

# **Extraction and Modification of hemicellulose from Wheat bran to produce entrapment materials for the controlled release of chemicals and bioactive substances**

*by*

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Thesis presented in partial fulfilment  
of the requirements for the Degree

The crest of Stellenbosch University, featuring a shield with various symbols, topped with a crown and a figure holding a torch. The motto 'Pectora roburant cultus recti' is inscribed on a ribbon below the shield.  
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## Abstract

Hydrogels are a network of cross-linked hydrophilic polymers that form an insoluble three-dimension structure capable of imbibing large amounts of fluid and they can be used as entrapment matrices for substances. However, synthetic polymers used for the formation of entrapment matrices cause health and environmental concerns, which leads to a need for environmental benign alternatives. Hemicellulose biopolymer is a potential replacement for the petroleum based materials as it is abundant, renewable and biodegradable. Agro-residues such as wheat bran are an abundant and affordable source of hemicellulose as they are not primary food products and would reduce environmental concerns related to their disposal. However, hemicellulose is bound to other components in a complex structure and extraction methods need to be carefully considered to maintain functional properties required for formation and performance of entrapment materials as slow release devices. Furthermore, hemicelluloses are water soluble due to the presence of side groups along the backbone and low molecular weight and that limits its application as insoluble entrapment matrices. The solubility of hemicellulose can be modified physically, chemically and enzymatically to produce insoluble entrapment matrices or hydrogels for the delivery of chemical and bioactive substances. The aim of this study was to develop insoluble entrapment matrices from soluble hemicellulose extracted from wheat bran agro-residue as delivery devices for chemical and bioactive substances. The type of hemicellulose extracted from wheat bran was arabinoxylan.

The extraction of soluble arabinoxylan from wheat bran was performed using alkaline method after pre-treatment of wheat bran to remove starch. A full factorial design was used to determine the effects sodium hydroxide (NaOH) concentration, solid loading, extraction time and temperature on xylan extraction. A face centered central composite design was subsequently used to optimize the significant ( $p < 0.05$ ) factors sodium hydroxide concentration, extraction time and temperature with yield and purity as the dependent variables. The highest arabinoxylan yield and purity in terms of arabinoxylan content was 63% and 53%, respectively. The extracted arabinoxylans contained no monomeric sugars indicating that they were in polymeric form and thus were suitable to form stable hydrogels.

The extracted hemicellulose was used to form insoluble hydrogels using: (1) coacervation method based on neutralizing an alkaline xylan solution with acid and (2) side chain removing enzyme  $\alpha$ -arabinofuranosidase that cleaved the arabinose side groups along the xylan backbone. The hydrogels were applied as delivery systems for gallic acid. The hydrogels were assessed for size, stability, encapsulation efficiency of gallic acid, antioxidant activity of encapsulated gallic acid and chemical structure. The wheat bran arabinoxylan hydrogels formed were in nanosize range (469 – 678nm), however chemically formed hydrogels were smaller compared to enzymatically formed hydrogels. Enzymatically formed hydrogels had smaller size distribution with a polydispersity index (PDI) of up to 0.3 whilst chemically formed hydrogels had a broad size distribution and PDI of 1. In addition, the zeta potential of enzymatically formed hydrogels was more negative compared to chemically formed hydrogels. The more negative zeta potential indicated agglomeration of hydrogels is less, hence enzymatically formed hydrogels were more stable than chemically formed hydrogels. The encapsulation efficiency was up to 72% and 59% for chemically and enzymatically formed hydrogels, respectively. It is worth noting that encapsulating gallic acid before and after formation of hydrogels resulted in differences in encapsulation efficiency. The enzymatically formed hydrogels better preserved the integrity of encapsulated gallic acid with an antioxidant activity of 91% as compared to 80% for chemically formed hydrogels. The release of gallic acid was sustained when the encapsulation of gallic acid occurred during the formation of hydrogels as compared to encapsulation after the formation of hydrogels. Despite enzymatic hydrogels having lower encapsulation efficiency, they showed sustained release of encapsulated gallic acid.

In conclusion, chemically and enzymatically modified wheat bran arabinoxylan hydrogels were formed with the ability to entrap and release gallic acid.

