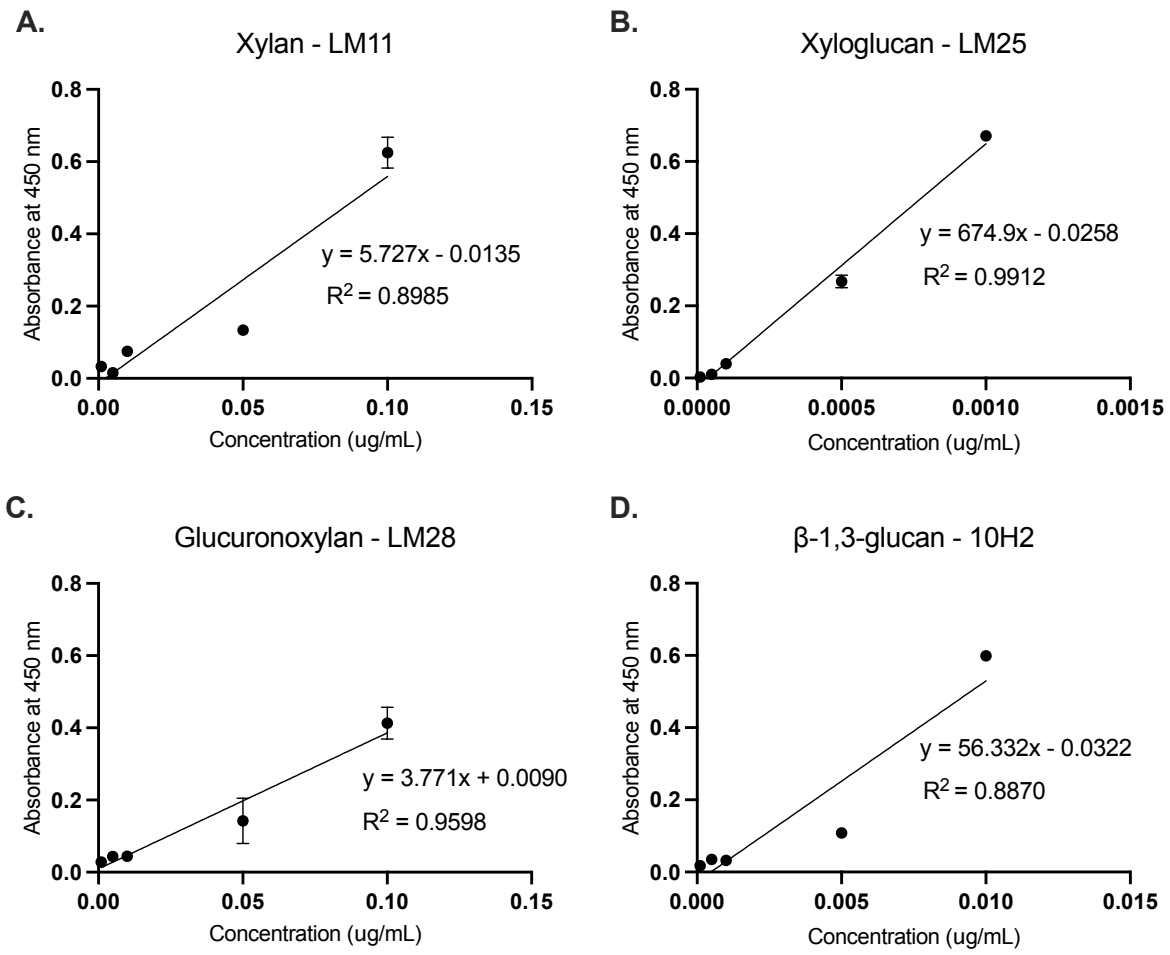


Figure 6. Standard curves showing absorbance of different cell wall polysaccharide concentrations detected using ELISA. ***A,*** *xylan using LM11,* ***B****, glucuronoxylan using LM28,* ***C****, xyloglucan using LM25 and* ***D****, ß-1,3-glucan (laminarin) using 10H2.*

3.2.4. Protein Assay

A standard curve was prepared using bovine serum albumin (BSA) with concentrations of 1.0, 2.5, 5.0, 7.5 and 10 µg/mL as per protocol from Bio-Rad (Figure 7). CDTA extracts of brewers' spent grain waste and brewers' spent grain protein pulp were prepared (as described in section 3.2.1.) and diluted 1:10 and 1:20 using PBS. 800 µL of standard/sample was mixed with 200 µL Coomassie Blue dye concentrate in 5 ml centrifuge tubes and vortexed for 10 seconds before being incubated at room temperature for 8 minutes. Each sample was transferred in duplicate into cuvettes and absorbance was read at 595 nm using PBS containing no BSA or test compound as the blank. Duplicate readings were averaged and used to calculate the amount of protein in µg/mg sample.

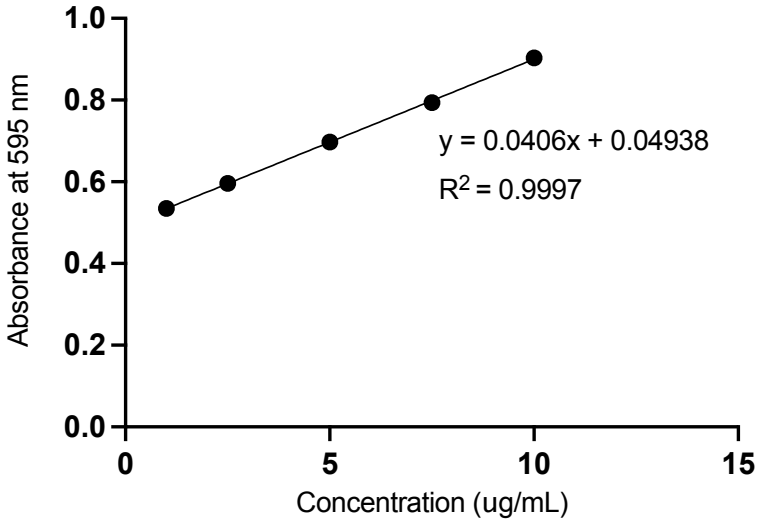


Figure 7. Bovine Serum Albumin (BSA) standard curve. *Absorbance vs concentration was detected using the Bio-Rad protein quantification kit.*

3.2.5. FTIR Spectroscopy

Commercial standard samples did not require any physical preparation due to the samples already being in powdered form. Agricultural wastes and pulps required grinding to reduce particle size using the tissue lyser grinding method (as described in section 0). Agricultural films required grinding and slicing using a coffee grinder to reduce the size of the film fragments.

FTIR analysis was carried out in the School of Chemistry at the University of Leeds by Natalia Sergeeva. FTIR spectra was obtained for all samples in solid phase on a Bruker Alpha Platinum ATR FTIR spectrometer within the frequency range of 550-4000 cm^{-1} using the microcrystalline cellulose (avicel) peak at 1029 cm^{-1} as the normalisation standard.

3.3. Cell Culture

RAW-Blue™ cells, derived from murine RAW 264.7 macrophages, express multiple PRRs including Dectin-1 and all TLRs except for TLR5. Upon activation of the PRRs by specific agonists a signalling cascade is triggered which leads to the activation of NF-kB and subsequent secretion of Secreted embryonic alkaline phosphatase (SEAP) (Figure 8) (Karki and Igwe, 2013).

3.3.1. Cell growth and maintenance

RAW-Blue™ cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 4.5 g of glucose/L) without sodium pyruvate, supplemented with L-glutamine, 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) Penicillin-Streptomycin. Growth medium for cell maintenance was supplemented with 200 µg/mL Zeocin. RAW-Blue cell cultures were incubated in a humidified 5% CO_2 incubator at 37 °C. Cells were grown to be 70-80% confluent before being passaged twice a week.

3.3.2. Preparation of test compounds

ß-glucan (laminarin, carboxymethyl (CM) curdlan, CM pachyman and CM cellulose) and other hemicelluloses (xylan, arabinoxylan and xyloglucan) stock solutions of 20 mg/mL were prepared by adding pre-warmed (37 °C) DMEM to sample powders and vortexing for 1 minute until fully hydrated (or until clear solutions resulted). Stock solutions were diluted sequentially with DMEM to final concentrations of 5000, 1000, 500, 100, 50, 10 µg/mL, vortexing for 5 seconds between each subsequent dilution.

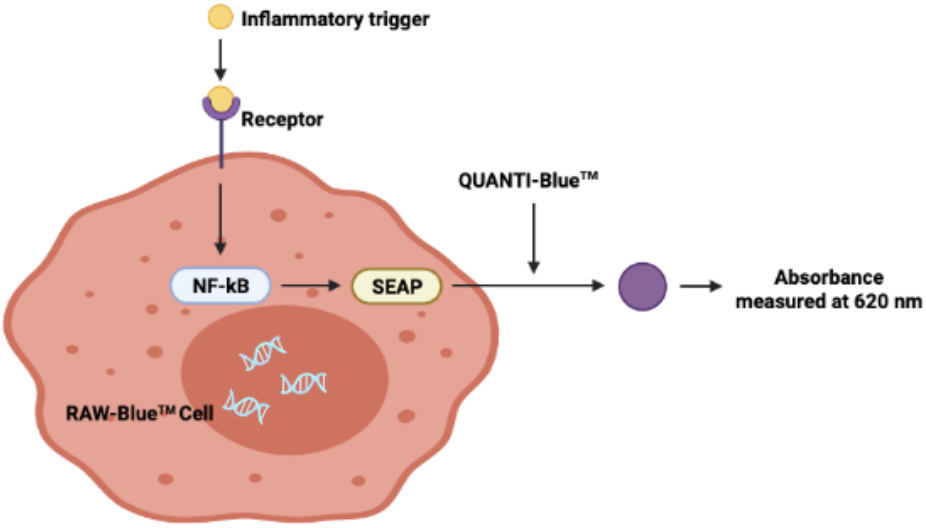


Figure 8. *RAW-Blue inflammatory reporter cells which respond to inflammatory triggers upon recognition and stimulation of receptors such as TLR4 and Dectin-1. RAW-Blue™ cells induce the activation of NF-KB, leading to the secretion of embryonic alkaline phosphatase enzyme (SEAP) which can be detected using QUANTI-Blue™ reagent. (Created with BioRender.com).*

3.3.3. QUANTI-Blue™ SEAP Reporter Assay

RAW-Blue cells (1.5 x 10^5) were seeded into 24 well plates and grown at 37 °C until approximately 80% confluent. Medium was aspirated and replaced by media containing test compound in duplicate wells at concentrations of 10-5000 µg/mL. Bacterial lipopolysaccharide (LPS), a TLR4 agonist, was used at a concentration of 10 ng/mL in duplicate wells as the positive control and DMEM, excluding any test compound, included in duplicate wells as the negative control. Plates were incubated for 24 hours at 37 °C before media from each well being transferred in duplicate to 96 well plates. QUANTI-Blue™, SEAP detection reagent, prepared as per manufacturer's instructions, was added to each well and plates were incubated for 1.5 hours at 37 °C before absorbance being read at 620 nm by a MultiSkan plate reader to determine SEAP levels.