## Opsomming

‘n Hidrojel is ‘n netwerk van kruisgebonde hidrofiliese polimere wat ‘n onoplosbare drie-dimensionele struktuur vorm. Dit is in staat om groot hoeveelhede vloeistof op te neem en kan gebruik word om verbindings vas te vang. Daar bestaan egter kommer oor die gesondheids- en omgewingsimpak van die sintetiese polimere wat gebruik word in die produksie van sulke vasvang matrikse. Derhalwe bestaan daar ‘n behoefte aan omgewingsvriendelike alternatiewe. Hemisellulose biopolimeer is ‘n potensiële vervanging vir petroleum gebasseerde materiale omdat dit volop, herwinbaar en biodegradeerbaar is. Landboukundige residue soos koringsemels is ‘n volop en bekostigbare bron van hemisellulose omdat dit nie primêre voedselprodukte is nie en daar bestaan minder kommer met betrekking tot hul omgewingsvriendelike wegdoening. Hemisellulose is egter gebind aan ander komponente in ‘n komplekse struktuur. Hemisellulose ekstraksiemetodes moet derhalwe deeglik oorweeg word om die funksionele eienskappe te behou wat benodig word vir die vorming en werksverrigting van verstrikkingsmatrikse as stadige vrystellingsapparate. Hemiselluloses het lae molekulêre gewigte en is verder ook wateroplosbaar weens die teenwoordigheid van sykettings al langs die polimeer ruggraat af. Hierdie eienskappe beperk die toepassing daarvan as onoplosbare verstrikkingsmatrikse. Die oplosbaarheid van hemisellulose kan fisies, chemies en ensiematies gewysig word om onoplosbare verstrikkingsmatrikse of hidrojels te produseer vir die aflewering van chemiese en bio-aktiewe substansie. Die doel van hierdie studie was om onoplosbare verstrikkingsmatrikse te ontwikkel vanuit oplosbare hemisellulose, geëkstraheer uit koringsemel landboukundige residue, om te dien as afleweringstelsels vir chemiese en bio-aktiewe substansie. Die tipe hemisellulose wat geëkstraheer is uit koringsemels was arabinoxilaan.

Die ekstraksie van oplosbare arabinoxilaan uit koringsemels is verrig deur gebruik te maak van ‘n alkaliese metode na afloop van vooraf behandeling van die koringsemels om stysel te verwyder. ‘n Volledige faktorale ontwerp is gebruik om die effek te bepaal van natriumhidroksied (NaOH) konsentrasie, soliede lading, ekstraksie temperatuur en ekstraksietyd op xilaan ekstraksie. ‘n Gesig-gesentreerde sentraal saamgestelde ontwerp is gebruik om die statisties noemenswaardige ( $p < 0.05$ ) faktore, naamlik natriumhidroksied konsentrasie asook ekstraksietyd en –temperatuur, te optimaliseer met opbrengs en suiwerheid as die afhanklike veranderlikes. Die hoogste arabinoxilaan opbrengs en

suiwerheid in terme van arabinoxilaan inhoud was 63% en 53%, onderskeidelik. Die geëkstraheerde arabinoxilane het geen monomeriese suikers bevat nie, wat aandui dat hulle in polimeriese vorm was en daarom geskik was om stabiele hidrojels te vorm.

Die geëkstraheerde hemisellulose is aangewend om onoplosbare hidrojels te vorm deur gebruik te maak van: (1) 'n faseskeidingsmetode gebaseer op die neutralisasie van 'n alkaliese xilaanoplossing met suur en (2) die syksetting verwyderende ensiem  $\alpha$ -arabinofuranosidase wat die arabinose syksettings afgesplyt het al langs die xilaan ruggraat af.

Die hidrojels is aangewend as afleweringstelsens vir gallusuur. Die hidrojels is geassesseer in terme van grootte, stabiliteit, inkapselingseffektiwiteit vir gallusuur, anti-oksidant aktiwiteit van gallusuur en chemiese struktuur. Die koringsemel arabinoxilaan hidrojels wat gevorm is, het grootte gehad wat gevarieer het in die nanogrootte gebied (469-678 nm). Chemies gevormde hidrojels was egter kleiner as ensiematies gevormde hidrojels. Ensiematies gevormde hidrojels het 'n kleiner verspreiding in grootte gehad met 'n polidispersie indeks (PDI) van tot 0.3, terwyl chemies gevormde hidrojels 'n wye verspreiding in grootte gehad het en 'n PDI van 1. Daarbenewens was die zeta potensiaal van die ensiematies gevormde hidrojels meer negatief in vergelyking met dié van chemies gevormde hidrojels. Die meer negatiewe zeta potensiaal het aangedui dat agglomerasie van hidrojels tot 'n mindere mate plaasgevind het, met die gevolg dat die ensiematies gevormde hidrojels meer stabiel was as die chemies gevormde hidrojels. Die inkapselingseffektiwiteit was so hoog as tot 72% en 59% vir chemies en ensiematies gevormde hidrojels, onderskeidelik. Dit is noemenswaardig dat verskille in inkapselingseffektiwiteit waargeneem is na gelang daarvan of die inkapseling van gallusuur plaasgevind het voor of na die vorming van hidrojels. Die ensiematies gevormde hidrojels het die integriteit van die geïnkapselleerde gallusuur beter bewaar met 'n anti-oksidant aktiwiteit van 91% in vergelyking met 80% vir die chemies gevormde hidrojels. Volgehoue vrystelling van gallusuur is waargeneem wanneer die inkapseling van gallusuur plaasgevind het gedurende die vorming van hidrojels in vergelyking met inkapseling na die vorming van hidrojels. Alhoewel ensiematies gevormde hidrojels laer inkapselingseffektiwiteit gehad het, het hierdie jels volgehoue vrystelling van geïnkapselleerde gallusuur getoon.