3.4. Statistical analysis

The mean and standard deviations of triplicate results in one independent experiment for ELISA, polysaccharide, and protein quantifications, and duplicate results in two independent cell culture experiments were calculated using Microsoft Excel. Due to Covid-19 restrictions, there were insufficient opportunities to carry out further repetitions, therefore the results reported cannot be deemed statistically significant.

If time had permitted, three repetitions of each independent experiment would have been carried out. The mean and standard deviations would be calculated, along with carrying out a one-way ANOVA to determine whether the means of these results are statistically significant from each other.

Chapter 4 - Results and Discussion

4.1. Glycome profiling and hemicellulosic epitope quantification of raw agricultural wastes

Immunochemical analysis of agricultural waste cell wall components was performed using monoclonal antibodies (mAbs) (Table 2). Three sequential extractions in 50 mM CDTA, 4 M KOH, and cellulase were carried out from the waste materials for analysis. The increasing strengths of these extractions were due to different polysaccharides being released in different fractions based on how heavily bound to cellulose fibrils they are. The initial extraction in CDTA extracted pectins and some hemicelluloses. The secondary stronger extraction in KOH extracted hemicelluloses and unesterified pectins. The final cellulase enzymatic digestion extracted components heavily bound to cellulose.

The initial screening was performed on the raw waste materials using a wide range of antibodies for a basic compositional polysaccharide epitope profile, highlighting which polysaccharide epitopes to quantify using commercial standards. Screening was also carried out to decide (alongside input from the industrial sponsor) which materials will be used to continue the main analysis.

Initial cell wall glycome profiling of raw waste materials highlighted the epitopes enriched within the wastes (Table 3)**.** The profiles of hemp and brewers' spent grain was more similar than that of sugarbeet, likely due to the differences in cell wall composition in the organs used to produce the waste in these species (for sugarbeet, the beet itself was used after industrial processing, whereas for hemp leaves and stalks were used). Both hemp and brewers' spent grain had strong xylan and glucuronoxylan signals but week signals for proteins (extensins and arabinogalactan proteins), callose and xyloglucans. Pectin concentration (except for homogalacturonan in the cellulase fraction) was also low. Sugarbeet had strong profile in the KOH extracts, with higher overall amounts of pectins, hemicelluloses and glycoproteins than either hemp or brewers' spent grain. The exception is xylan and glucuronoxylan, which remained higher in hemp in comparison to sugarbeet. Sugarbeet released a high amount of pectins and proteins after cellulase extraction, however a much weaker profile for hemicelluloses. Xylan and glucuronoxylan remained high in the hemp cellulase extracts indicating these hemicelluloses are strongly bound to cellulose in hemp. Brewers' spent grain waste also had a strong detection of glucuronoxylan in the cellulase extract, even stronger detection than in the KOH extract, suggesting the glucuronoxylan is heavily bound to cellulose in brewers' spent grain. The detection of xylan/arabinoxylan epitope was also strong in both hemp extracts and the KOH extract of sugarbeet. Xyloglucan epitope appeared more strongly detected in the KOH extract of sugarbeet.

Table 3. Cell wall glycome profiling of KOH and cellulase extractions from sugarbeet, hemp and brewers' spent grain wastes. *Data represented in a heat map with dark green representing the highest relative absorbances and red the weakest absorbances at 450 nm detected using ELISA.*

	Antibody	Polysaccharide Epitope	Sugarbeet Waste		Hemp Waste		Brewers Grain Waste	
			KOH	Cellulase	KOH	Cellulase	KOH	Cellulase
Pectins	LM5	Beta-(1,4)-D-galactan	0.2658	0.4054	0.0634	0.0877	0.0170	0.0704
	LM6	Alpha-(1,5)-L-arabinan	0.6439	0.6978	0.1501	0.2260	0.1052	0.1949
	LM19	Homogalacturonan (Unesterified)	0.3348	0.5124	0.1597	0.3290	0.0665	0.2888
Hemicelluloses	LM11	Xylan/arabinoxylan	0.4610	0.0246	0.6944	0.4784	0.2056	0.2210
	LM23	Xylosyl residues	0.1065	0.0146	0.1569	0.0151	0.0150	0.0062
	LM25	Xyloglucan	0.4689	0.1904	0.1032	0.0717	0.1197	0.1653
	LM28	Glucuronoxylan	0.5977	0.0643	0.7861	0.7851	0.2802	0.6199
	10H2	Callose	0.1895	0.0495	0.0787	0.0861	0.0220	0.0385
Glycoproteins	LM1	Extensin	0.3888	0.3306	0.0091	0.1501	0.0000	0.0328
	JIM13	Arabinogalactan	0.2493	0.2657	0.2331	0.2638	0.0000	0.0532

Focusing on the proteins, the glycoprotein arabinogalactan epitope gave good signal intensity in both KOH and cellulase extracts of sugarbeet and hemp but was absent in the brewers' grain extracts. Extensin epitope was detected in both sugarbeet extracts but it was low in hemp and brewer's grain. The pectic profile was strongest in sugarbeet which was consistent with the literature (Rombouts and Thibault, 1986). α-arabinan side chains for RGI was the most strongly detected epitope in the sugarbeet extracts suggesting similar levels of loosely bound, and cellulose-bound α-arabinan in the sugarbeet waste. The pectic profiles of both the hemp and brewers grain wastes suggest low concentration of pectins in these materials although mild detection of homogalacturonan epitope occurred in the cellulase extract of the hemp and brewers grain waste and of alpha-arabinan RGI epitope in the cellulase extract of hemp waste.

Following initial immunochemical analysis of hemp, sugarbeet and brewers' spent grain wastes, a second profile analysis was carried out to verify the differences. The CDTA fraction and a new sample (tomato waste) were included in this analysis (Figure 9). Hemicellulosic mAbs LM11, LM21, LM25 and LM28 produced relatively high signals in the initial analysis, as well as 10H2, specific to callose, a ß-1,3-glucan of interest in this investigation. A pectic mAbs, LM19 and glycoprotein mAbs, JIM13 were also used in the analysis.

Unesterified homogalacturonan (LM19) was moderately detected in all three wastes in the CDTA extract, along with xylan (LM11) in the tomato waste and arabinogalactan in sugarbeet waste. In the KOH extract, xylan and glucuronoxylan (LM28) were strongly detected in both hemp and tomato waste with weak signals of both epitopes in sugarbeet waste. Similarly, in the cellulase extracts xylan and glucuronoxylan were strongly detected in hemp and tomato wastes however slightly lower detection levels of xylan in the cellulase extracts compared to the KOH extract.

Although cell wall glycome profiling using mAbs can be useful to compare epitope levels between waste samples, due to the varying affinity of antibodies, it is not possible to establish direct comparison between different antibodies (Rydahl et al., 2018). Quantification using calibration curves produced using commercial standards is required to compare quantitatively between antibodies. Standard calibration curves using commercial compounds of xylan, glucuronoxylan, xyloglucan and ß-1,3-glucan were produced using 5 concentrations. The linear domain of each curve lay between 0.00001-0.1 mg/mL (Figure 6). A linear model was used to generate standard curves, however the absorbance results at 0.05 µg/mL for xylan and glucuronoxylan, 0.005 µg/mL for ß-1,3-glucan and 0.0005 µg/mL for xyloglucan are below the curve. This means that the quantification results at these concentrations may be overestimated.

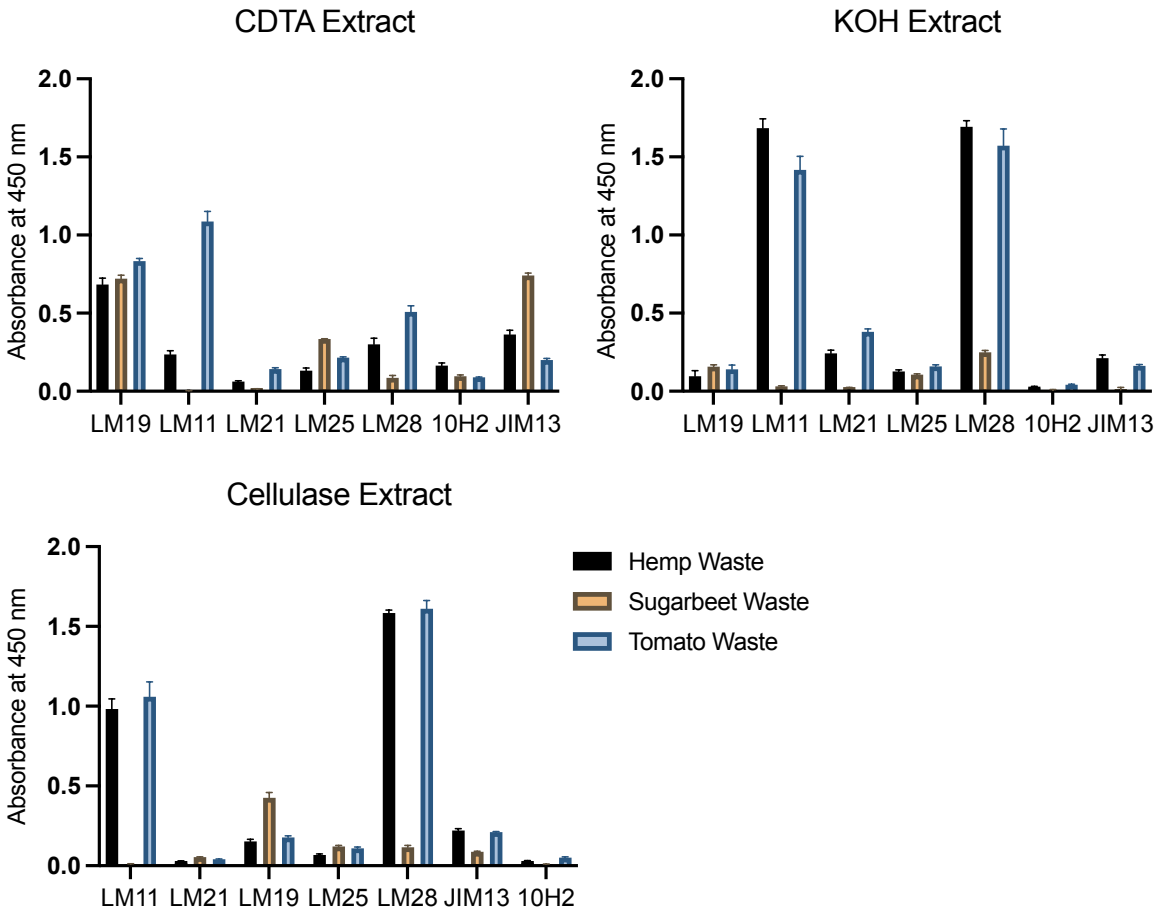


Figure 9. Detection of specific cell wall polysaccharides in raw waste materials*. Absorbance was measured following ELISA detection of the cell wall polysaccharide epitopes in CDTA, KOH and cellulase extracts of raw hemp, sugarbeet and tomato wastes using mAbs for pectins, hemicelluloses and glycoprotein described in Table 2. Error bars represent the standard deviation of triplicate results.*

The standard curves allow us to quantify the amounts of the different polymers in the waste material. Quantification data in hemp, sugarbeet and tomato wastes are shown in Figure 10. Tomato and hemp waste had the highest quantity of both xylan and glucuronoxylan epitopes in all CDTA, KOH and cellulase extracts. Sugarbeet waste contained less of the two epitopes in all extracts. Comparatively sugarbeet waste contained more glucuronoxylan than xylan in all fractions. Overall, there was similar amounts of xylan and glucuronoxylan extracted in each extract suggesting that there are similar amounts of these components free, lightly bound, and heavily bound to the cell walls of all three wastes. On the other hand, the sugarbeet waste had the highest amount of xyloglucan (when compared to tomato and hemp waste) in the CDTA extract. The levels of ß-1,3-glucan was similar in all three wastes. There was more xyloglucan and ß-1,3-glucan extracted with CDTA compared to the KOH and cellulase suggesting the polysaccharides are loosely bound to the cell wall.

Although generating standard curves from commercial standards can be a useful tool for indicating relative levels of polysaccharide epitope levels within samples, mAbs are specific to one epitope (Cornuault et al., 2014). As polysaccharides may have multiple different epitopes, with some not having corresponding antibodies for analysis, it cannot be concluded that the quantifications reported are the total amount of polysaccharide within each sample.

Other epitopes may also have different abilities to bind to cellulose within the cell wall domain and therefore the differences in detection of epitopes between the CDTA, KOH and cellulase extracts may not be entirely representative. Taking into consideration the disclaimer, we can still compare the different waste materials regarding the amount of polysaccharide present detected by the epitope specific antibody. Overall, there was a higher amount of glucuronoxylan and xylan (in the order of 100 µg/mg in hemp and tomato) than xyloglucan and ß-1,3-glucan (at least 10-100 less) in all three wastes. The higher levels of glucuronoxylan compared to xylan in the KOH and cellulase extracts of all three wastes compared to xylan are consistent with the literature, as hemp, sugarbeet and tomato are all dicots and glucuronoxylan is the major non-cellulosic polysaccharide in their secondary cell walls. The low levels of xyloglucan in the wastes were inconsistent with the literature, as xyloglucan has been reported to be the major hemicellulose present in the primary cell walls of dicots (Scheller et al., 2010), however, as the distinction between primary and secondary cell walls in the present study was not carried out conclusions should not be made. The overall quantity of xyloglucan in the wastes may also differ as only one epitope of xyloglucan was quantified in this study.

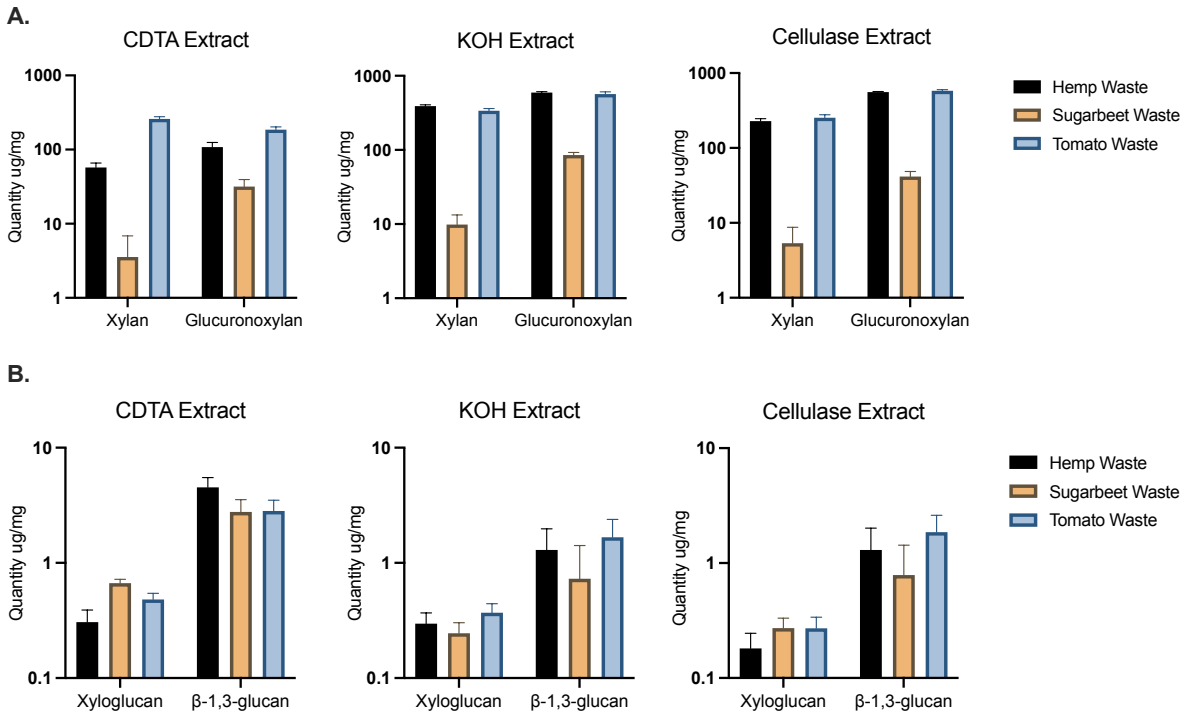


Figure 10. Quantification of hemicellulosic epitopes in raw waste materials*. Amount (µg/mg of total cell wall) of* ***A,*** *xylan with LM11 and glucuronoxylan with LM28,* ***B****, xyloglucan with LM25 and ß-1,3-glucan with 10H2. Quantification of each epitope shown in CDTA, KOH and cellulase extracts of raw hemp, sugarbeet and tomato waste materials. Monoclonal antibody-based ELISA was performed using standards of increasing concentrations with the specific primary antibody for the epitope, to generate a standard linear regression curve with equation to calculate the quantity of each epitope in the sample (Figure 6). Error bars represent the standard deviation of triplicate results.*

In the present study, the hemp and tomato wastes consist of stalk and leaf, whereas the sugarbeet waste consists of the beet itself. The tomato and hemp waste both comprise of similar parts of the plant and are both dicots, which could offer a partial explanation for the similar epitope quantification profiles. Hemp and tomato wastes were richer in xylan and glucuronoxylan epitopes than sugarbeet whereas similar levels of epitopes of xyloglucan and ß-1,3-glucan were found in all three wastes. With xylan and glucuronoxylan in higher quantities than xyloglucan and ß-1,3-glucan, it could be suggested that raw hemp and tomato

wastes are valuable sources for extraction of these hemicelluloses. On the other hand, sugarbeet waste was higher in pectins and glycoproteins although in this study exact quantification of these components was not carried out.

4.2. Comparison of the composition of raw wastes, partially processed pulps and fully processed films

To extract cellulose from the raw waste materials various processing steps occur. The materials investigated included raw wastes, partially processed pulps of tomato and hemp as well as a fully processed standard film and film containing 5% (w/w) of hemp pulp. It is important to investigate the effects these processing steps have on the polysaccharide epitope profile of the materials as residual polysaccharides could alter the properties of the final materials. The presence of components other than cellulose could highlight the need for further processing steps to remove components heavily bound to cellulose in the wastes.

First, the composition of polysaccharides in the waste and extracted pulps was compared. Qualitative analysis of raw tomato waste compared to two partially processed tomato pulps was carried out (Figure 11). The antibodies LM19 (detects homogalacturonan pectin), LM11 (xylan epitope), LM21 (mannan), LM25 (detects xyloglucans), LM28 (detects glucuronoxylan) and 10H2 (to detect ß-1,3-glucans) were selected for this analysis. There were reductions in the absorbance detected using ELISA with the LM11, LM21 and LM28 antibodies when comparing waste and tomato A and tomato E pulps (two separate extraction processes). This is consistent with an effective purification of cellulose from the waste.

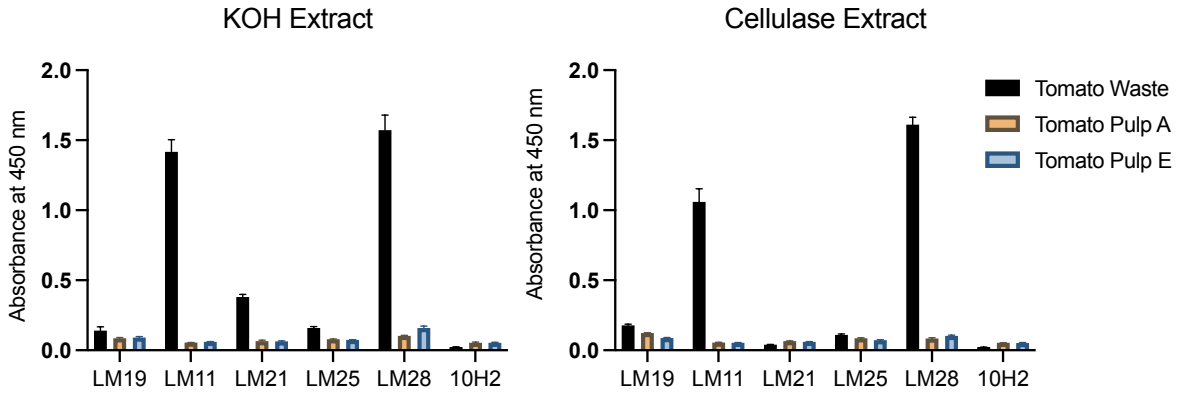


Figure 11. Detection of specific cell wall polysaccharides in raw tomato waste and two partially processed tomato pulps. *Absorbance was measured following ELISA detection of the cell wall polysaccharide epitopes in KOH and cellulase extracts using mAbs for pectins, hemicelluloses and glycoproteins described in Table 2. Error bars represent the standard deviation of triplicate results.*

To quantify the amounts of xylans, xyloglucans and ß-1,3-glucans in the waste and pulps, standard curves were used as described in material and methods (Figure 12). In both the KOH and cellulase extracts there was a reduction of epitopes of xylan and glucuronoxylan in both tomato A and E pulps compared to the raw waste. The amount of xylan decreased from ~500 µg/mg of total waste to less than 10 µg/mg in the pulps. Similarly, the amount of glucuronoxylan decreased from ~800 µg/mg in the waste to less than 50 µg/mg in the pulps. There was no difference in xylan and glucuronoxylan epitope levels between the two pulps apart from in the KOH extract where tomato E was a little higher in glucuronoxylan than tomato A. There was also slightly more glucuronoxylan epitope in the KOH extract of tomato

E than in the cellulase extract. There was no difference in the xyloglucan or ß-1,3-glucan epitopes but these hemicelluloses were already low represented in the waste.