In hierdie studie is dus chemies en ensiematies gewysigde koringsemel arabinoxilaan hidrogele gevorm, wat die vermoë het om gallusuur vas te vang en vry te stel.

## **Dedication**

To my beloved parents Mr Clement Matavire and Mrs Grammy Matavire for their continuous love and support.

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## Abbreviations and symbols

Ara	Arabinose
AX	Arabinoxylan
AGX	(Arabino)glucuroxylan
AFB	$\alpha$ -Arabinofuranosidase
Araf	$\alpha$ -Arabinofuranose
Agu1	$\alpha$ -Glucuronidase
DP	Degree of polymerization
DS	Degree of substitution
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EGCG	Epigallocatechin gallate
FA	Ferulic acid
FT-IR	Fourier infrared spectrophotometer
GAX	(Glucurono)arabixylans
GX	Glucuronoxylans
HEMA	2-Hydroxyethyl methacrylate
HX	Homoxylans
HCL	Hydrochloric acid
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
ICP-MS	Inductively coupled plasma mass spectrometry
KOH	Potassium hydroxide
MTT	3-(4,5-Dimethylthiazol-2-yl)-,5-diphenyltetrazolium bromide
MeGlcA	4-O-Methylglucuronic acid
MW	Molecular weight
NREL LAP	National Renewable Energy Laboratory Analytical Procedure

NaOH	sodium hydroxide
NaBH <sub>4</sub>	sodium borohydride
PBS	phosphate buffer
PNP	p-nitrophenyl $\alpha$ -L-arabinofuranosidase
PEG	Plasticiser polyethylene glycol
TC	terephthaloyl chloride
SEM	Scanning electron microscope
SEC	Size exclusion chromatography

## Chapter 1: Introduction

Hydrogels are formed by crosslinking of hydrophilic polymers to form insoluble 3D network structures, which have an ability to swell more than 20% of their dry weight by absorbing water, chemical or biological substances. The absorbed liquids are retained by entrapment or adhesion on the surface area of the 3D-network. These three-dimensional structures have several industrial applications such as in oil recovery, pharmaceutical, textile and water treatment (Laftah, Hashim & Ibrahim, 2011; Ullah, Othman, Javed, Ahmad & Akil, 2015) due to their ability to take up substances and release them. In the medical and pharmaceutical field, hydrogels are used as drug delivery systems for controlled release of drugs to increase its therapeutic effect through controlled release. Thus these hydrogels may protect drug from degradation, target drugs to specific sites of action, and ameliorate the side effects or toxicity of the drugs (Soppimath, Aminabhavi, Dave, Kumbar & Rudzinski, 2002). The challenges of hydrogels include non-biodegradability, unfavorable thermal and mechanical properties, and toxicity (Ullah *et al.*, 2015). Furthermore, many entrapment matrices are formed using synthetic polymers that pose environmental concerns such as non-biodegradability, non-renewability, greenhouse emissions and limited fossil reserves. Therefore, there is need for alternative natural, biodegradable and renewable polymeric materials for the formation of entrapment materials. Plant biomass would be a good alternative to synthetic polymers because of its renewability and abundance (Fonseca Silva, Habibi, Colodette & Lucia, 2011; Peng & She, 2014).

There is a range of plant biomass sources, but agro-residues would be the most suitable source of natural polymers because of their low-cost, that they are not primary food components and that their use would reduce pollution caused by their disposal. The use of agro-residues such as wheat straw, corn bran and rice husk to obtain natural biodegradable polymers for formation of entrapment matrices would add value to the agro-residues (Deutschmann & Dekker, 2012).