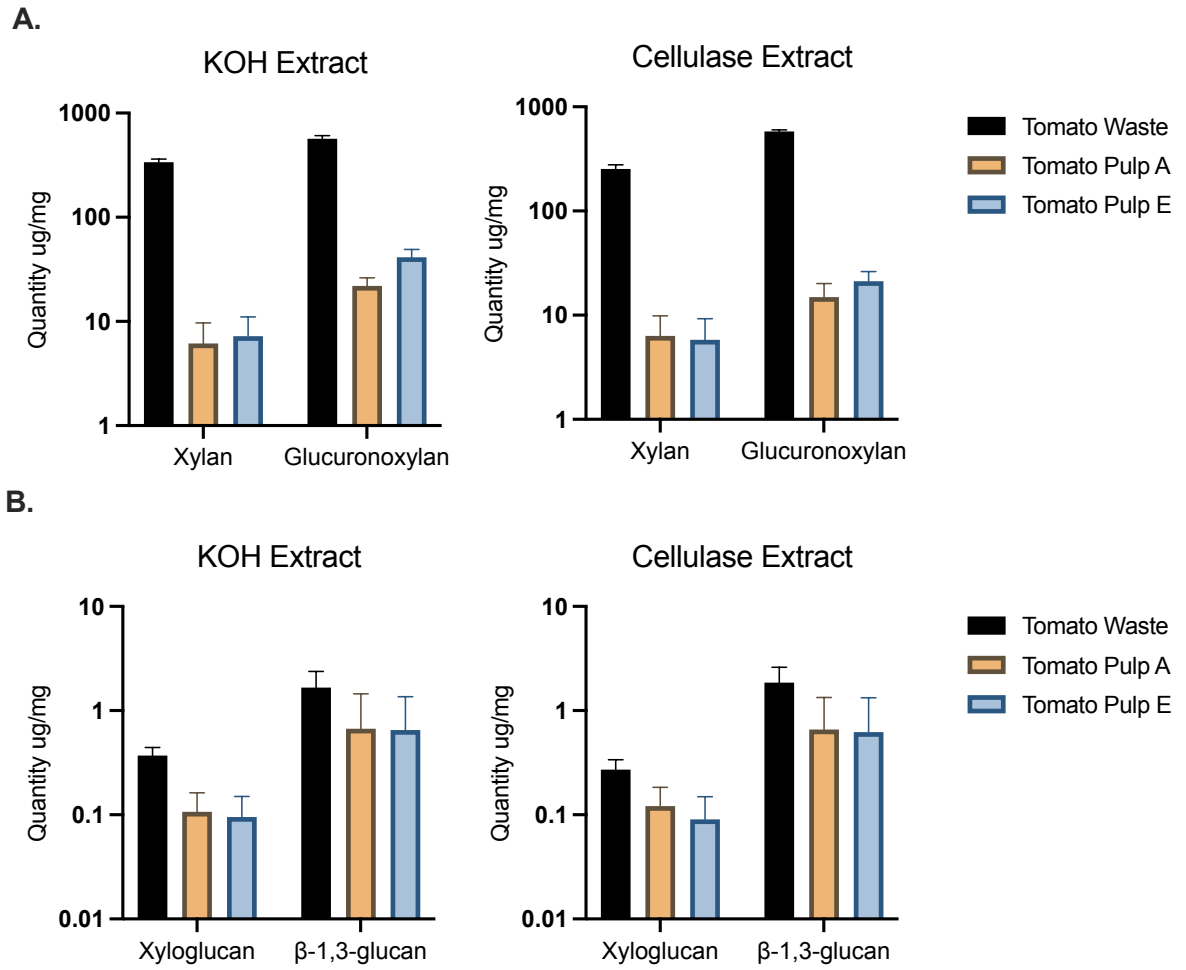


Figure 12. Quantification of hemicellulosic epitopes in raw tomato waste compared to two partially processed tomato pulps. *Amount (µg/mg of total cell wall) of **A**, Xylan with LM11 and glucuronoxylan with LM28, and **B**, xyloglucan with LM25 and ß-1,3-glucan with 10H2. Quantification of each epitope shown in KOH and Cellulase extracts. Monoclonal antibody-based ELISA was performed using standards of increasing concentrations with the specific primary antibody for the epitope, to generate a standard linear regression curve with equation to calculate the quantity of each epitope in the sample (Figure 6). Error bars represent the standard deviation of triplicate results.*

The ELISA profile analysis of hemp waste compared to extracted hemp pulp was also carried out (Figure 13). As before, relevant antibodies against pectins (LM19), xylan (LM11), mannan (LM21), glucuronoxylan (LM28), xyloglucan (LM25), ß-1,3 glucans (10H2) and arabinogalactan proteins (JIM13) were used. There was a reduction in the xylan (LM11), mannan (LM21), glucuronoxylan (LM28) and arabinogalactan (JIM13) in the hemp pulp compared to raw hemp waste. There was no difference between the raw waste and pulp in epitopes low represented in the hemp waste, including LM19, LM25 and ß-1,3-glucan (10H2).

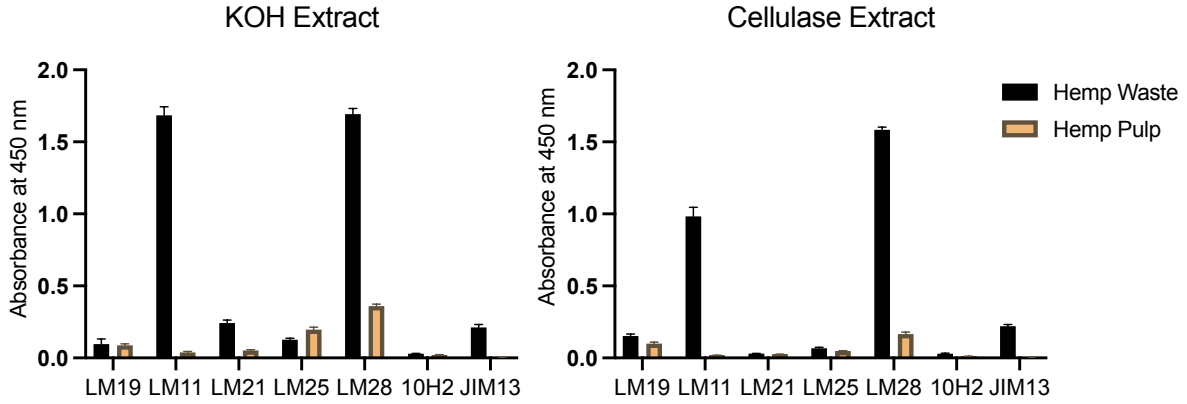


Figure 13. Detection of specific cell wall polysaccharides in raw hemp waste and partially processed hemp pulp. *Absorbance was measured following ELISA detection of the cell wall polysaccharide epitopes in KOH and cellulase extracts using mAbs for pectins, hemicelluloses and glycoproteins described in Table 2. Error bars represent the standard deviation of triplicate results.*

Next, we quantified the amount of the selected hemicelluloses using standards in raw waste, pulps and included a newly obtained film containing 5% (w/w) of hemp (produced by the company Futamura) (Figure 14). KOH and cellulase extracts displayed a reduction in xylan and glucuronoxylan epitopes in the hemp pulp compared to the raw hemp waste. Similar as in tomato, there was a decrease of these polysaccharides detected in the pulp, with ~500 µg/mg in the raw waste and ~10 µg/mg and ~100 µg/mg of xylan and glucuronoxylan respectively in the pulp. Further reduction from ~10 to 0.5 µg/mg of material was observed in the film, consistent with the films containing only 5% of the pulp. There was no difference between the xyloglucan and ß-1,3-glucan epitope levels in KOH and cellulase extracts of the raw hemp waste and hemp pulp as these appear in low concentrations. These polysaccharides were basically undetected in the hemp film, except for trace amounts of xyloglucans.

The data suggest that the processing methods involved in producing hemp pulp from hemp waste and hemp film from hemp pulp were effective in eliminating xylan, glucuronoxylan and xyloglucan epitopes to just traces as well as completely eradicating ß-1,3-glucan from the final film product. The reduced presence of xylan in the processing of hemp waste to pulp was consistent with the results of a study conducted by Beluns et al. (2021), where an untreated hemp waste sample contained 2.95% overall density percentage (odp) of xylan whereas a treated hemp sample contained 2.39% odp. Although the method for hemp treatment used by the company Futamura was not revealed, the raw waste was hemp stalks similar as in Beluns et al. (2021). In chemical composition tests, it was also found that although acid insoluble lignin had been reduced from 5.98% odp in the raw hemp waste to 4.30% odp in the treated hemp, some remained. As the present study also utilised hemp stalk and no lignin analysis within the waste products was carried out it cannot be confirmed that the processing step from raw waste to pulp removed all the lignin. The comparison of the hemp lignin content compared to wood in the study, reported 19.29% odp in the raw wood, compared to 24.06% odp in the treated wood suggesting the process was unsuccessful in removing lignin. The high lignin content of wood compared to hemp highlights the benefit of utilising non-woody lignocellulosic wastes, as less chemical and energy is required to remove the lower percentage of lignin which contributes to the energy requirements of the overall process (Ates et al., 2020).