Agro-residues are rich in lignocellulose, which is composed of three main components, which are cellulose, hemicellulose and lignin that can be potential sources of natural polymers. Cellulose, which is most abundant polymeric material has already a wide range of applications and hemicellulose the second most abundant polysaccharide in nature (making up to 20 –

35% of the biomass) would be a biopolymer that could be used to form entrapment materials. Hemicelluloses have the advantage of being biodegradable, non-toxic, biocompatible and safe. Hemicelluloses have been found to have pharmacological applications such as scavenging cholesterol and bile acids, induction of immune response, cough-suppressing effect and improving food digestion (Lopez, Levrat, Guy, Messenger, Demigné & Rémésy, 1999; Prisenžňáková, Nosáľová, Hromádková & Ebringerová, 2010; Zhou, Liu, Guo, Wang, Peng & Cao, 2010; Hu & Yu, 2013). In addition, hemicelluloses are attractive biopolymers due to their structural variability and their physicochemical properties that can be modified to form different high value entrapment matrices (Zhang, Pitkänen, Douglade, Tenkanen, Remond & Joly, 2011). Of the different types of hemicelluloses, xylan is the most abundant and hence will be readily available for exploitation.

The xylan polymer is reported to be made up of  $\beta$ -1, 4-linked xylose residues that are mainly substituted with arabinose, glucuronic acid and or 4-O-methylglucuronic acid, and acetic acid along the backbone (Saha, 2003; Eduardo da Silva, Rodrigues Marcelino, Christine Salgado Gomes, Eleamen Oliveira, Nagashima Jr & Sócrates Tabosa Egito, 2012). However, the presence of side groups such as arabinose along the xylose backbone and the hemicellulose's low molecular weight result in xylan being water soluble thereby limiting their application as insoluble entrapment materials (Ebringerová, Hromádková & Heinze, 2005; Zhu Ryberg, Edlund & Albertsson, 2011). Furthermore, the xylan is bound to other components of the lignocellulose in a complex structure by strong bonds (Otieno & Ahring, 2012; Peng, Peng, Xu & Sun, 2012) and it is important to carefully select an extraction method that is suitable to obtain high molecular weight xylan in polymeric form. In addition, a high extraction yield of xylan would be beneficial to meet a high demand of the entrapment materials. Xylan is co-extracted with other components of lignocellulose and these contaminants may affect the modification of xylan into entrapment materials for the slow release of substances. Optimizing the extraction of polymeric xylan for high yield and xylan content would be beneficial.

The soluble hemicellulose extracted from agro-residues can be modified into insoluble entrapment materials for the controlled release of chemicals using chemical, physical or biological methods (Gomes et al. 2015; Chimphango et al. 2012b). The partial removal of substituents on the xylan backbone or altering the substitution pattern leads to precipitation

of xylan forming insoluble hydrogels (Andrewartha, Phillips & Stone, 1979) that can function as entrapment matrices for controlled release of substances. When the substitution pattern is altered, the unsubstituted linear regions of the xylan chains associate with each by hydrogen and hydrophobic bonding (Linder, Bergman, Bodin & Gatenholm, 2003; Kabel, van den Borne, Vincken, Voragen & Schols, 2007) forming insoluble 3D-network. Chemical methods of modification often use toxic chemicals that can damage the entrapped material and therefore a mild chemical method would be suitable for the formation of entrapment materials for the slow release of sensitive substances. Enzymatic modification of soluble xylan into insoluble entrapment matrices for the slow release of substances are also non-toxic to incorporated substance and may allow for the *in situ* encapsulation (Chimphango, van Zyl & Görgens, 2012b). This project was focused on isolating soluble hemicellulose from wheat bran agro-residue using mild alkaline method, after which it was modified chemically and enzymatically to form entrapment matrices for the slow release of chemical and bioactive substances. These entrapment matrices were evaluated in terms of their stability, encapsulation efficiency and ability to protect encapsulated material and performance as delivery systems of substance.