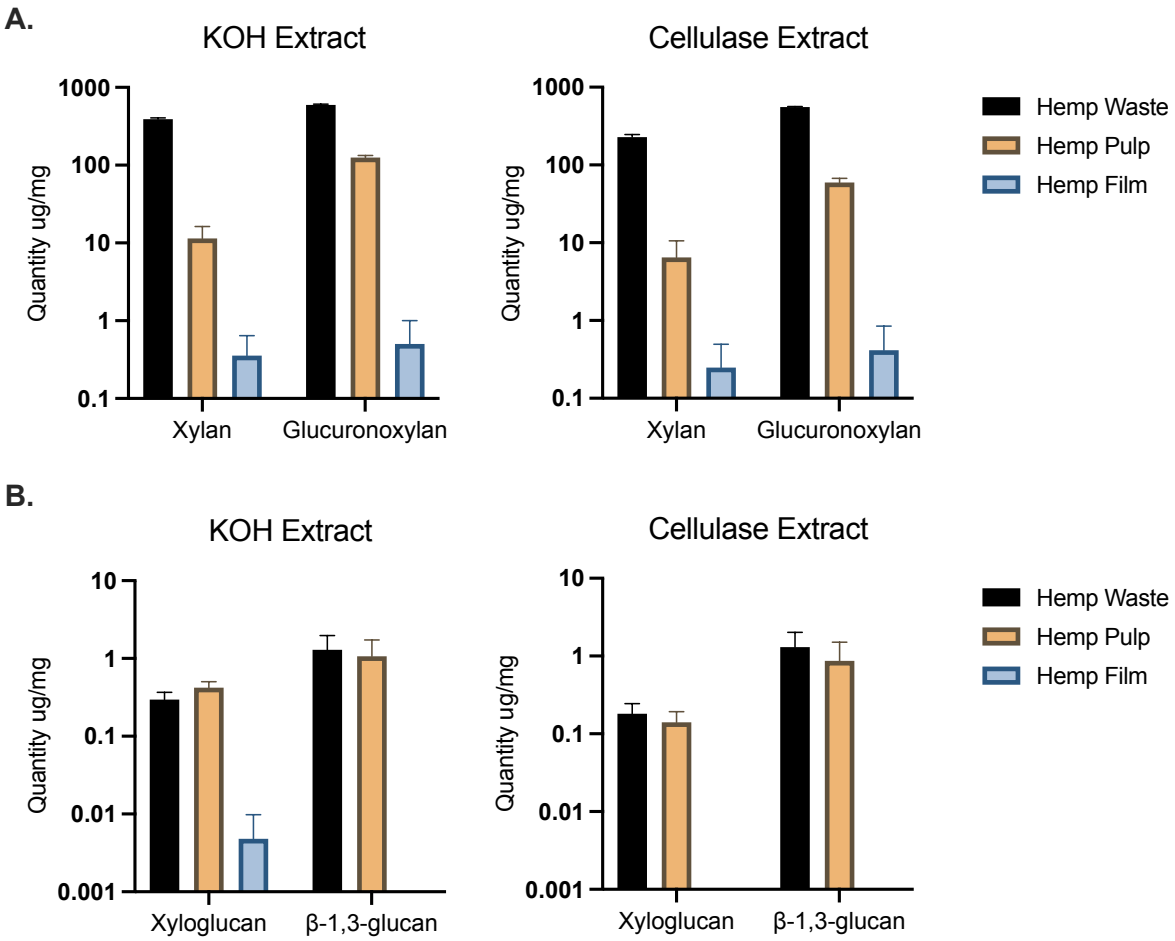


Figure 14. Quantification of hemicellulosic epitopes in raw hemp waste compared to partially processed hemp pulp and fully processed hemp film. *Amount (µg/mg of total sample) of **A**, Xylan with LM11 and glucuronoxylan with LM28, and **B**, xyloglucan with LM25 and ß-1,3-glucan with 10H2. Quantification of each epitope shown in KOH and cellulase extracts. Monoclonal antibody-based ELISA was performed using standards of increasing concentrations with the specific primary antibody for the epitope, to generate a standard linear regression curve with equation to calculate the quantity of each epitope in the sample (Figure 6). Error bars represent the standard deviation of triplicate results.*

Another study conducted by Merkel et al. (2014), reported that the overall hemicellulose content in hemp straw reduced after initial extraction of cellulose fibers from 28.01% overall hemicellulose content to 6.09% during initial processing of processed hemp. After enzymatic treatment this further reduced to 2.68%. Although individual hemicelluloses were not quantified, the results are consistent with those of the present study where the hemicelluloses epitopes, xylan, glucuronoxylan and ß-1,3-glucan presence all reduced from raw hemp waste to hemp pulp. The exception in the present study was an epitope of xyloglucan where no change occurred during initial processing, however compared to xylan and glucuronoxylan epitope levels, a minor quantity of xyloglucan was present.

To summarize, the two studies by Beluns et al. (2021) and Merkel et al. (2014), agreed with the results from this work, suggesting that hemicelluloses including xylans, were still likely present after the initial processing of the waste. Next, 5% hemp film and standard (wood) film were compared to assess whether the remaining hemicelluloses in hemp pulp were enough to be detected when incorporated into the film.

Comparison of two films, a standard film produced solely from wood (bahia-eucalyptus hardwood) cellulose pulp, and a film containing 5% hemp pulp was carried out (Figure 15). The quantities of xylan and glucuronoxylan were very similar between the two films in either the KOH or cellulase extract suggesting traces of these hemicelluloses are also present in eucalyptus wood. There were only traces of xyloglucan in the hemp and standard films and no ß-1,3-glucan was detected. The similarity between the epitope profiles of the two films suggests that the addition of 5% (w/w) of hemp pulp did not alter the composition of the standard film to be detectable using this assay.

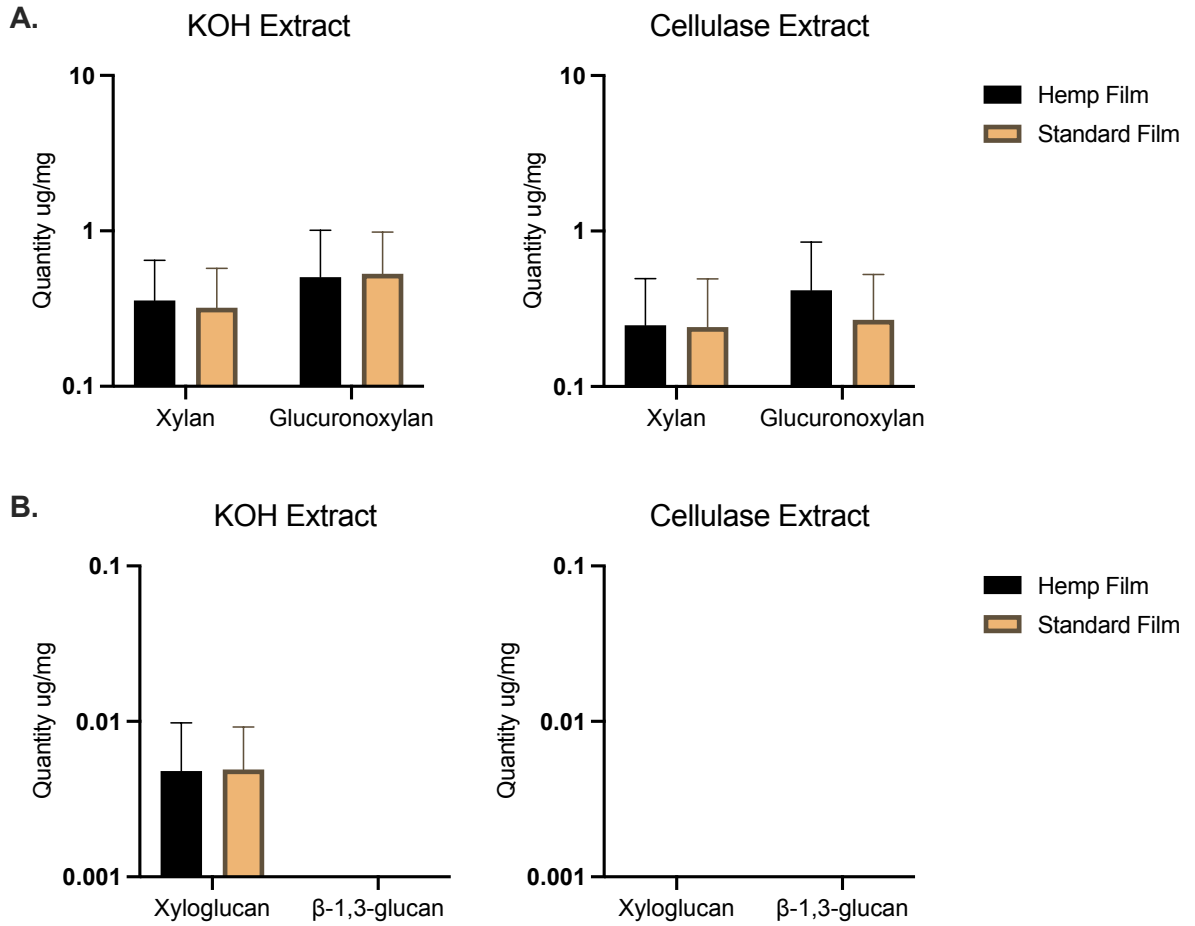


Figure 15. Quantification of hemicellulosic epitopes in a 5% hemp film compared to standard film. *Amount (µg/mg of total sample) of **A**, xylan and glucuronoxylan, and **B**, xyloglucan and ß-1,3-glucan in fully processed hemp and standard films shown in KOH and cellulase extracts of each sample. Error bars represent the standard deviation of triplicate results.*

To further evaluate the similarities and differences, a complimentary technique for the structural characterisation of lignocellulosic based materials was used. FT-IR spectroscopy allows the assignment of specific wavelengths to bonding or functional group structures according to the location, shape and intensity of the bonds, vibrational and stretching profile (Bian et al., 2012).

The FT-IR spectra of the pulps are shown in Figure 16A, and the spectra of two fully processed films in Figure 16B. The hemp pulp, as previously mentioned was a product from partial processing of hemp waste whereas the bahia pulp is a product of eucalyptus, which has

undergone bleaching and contains approximately 95% cellulose. The standard film was produced from bahia pulp and hemp film contained 5% (w/w) hemp pulp.