## 1.1 Study Approach

Chapter 2 of this thesis reviews the literature in relation to sources of biopolymers that can replace synthetic polymers in the production of entrapment matrices. Hemicellulose was identified as a suitable alternative to synthetic polymers. The isolation, properties, modification and application of hemicellulose were reviewed. The review of literature lead to the identification of problem, aim and objectives of the study outlined in chapter 3. This study was approached in two phases, which are elaborated in chapters 4 and 5. The first phase xylan was obtained from wheat bran for the formation of insoluble biodegradable entrapment matrices (Chapter 4). The industrial wheat bran was characterized for chemical composition. The wheat was destarched and xylan was extracted using alkaline solution. The extraction process of xylan from wheat bran was optimized for the parameters temperature, NaOH concentration, time and solid loading. The performance indicators for extraction optimization were yield and xylan content. Subsequently, the isolated soluble xylyans were modified into insoluble hydrogels using a chemical method and side chain removing enzymes (chapter 5). The hydrogels produced by the two different methods were compared in terms of

morphology, size and chemical composition. Furthermore, the entrapment and controlled release of gallic acid from xylan hydrogels produced by the two different methods were investigated. Chapter 6 is a main conclusion of the study that summarises the main findings and suggests recommendation for further study.

## Chapter 2: Literature review

### 2.1 Sources of biodegradable entrapment materials

#### 2.1.1 Lignocellulose biomass

Lignocellulose biomass is a suitable source of biodegradable polymers that can be used as entrapment materials for the slow release substances. In addition, the lignocellulosic material is renewable and abundant. Lignocellulosic biomass is the non-starch, fibrous part of plant material with a complex structure (Otieno & Ahring, 2012) and its major components are cellulose (35-50%), hemicellulose (20-35%) and lignin (10-25%). Lignocellulosic biomass also contains ash (3–10%) as well as other components such as extractives (Saha, 2003; Otieno & Ahring, 2012).

The most abundant component of the lignocellulose biomass is cellulose, a linear homogenous polymeric chain of over 10 000 D-glucose units that are connected by 1, 4- $\beta$ -glycosidic linkages. The number of sugar units in a polymeric chain is considered to be the degree of polymerization (DP) (Le Floch, Jourdes & Teissedre, 2015). Although the cellulose has a high DP that would be advantageous for formation of stable entrapment materials, cellulose has many other high value applications. On the other hand, hemicellulose a short, heterogeneous branched polysaccharide composed of pentoses (xylose, arabinose), hexoses (mannose, glucose, and galactose) and sugar acids has less applications and can be used to form entrapment materials. The DP of hemicellulose is generally from 70 to 200 (Peng & She, 2014), which is lower compared to that of cellulose and that limits its application. Lignin the third abundant component of the hemicellulose is made up of an irregular polymer of aromatic alcohols known as monolignols such as p-coumaryl, coniferyl and sinapyl alcohol with different type of linkages between the monomeric units (Le Floch et al., 2015; Shen, Jin, Hu, Xiao & Luo, 2015; Yeo, Seong & Hwang, 2015). Figure 2.1 shows structure of lignocellulosic biomass containing the major components, which are cellulose, hemicellulose and lignin. The lignocellulosic biomass components are held together in a complex structure by four basic bonds that include  $\beta$ -bonds,  $\alpha$ -bonds, ether bonds and the hydrogen bonds (Otieno & Ahring, 2012). The structure of lignocellulose is very complex presents a challenge in isolation of hemicellulose with high purity and yield and without the depolymerisation of hemicellulose (Peng, Peng, *et al.*, 2012) therefore, extraction methods that are selective for hemicelluloses

are needed. There are several sources of lignocellulosic biomasses that include wood, municipal solid wastes, pulp and paper industry wastes forestry residues and agro-residues.