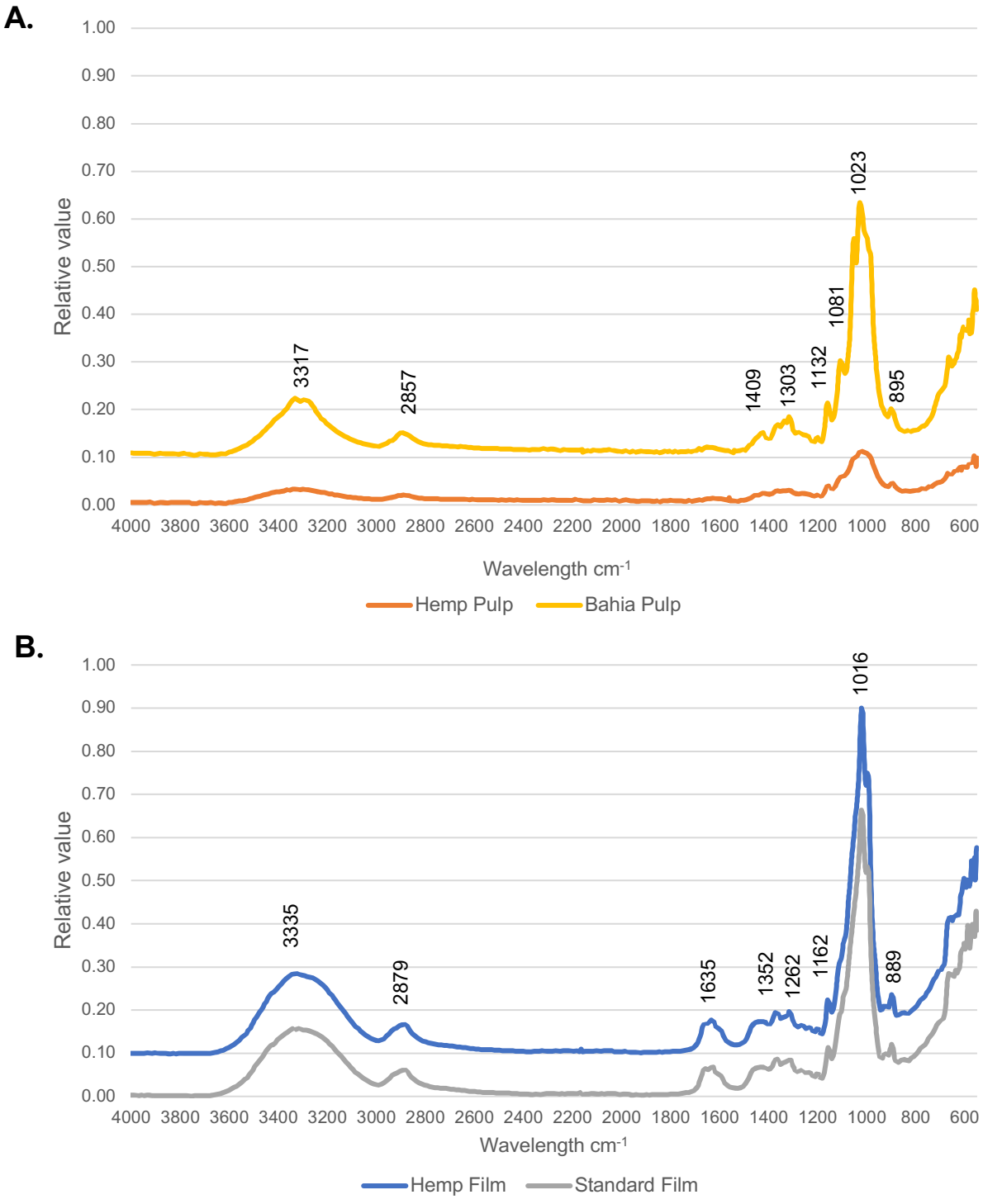


Figure 16. FTIR spectra of pulps and films. *Relative values for % transmittance of* ***A,*** *hemp and bahia wood partially processed pulps, and* ***B,*** *5% hemp and standard films. Spectra have been stacked and normalized to the C-O stretching band of microcrystalline cellulose (avicel).*

The peak intensities of the bahia pulp compared to hemp pulp were much stronger across the spectra. In comparison, the peak intensities of the hemp and standard films were very similar.

The broad peaks at 3317 cm^{-1} and 3335 cm^{-1} were due to hydroxyl (OH) group stretching vibrations. The two peaks in Figure 16B at 2879 and 1352 cm^{-1} can be assigned to CH bending and CH_2 stretching vibrations (Chen et al., 2014), however only the CH bending peak was visible in the pulps at 2857 cm^{-1} in Figure 16A. The mild peak in bahia pulp at 1409 cm^{-1} can be assigned to CH_2 scissoring. This peak is not present in either of the film spectra however is characteristic of cellulose, suggesting it might have moved or it might have been absorbed by the peak at 1352 cm^{-1} (Abidi et al., 2014). The peaks at 1635 cm^{-1} in the hemp and standard films are due to O-H bending which is characteristic of absorbed water. This peak was not present in either of the pulps. The peaks at 1303 cm^{-1} in the pulps and 1262 cm^{-1} in the films can be assigned to C=O stretching. The peak at 1132 cm^{-1} in the pulps and 1162 cm^{-1} in the films was due to C-O-C bond stretching vibrations (Abidi et al., 2014). A peak only present in bahia pulp at 1081 cm^{-1} but also characteristic in cellulose at 1079 cm^{-1}, can be assigned the anti-symmetric in-plane stretching band. The most intense peak evident in all spectra at 1023 cm^{-1} in the pulps and 1016 cm^{-1} in the films can be assigned to the C-O contribution of glycosidic linkages, characteristic in polysaccharide chains. The peaks at 895 cm^{-1} in the pulps and 889 cm^{-1} in the films are characteristic of ß-linkages (Bian et al., 2012), highlighting that a large proportion of the component backbones within the samples are ß-linked. The spectra of the two films were extremely similar which further suggests that the addition of 5% (w/w) hemp pulp did not alter the film composition to a detectable level.

In summary, the processing of raw wastes into pulp was successful in reducing the presence of hemicellulosic epitopes of xylan, glucuronoxylan in both tomato and hemp. The presence of an epitope of xyloglucan was also reduced in tomato pulp however this polysaccharide and the ß-1,3-glucan in waste and pulp were only present at trace level. Overall, the processing was successful in eliminating xylan and glucuronoxylan hemicelluloses thus purifying the cellulose samples. Further integration of hemp pulp into 5% (w/w) film showed just traces of hemicellulosic epitopes with ß-1,3-glucan completely removed from the sample and this material showed no distinguishable features in terms of composition when compared to standard films. The profiles were supported by FT-IR analysis. These results suggest that the overall processing of waste to final film is successful in cleaning cellulosic film to the extent where just trace hemicellulose components remain. This indicates that hemp waste (and potentially tomato waste) can be used in the production of sustainable films reducing the economic and environmental cost of sourcing wood and of discarding waste in landfills.

4.3. Comparison of the composition of raw wastes and processing by-products

The effect of processing has shown to reduce the quantity of hemicellulosic epitopes in pulps and films. During industrial processing of raw brewers' spent grain, two by-products were produced. One by-product was thought to have extracted mainly ß-glucans and the other, mainly proteins. The composition of these two by-products could be of importance as a valuable source of materials/components for other industrial applications, improving the sustainability of the process even further.

The composition of raw brewers' spent grain waste and ß-glucan by-product was studied with a range of mAbs specific to pectic and hemicellulosic epitopes (Figure 17). In the KOH extract, epitopes of xylan (LM11) and glucuronoxylan (LM28) were relatively higher in the by-product than the raw waste, whereas in epitopes of α-arabinan-RGI pectin (LM6), homogalacturonan (LM19), mannan (LM21), and xyloglucan (LM25), there was no difference between the raw waste and the by-product. In the cellulase extract there was only higher levels of xylan epitope (LM11) in the by-product compared to the raw waste and reduction in levels of epitopes of

homogalacturonan (LM19) and glucuronoxylan (LM28) and no difference in epitope detection of α-arabinan (LM6), mannan, (LM21) and xyloglucan (LM25). The results suggest that there is effective concentration of xylans in the brewers' spent grain by-product compared to the raw waste, thus potentially this could be the main polysaccharide to be extracted from this material.

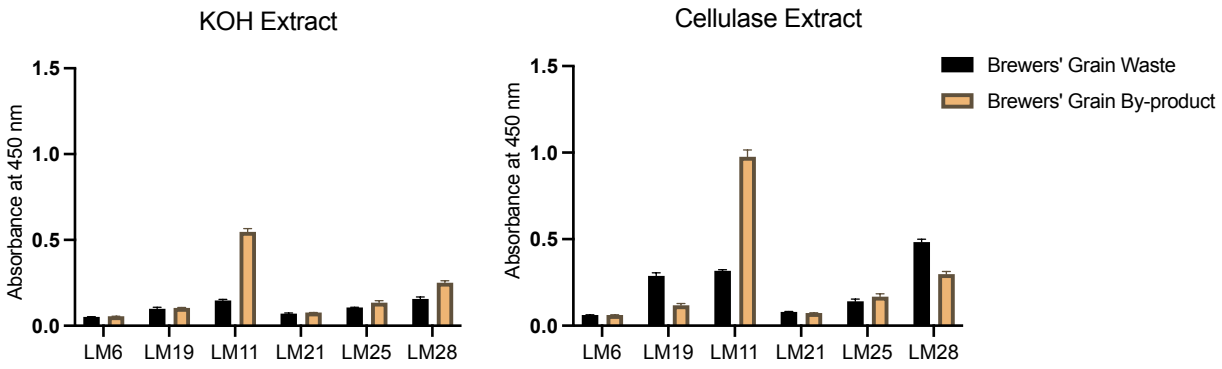


Figure 17. ELISA analysis of polysaccharide composition in raw brewers' spent grain waste and processing by-product. *Overview of the cell wall polysaccharide epitope signals in KOH and cellulase extracts using mAbs for pectins, hemicelluloses and glycoproteins described in Table 2. Error bars represent the standard deviation of triplicate results.*

Quantitative analysis in brewers' spent grain waste compared to a processing by-product (Figure 18) indicated that in the KOH extract there was 99.79 µg/mg and 62.53 µg/mg of xylan and glucuronoxylan respectively in the brewers' grain by-product compared to only 20.16 µg/mg and 33.42 µg/mg in the raw waste. The data further supports that the processing method is effective in concentrating these epitopes within the by-product. There was no difference in epitope levels of xyloglucan which was present in trace amount. In the cellulase extract xylan was still present in the by-product at higher levels than in the raw waste (184.89 µg/mg vs. 53.04 µg/mg). The amount of xylan epitope in the cellulase extract of the by-product was higher than in the KOH extract, suggesting xylan was better extracted when digesting the remaining cellulose. Glucuronoxylan in the by-product was also extracted with cellulase and only trace amounts of xyloglucan.

It was reported by Mussatto and Roberto (2005), that brewers' spent grain contains around 21.8% arabinoxylan as the major hemicellulose present in the grain. In this investigation the major component of brewers' spent grain waste was glucuronoxylan and in the by-product, xylans. Although quantification was carried out using a standard curve generated using commercial xylan and the specific mAbs, LM11, this antibody also detects arabinoxylan, so the reported quantity of xylan could also represent arabinoxylan present in the sample. The curve of calibration used for calibration was made using commercial xylan (not arabinoxylan) thus it could explain why the reported level of xylan/arabinoxylan is lower in this investigation than reported in the literature.

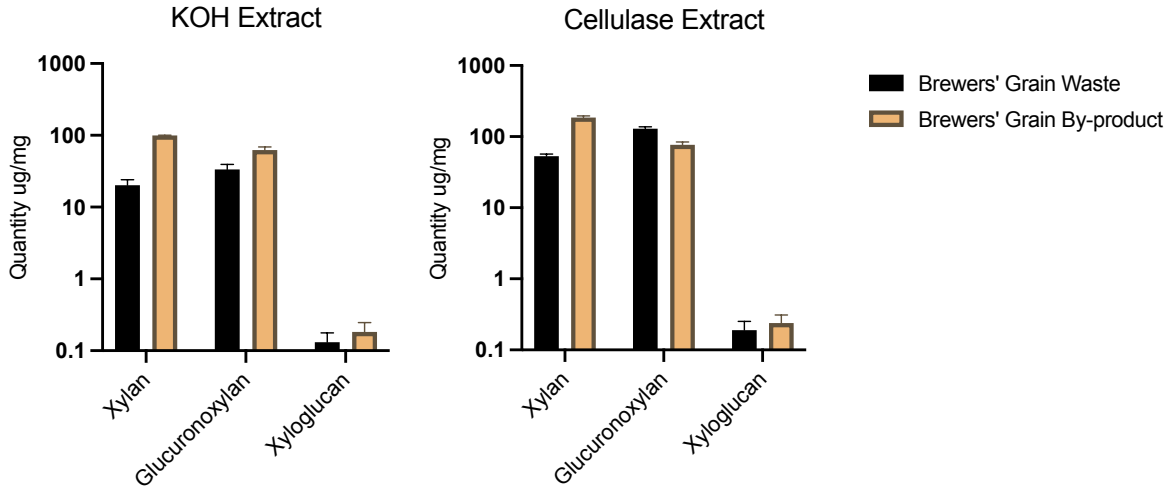


Figure 18. Quantification of hemicellulose epitopes in raw brewers' spent grain waste compared to partially processed brewers' spent grain pulp*. Amount (µg/mg of total sample) of xylan with LM11, glucuronoxylan with LM28 and xyloglucan with LM25. Quantification of each epitope shown in KOH and cellulase extracts. Monoclonal antibody-based ELISA was performed using standards of increasing concentrations with the specific primary antibody for the epitope, to generate a standard linear regression curve with equation to calculate the quantity of each epitope in the sample (Figure 6). Error bars represent the standard deviation of triplicate results.*

In an attempt to optimize the use of brewers' spent grain waste, a protein extraction was performed by Futamura. Protein quantification in brewers' spent grain waste was carried out and compared to the company protein extraction (Figure 19). The brewers' spent grain by-product was higher in protein than in the raw waste, suggesting that the extraction method used by the company was successful in concentrating protein from the brewers' spent grain waste. The amount of protein obtained was of ~50 µg/mg of extract. The results are consistent with the results from a study conducted by Mishra et al. (2016). The results highlighted the production of a protein rich liquor during the alkaline treatment of brewers' spent grain waste to produce cellulose nanofibrils. Other studies conducted by Kanauchi et al. (2001) and Mussatto and Roberto (2005), reported a protein content of around 24% and 15.2% (w/w) respectively in the dry mass of spent brewers' grain. These figures are much higher than those obtained in this investigation with only 0.16% (w/w) in raw spent brewers' grain waste and 3.82% (w/w) of protein in the extract. A potential explanation for the lower protein levels could be the methods used for the protein extraction but also the source of the waste material. There are various brewers' spent grain protein extraction methods documented which differ in the resulting protein yields. Extraction methods include alkaline extraction, acid extraction, enzyme treatment and filtration with ultrasonic assisted treatment and pulsed field electric treatments also being utilised as more novel methods (Zannini et al., 2021). Using a combination of methods alongside pre-treatment has been shown to offer the optimum protein extraction yield (Tang et al., 2009; Rommi et al., 2018; Qin et al., 2018). The method, or combination of methods, used in the waste processing to obtain the protein by-product in this investigation could be a factor that need further optimization.

Characterisation of the proteins present in the pulp was not carried out, however Brijs et al. (2006), reported that brewers' spent grains contained hordeins, albumins, globulins and glutelin. A high level of amino acids have also been reportedly present in the grains, with histidine and glutamic acid the most abundant non-essential amino acids and lysine the most abundant essential amino acid (Jacob et al., 2012). The high level of lysine can be of particular

interest due to limited levels in other cereal foods which are used for human consumption (Al-Aseeri et al., 2003). Brewers' spent grain has previously been used in animal nutrition and as a food applicant in bread, biscuits, and snacks, where the high protein levels and essential amino acid profile can be beneficial (Zannini et al., 2021). Brewers' spent grain protein could also be used as an additive in these same applications as well as in the production of biodegradable film composites. Lee et al. (2015) used brewers' spent grain protein to produce a composite, biodegradable film with chitosan. The films also had antimicrobial and antioxidant activity.

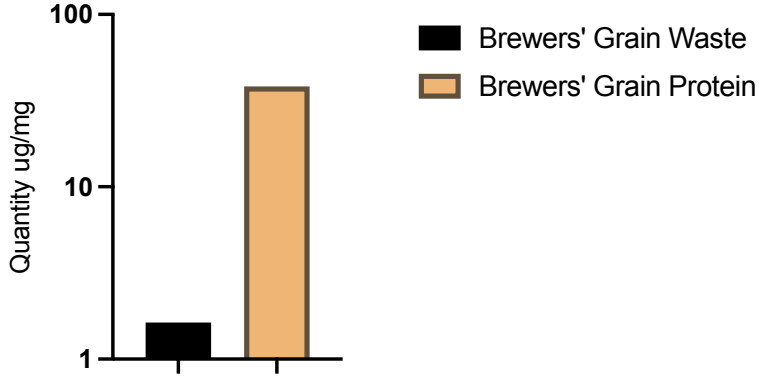


Figure 19. Protein determination of raw brewers' spent grain waste compared to a processing by-product of brewers' spent grain. *Amount (µg/mg of total sample) was determined using a Bio-Rad Assay using BSA standards of increasing concentration to generate a standard linear regression curve with equation (Figure 7) to calculate the quantity of protein in each sample. Error bars represent the standard deviation of triplicate results.*

In summary, although the amount of protein present in the brewers' spent grain waste and protein pulp by-product were not very high, the process was successful in extracting some proteins from the waste which have the potential to be utilised in various industrial applications. The presence of xylan/arabinoxylan and glucuronoxylan in the ß-glucan processing by-product pulp of brewers' spent grain highlights a potential source for the extraction of these hemicelluloses. The utilisation of proteins and hemicelluloses of industrial value from processing by-products improves the efficacy of the lignocellulosic waste processing line increasing the overall sustainability.

4.3. Assessment of the immune response for specific hemicellulose structures present in waste materials

To investigate the effect of ß-1,3-glucans, and plant hemicelluloses of interest, quantified in waste materials, on the induction of inflammation, a murine reporter cell model was employed (Figure 8). These initial experiments utilised commercial purified compounds rather than cell wall extracts to pinpoint the potential source of the effect.

Xylan extracted from beechwood, arabinoxylan extracted from wheat, and xyloglucans extracted from tamarind seed were elected, as these hemicelluloses were present at relatively high level in the agricultural wastes. All three hemicelluloses, xylan, xyloglucan and arabinoxylan showed a positive correlation between concentration and SEAP release (Figure 20A). Xyloglucan gave the greatest fold increase of SEAP release at each concentration with a maximum fold increase of 9.1 at 5000 µg/mL compared to xylan with a maximum of 6.4 at

5000 µg/mL and arabinoxylan with a 2.7-fold increase at 5000 µg/mL. The positive control, LPS (TLR4 agonist), induced a 12.5-fold increase.

The increased inflammatory signal observed in xyloglucan was inconsistent with a study conducted by Lin et al. (2018), where tamarind xyloglucan gave no significant inflammatory signal compared to a control. However, the concentration of xyloglucan was low at 300 mg/kg (equivalent to 0.3 µg/mg) and no dose increase was included. In the present study, at the equivalent of 0.3 µg/mL xyloglucan, there was no difference in inflammatory signal compared to the control so direct comparisons between the present study and previous study cannot be made. Less has been reported about the other hemicelluloses in the literature.

Laminarin, curdlan and pachyman (ß-1,3-glucans from brown alga, bacterial and fungal origins (Table 1)) were also tested to mimic the effects of callose which appears at low quantity in plant cell walls. Curdlan, pachyman and cellulose are all water-insoluble ß-glucans (Gidley and Nishinari, 2009; Lean, 2006), however for the purpose of this investigation the components were required to be in solution for exposure to cultured cells. Modified carboxy-methyl (CM) curdlan, pachyman and cellulose (as a biocompatible control) were therefore used in this investigation.

Laminarin and CM curdlan were both successful in inducing inflammation in RAW-Blue™ cells (Figure 20B), with a positive correlation between concentration and SEAP release. Although laminarin induced the greatest fold increase of 8.0 at the highest concentration of 5000 µg/mL compared to the negative control, CM curdlan induced a greater fold increase of SEAP release at lower concentrations, before plateauing between 1000 and 5000 µg/mL. The positive control, LPS, induced a 12.4-fold increase. CM pachyman induced a very small fold increase of 1.5 at the highest concentration of 5000 µg/mL. The biocompatibility of CM cellulose has been described thus was used as control. Not surprisingly, this compound produced only a 1.4-fold increase in SEAP release at the highest concentration.