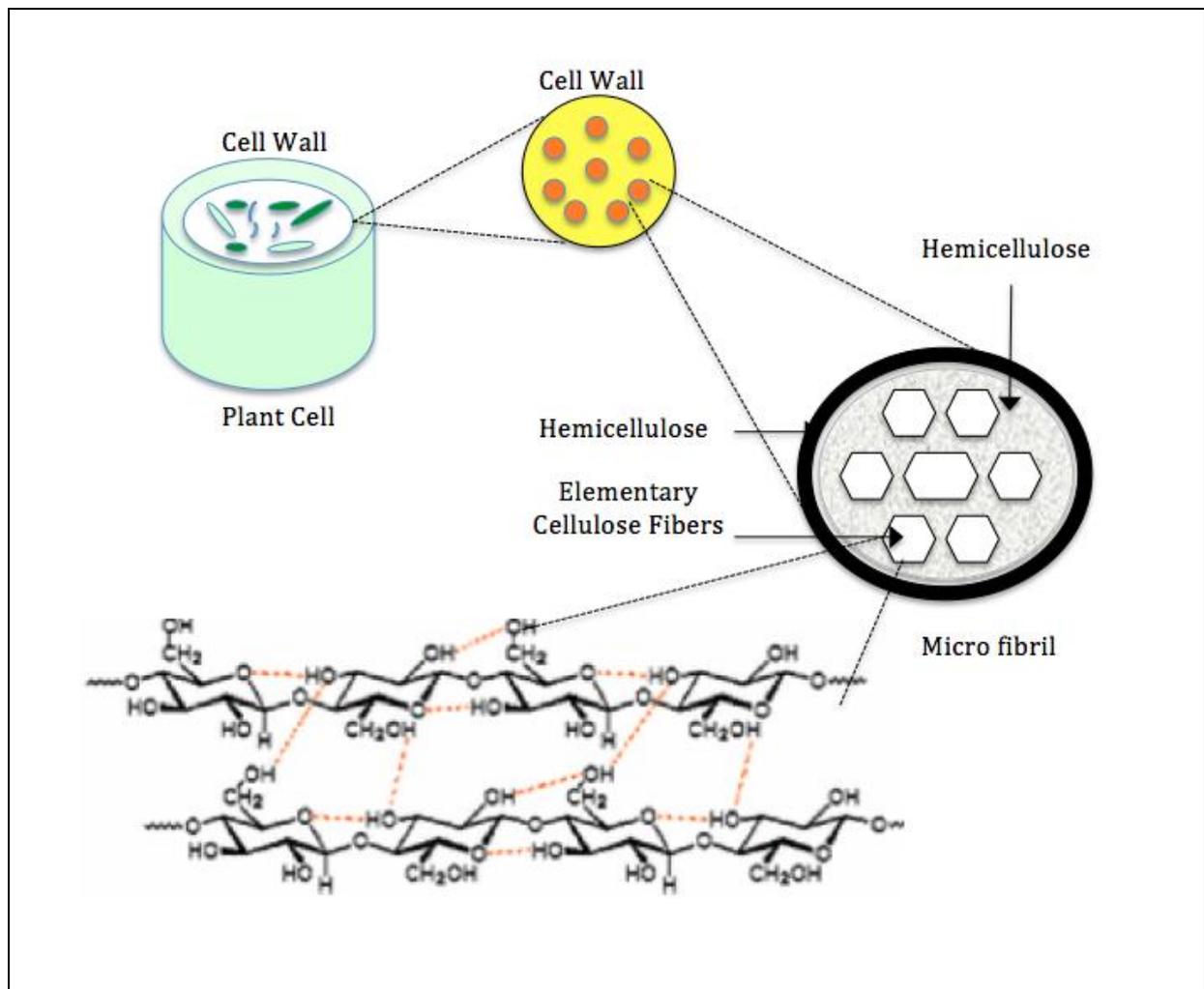


Figure 2.1: Structure of lignocellulose showing cellulose, hemicellulose and lignin (redrawn from Zhang, 2008).

### 2.1.2 Agro-residues

Agro-residues are one of the best sources of lignocellulosic material because of their abundance and this makes them ideal as source of biopolymers for the formation of biodegradable entrapment matrices. A great deal of agricultural residues is produced annually worldwide from harvesting and different industrial processes. Some of these agro-residues are disposed by burning, which causes air pollution and other environmental hazards (Ahmed, Cavalli, Wardell, Bushell & Hay, 2012). The utilization of biopolymers from agro-residues would add value to the agro-residue and reduce the environmental concerns

associated with their disposal (Höijje, Gröndahl, Tømmeraas & Gatenholm, 2005; Ahmed *et al.*, 2012). The use of agro-residues to generate biopolymers for the production of entrapment matrices would also relieve the pressure from fossil reserves and subsequently reduce the negative impact of the latter on human health and environment. The composition of lignocellulosic components may vary with each type of agro-residue as illustrated in Table 2.1. Agro-residues such as straws, cobs, stems, seeds and stalks from different crops and fruits are being used to produce high value products that include ethanol, adsorbents, chemicals and paper (Daifullah, Girgis & Gad, 2003; Ahmed *et al.*, 2012; Gupta & Verma, 2015). Based on Table 2.1, wheat bran has a higher hemicellulose content as compared to other residues. Furthermore, wheat bran has a higher composition of hemicellulose and lower cellulose and lignin content. The low cellulose and lignin content would be advantageous in obtaining less contaminated hemicellulose for the formation of entrapment matrices. In this study, wheat bran was selected as a source of hemicellulose for the production of biodegradable entrapment materials.