ß-glucans have been shown to have immunomodulatory and anti-inflammatory effects before. An investigation carried out by Luo et al. (2020), studied the effects of high and low molecular weight ß-glucans from *Agrobacterium* sp. ZX09 on anti-inflammatory activity in weaned pigs. Pigs were supplemented with high or low molecular weight ß-glucans for 21 days with a control group receiving no ß-glucan supplementation. When injected with LPS the effects of the supplementation on NF-kB activation were compared. Both high molecular weight and low molecular weight ß-glucans inhibited the LPS treatment induced NF-kB activation compared to the positive LPS control, however there was no significant difference in the inhibition between the high and low molecular weight ß-glucans. Specifically for ß-1,3-glucans, Luo et al. (2020) showed that laminarin (low molecular weight ß-glucan) and CM curdlan (high molecular weight) were effective in inducing inflammatory signaling in macrophages. CM pachyman is also a low molecular weight ß-glucan, however only minor inflammatory signal was detected. Nishitani et al. (2013) investigated the effect of treatment with a different ß-1,3;1,6-glucan, lentinan on LPS induced NF-kB increase. Lentinan was used at a single concentration (500 µg/mL) and in combination with LPS and found successful in reducing the LPS induced NF-kB increase in a gut inflammation model. The authors failed to determine the dose dependent effect or the effects of lentinan in isolation on the activation of NF-kB.

It has also been reported that particulate ß-glucans induce more pronounced immune effects than soluble ß-glucans (Dokter-Fokkens et al., 2016). It has been suggested by Reyes et al. (2011), that this is partly due to particulate ß-glucans having the ability to induce Dectin-1

clustering whereas soluble ß-glucans cannot. Laminarin is a water-soluble ß-glucan and induced the strongest inflammatory signal. Soluble CM curdlan also produced an inflammatory signal. As CM curdlan and CM pachyman were not tested in their native structures, the signals produced may not be representative of their effects as particulate ß-glucans.

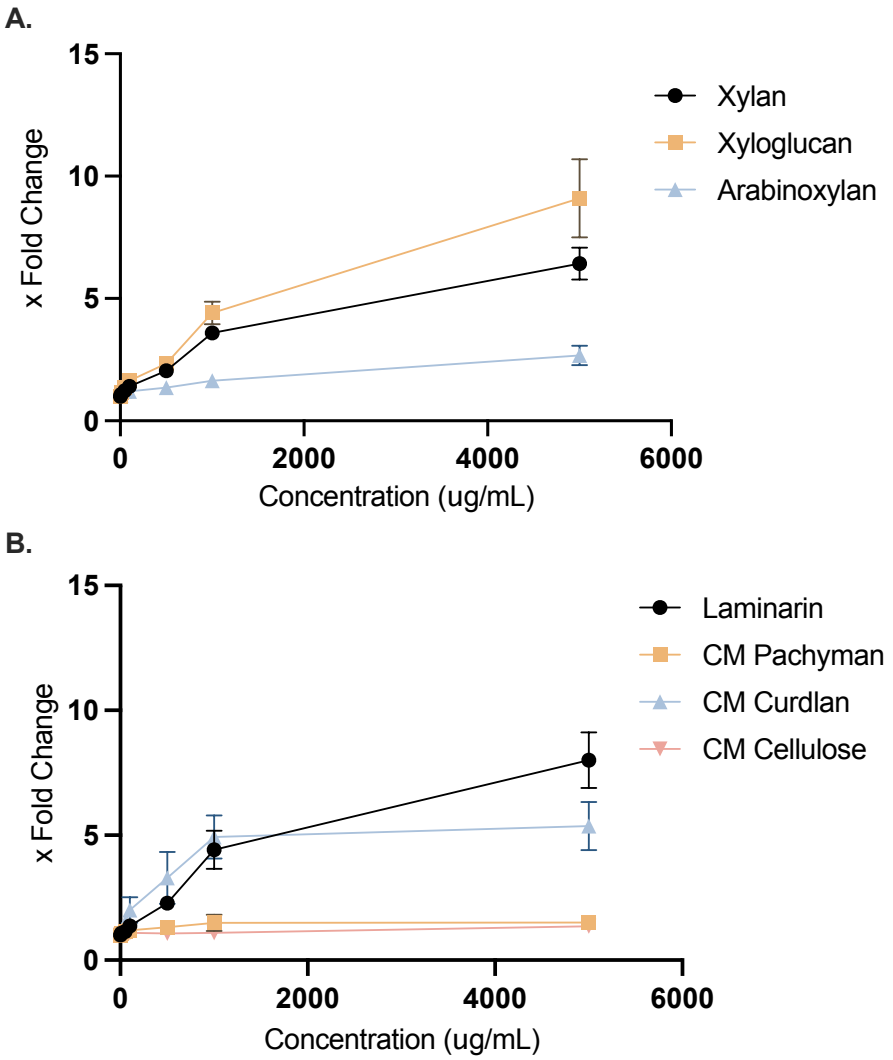


Figure 20. Dose-dependent effects of A, hemicelluloses: Xylan, Xyloglucan and Arabinoxylan and B, ß-glucans: Laminarin, CM Pachyman, CM Curdlan and CM Cellulose, on activation of an inflammatory response in RAW-Blue™ cells. *Data show fold change for individual test component absorbance against baseline values (620 nm). The data represent mean with standard deviation of 2 independent experiments performed in duplicate.*

In the present investigation, sample components were tested individually, however, given the pro-inflammatory capacity of some samples, further tests should be undertaken to study the interaction with known inflammatory triggers such as LPS. This could help establish whether ß-glucans have an additive effect or may moderate inflammatory response. In addition, the specificity of samples to activate inflammatory response via Dectin-1 receptor should be confirmed as well as findings established using *in vivo* models.

There was no visual toxicity observed in these preliminary experiments but follow up investigations should be conducted with a quantitative 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay to determine whether cell viability was

affected upon incubation with ß-1,3-glucan, and other hemicellulose compounds. In this study, concentrations between 10 - 5000 µg/mL were tested in the cell culture experiments, therefore, if cell viability permits, further investigations should be carried out at higher sample concentrations. This would be to firstly, analyse whether the SEAP release continues to increase to a plateau, and secondly, observe whether increasing concentrations cause decreased SEAP release. Possible barriers to conducting experiments at higher concentrations are the solubility of the test components alongside the pH change caused by increased concentrations of test components in solution.

A study conducted by Akaras et al. (2020), reported that ß-1,3-glucan enhanced toxicity in the testis of rats treated with bortezomib, a proteome inhibitor used in cancer treatment. The damage to the testis following treatment with bortezomib, was not eliminated by the addition of ß-1,3-glucan at a concentration of 75 mg/kg (equivalent to 0.075 µg/mg). It was further reported in the study that the ß-1,3-glucan enhanced the toxicity caused by bortezomib in the testis by decreasing levels of antioxidative enzymes which aid damage prevention. It was concluded that further studies on testis tissue should be carried out to ensure ß-1,3-glucan is safe to use as an antioxidant.

In conclusion, the plant derived hemicelluloses xyloglucan and xylan, and the ß-1,3-glucans laminarin and CM curdlan were effective in inducing inflammatory signaling in macrophages. The variation of inflammatory activation by high and low molecular weight ß-glucans in this investigation could suggest that the structure of these glucans might partly influence the response. These polysaccharides have the potential to be utilised in applications to enhance immunomodulation, although further investigations should be carried out to determine the immune response in a more complex scenario, e.g., in the presence of inflammatory triggers such as LPS. Investigations should be carried out to ensure the safety of pulps extracted from agricultural waste as adverse effects could occur if used in applications involving contact with mammalian cells. If shown to have anti-inflammatory effects similar to other ß-glucans, the hemicelluloses could be valuable in reducing the onset of inflammation in humans and animals.

Chapter 5 - Conclusions

5.1. Summary

In conclusion, alongside rich sources of cellulose, hemp and tomato wastes appear as good sources for the extraction of hemicelluloses including xylan, glucuronoxylan and xyloglucan and ß-1,3-glucan in smaller quantities and although sugarbeet waste was not as rich in hemicelluloses, they were still present in moderate amounts. The by-product pulps of brewers' spent grain were concentrated in certain hemicelluloses including xylan/arabinoxylan and protein which offers an extra source for extraction of hemicelluloses and proteins adding value to the by-product which could otherwise be wasted. Extracted hemicelluloses and proteins have the potential to be used in packaging, food, and biomedical applications, however the most effective extraction method/s for optimal yield and efficiency should be investigated.

The processing of the hemp waste into hemp pulp reduced the presence of hemicelluloses, resulting in a relatively clean pulp. Processing of the hemp pulp into film further reduced the presence of these hemicelluloses resulting in hemp film with just traces of xylans and xyloglucan. Although tomato pulp was not further processed into film the initial processing of

the waste into pulp was successful in reducing the presence of hemicelluloses. These results, alongside FTIR analysis, highlight that the processes used from waste to final product are successful in removing other cell wall components so that a clean cellulosic product remains.

The hemicelluloses xyloglucan, xylan and arabinoxylan, and the non-plant derived ß-1,3-glucans, laminarin and CM curdlan, all induced an inflammatory signal in a dose dependent manner in a mouse inflammatory reporter model. Xyloglucan and laminarin induced the greatest fold increases in the experiments at the highest concentration of 5000 μg/mL.

Lignocellulosic waste materials are not only a sustainable source for the extraction of cellulose with many industrial applications but also a source for extraction of hemicelluloses which also have various applications including as immunomodulators, functional food ingredients, in biomedical technologies and in modification of biodegradable materials. The processing of these wastes can lead to functional cellulosic materials but also by-products, containing hemicelluloses and proteins.

5.2. Future work

Following this project, various areas have been identified that have the potential for further investigation. The interactions of different hemicellulose and pectin epitopes with cellulose could be investigated, using a wider range of mAbs to obtain a more detailed glycome profile of the cell wall. This more detailed glycome profiling could be carried out for agricultural wastes studied in this project, alongside other sources that have yet to be studied in detail. This could help identify further sustainable sources for the extraction of cellulose, hemicelluloses, and other cell wall components. Other analytical techniques such as NMR could be employed to further investigate the interactions of hemicelluloses and pectins with cellulose identifying functional groups unbound or involved in linkages.

The interactions of cellulose and other cell wall biopolymers could also be investigated by forming composite hydrogels. This has previously been carried out with callose in a study by Abou-Saleh et al. (2018), however other cell wall hemicelluloses and pectins could also be investigated. This could offer the potential to favourably modify the mechanical properties of cellulosic materials such as films.

Following initial cell culture experiments with hemicelluloses and non-plant derived ß-glucans, cell viability assays should be carried out to ensure cytotoxicity doesn't occur at the concentrations tested in this project. If there is no onset of cytotoxicity observed, higher concentrations of test compounds could be exposed to the cells however a barrier to this, as previously mentioned, is the solubility and pH of test compounds in high concentrations. Further experiments could also be carried out in the presence of LPS to investigate whether the cell wall polysaccharides can inhibit an LPS induced inflammatory response, and to what extent.

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