Table 2.1: Composition of some agro-residue lignocellulosic biomass as % dry mass

<b>Biomass</b>	<b>Cellulose</b>	<b>Hemicellulose</b>	<b>Lignin</b>	<b>Reference</b>
<b>Corn cob</b>	35	34.8	14	(Yang, Xu, Wang & Yang, 2005)
<b>Rice husk</b>	35	33	23	(Johar, Ahmad & Dufresne, 2012)
<b>Wheat bran (starch free)</b>	14.8	49.7	5.3	(Jacquemin, Zeitoun, Sablayrolles, Pontalier & Rigal, 2012)
<b>Wheat straw</b>	29.2	34.9	17.8	(Jacquemin <i>et al.</i> , 2012)
<b>Wheat bran</b>	11	51.7	3 – 10	(Maes & Delcour, 2002)
<b>Maize bran</b>	18.2	42.8	0.7	(Chanliaud, Saulnier & Thibault, 1995)
<b>Corn fibre</b>	20 - 22	37	-	(Hespell, 1998)

Wheat bran is a by-product during the milling process of wheat grain to produce white flour and it consists of some tissues of the wheat kernel, aleurone layer, endosperm and pericarp with testa attached. It is reported that wheat bran is produced in large quantities of about 150 million tonnes annually worldwide (Prückler, Siebenhandl-Ehn, Apprich, Höltinger, Haas, Schmid & Kneifel, 2014) and hence it is an abundant source of hemicellulose for applications such as entrapment matrices. Although wheat bran is used as low value animal feed, small

amounts are used for human consumption as a source of dietary fibre (Bergmans, Beldman, Gruppen & Voragen, 1996a; Prückler *et al.*, 2014). The economic value of wheat bran as animal feed is decreasing and therefore, it can be used for the production of high value materials (Bergmans *et al.*, 1996a) such as formation of entrapment materials. In addition to hemicellulose, cellulose and lignin, wheat bran also contains constituents such as proteins, phenolic acids, flavonoids,  $\beta$ -glucans, phytic acid and ash (DuPont & Selvendran, 1987; Bergmans, Beldman, Gruppen & Voragen, 1996b; Apprich, Tirpanalan, Hell, Reisinger, Böhmdorfer, Siebenhandl-Ehn, Novalin & Kneifel, 2014).

## 2.2 Hemicellulose

Hemicellulose is the second most abundant plant cell wall polysaccharides in nature hence it is attractive to use as a renewable raw material for the formation of entrapment materials (Edlund & Albertsson, 2008). Hemicellulose structure is reported to be constituted of  $\beta$ -(1 $\rightarrow$ 4)-linked xylose chain and can be substituted with mainly O-acetyl groups, with L-arabinose, or D-glucuronic acid residues and minor components such as D-galactose, *p*-coumaric, and ferulic acid (FA) moieties linked to L-arabinose residues (Deutschmann & Dekker, 2012). There are four structurally different classes of hemicellulose, which are xylans, mannas,  $\beta$ -glucans and xyloglucans and these differ in the type and distribution of side-chains and glycosidic bonds along the main backbone. The predominant hemicellulose in hard wood is acetylated xylans and a small percentage of mannan while soft woods contain mainly galactoglucomannans and the arabinoxylans in Gramineae. Different biomasses vary in hemicellulose content and chemical structure (Ebringerová *et al.*, 2005; Peng, Peng, *et al.*, 2012; Peng & She, 2014).

### 2.2.1 Xylans

The diversity and complexity of xylan structure is beneficial for the production of many useful products. It is therefore, beneficial to exploit them for the development of entrapment matrices for the controlled release of chemical and bioactive substances. Furthermore, xylans are the most common and abundant hemicelluloses making up to 20 to 30 % of the biomass in dicotyl plants (hardwoods and herbaceous plants) and 50% in some monocotyl plants (grasses and cereals). Wheat bran which is a by-product of wheat contains mainly xylans. Xylans are reported as heterogenous polymers of 1,4-linked  $\beta$ -D-xylose units substituted with O-acetyl,  $\alpha$ -L-arabinose,  $\alpha$ -1,2-linked glucuronic acid or 4-O-methylglucuronic acid

(MeGlcA) side groups. The different types of xylan include homoxylans (HX), glucuronoxylans (GX), arabinoxylans (AX), (arabino)glucuroxylan (AGX), (glucurono)arabinoxylans (GAX), and complex heteroxylans (Ebringerová & Heinze, 2000; Ebringerová *et al.*, 2005).

The different xylans and their properties have been reviewed by Ebringerová and co-workers (Ebringerová & Heinze, 2000; Ebringerová *et al.*, 2005). Homoxylans occur in seaweed and are unsubstituted along the xylose backbone. Glucuronoxylans which are the main components of hard woods have a backbone consisting of 4-O-methylated and non-methylated glucuronic acid substituted at position 2. Glucuronoxylans are also substituted with acetyl groups in their native form and these groups are degraded during the alkaline extraction from lignocellulose biomass. (Arabino)glucuronoxylans (AGX) and (glucurono)arabinoxylans (GAX) consist of single MeGlcA and  $\alpha$ -L-Arabinose residue substituted at position 2 and 3 respectively of the xylose backbone and ferulic acid (FA) attached at position 5 of Arabinose in their native form that is lost during alkaline extraction. AGX occur mainly in lignified cell walls of cereals and grasses, whilst GAX occur in non-endospermic tissues of cereal grains. Arabinoxylans (AX) are the main hemicelluloses represented in the cell walls of flour and bran of cereal grains such as wheat and corn. AXs have a linear xylose backbone that is substituted by  $\alpha$ -L-Arabinose residue at position O-3 or O-2 and may contain phenolic acids such as FA and coumaric acid attached at position 5 of the  $\alpha$ -L-arabinose. AX can either be water-insoluble or water-soluble. Water-soluble AX have higher ratio of Arabinose (Ara) to xylose (Xyl) of 0.3 – 1.2, whereas water-insoluble have a lower Ara:xyl ratio of 0.2 – 0.3. Lastly, heteroxylans are structurally complex with a xylose backbone substituted with mono- or oligoglycosyl side chains instead of single substituents. These complex xylans occur in cereals, gum exudates, seeds and mucilages. Xylans from different sources vary in composition as well as degree of polymerisation. The degree of polymerisations of xylans in hardwoods between is 150 and 200 whilst that of softwood xylans varied from 70 to 130 (Saha, 2003).

### **2.2.2 Physiochemical and functional properties of Xylans**

The physiochemical properties of xylans are mostly influenced by their structural and molecular properties and therefore, the different xylans may vary in their properties (Ebringerová & Heinze, 2000). These various properties of xylans have been reviewed and documented (Ebringerová & Heinze, 2000; Ebringerová *et al.*, 2005).

### 2.2.2.1 Molecular weight and molecular weight distribution

Xylans have smaller molecular weight and subsequently lower degree of polymerisation (DP) as compared to other biopolymers such as cellulose. The ability of arabinoxylans to form hydrogels was found to be dependent on their molecular weight. In a study, higher molecular weight arabinoxylans resulted in increased effectiveness of crosslinking of chains to form strong gel networks with high storage modulus and consequently increasing capacity to entrap and hold water (Izydorczyk & Biliaderis, 1992). However, precipitation of xylans using recombinant enzymes was observed at a low DP of 10 (Chimphango, van Zyl, *et al.*, 2012a). The molecular weight (MW) values of xylans vary based on the method of their estimation rather than method of extraction from feedstock. The values of the molecular weights of arabinoxylans obtained by gel filtration methods are higher as compared to those found by ultracentrifugation (Ebringerová & Hromádková, 1999). The MW values of AX and HX of cereals were shown to vary between 64 000 and 380 000 g/mol whilst MW of MGX and AGX were 5 000 to 130 000 g/mol and 30 000 to 370 000 g/mol (Ebringerová *et al.*, 2005). Xylans were extracted from wheat bran using calcium hydroxide and barium hydroxide for 16 hrs at temperatures 20°C to 95°C. The different extracts were found to have similar molecular weights and distributions (Bergmans *et al.*, 1996a). The study conducted by Methacanon and co-workers on the effect of different process parameters on extraction of hemicellulose from Vetiver grass confirmed that the extraction variables showed no significant effect on the molecular weight of hemicellulose obtained (Methacanon, Chaikumpollert, Thavorniti & Suchiva, 2003). However, when barley husks were subjected to four different pre-treatment methods, which are HCL pre-treatment, HCL pre-treatment with ethanol delignification, HCL pre-treatment with chlorite delignification and enzyme pre-treatment before extracting AX, the molecular weights in aqueous system were 35 100, 34 100, 39 100, and 40 400 g/mol respectively. This led to a conclusion that the extraction method significantly influenced the molecular weight distribution (Bergmans *et al.*, 1996a; Höije *et al.*, 2005) and it will be important to select extraction methods that would not degrade the hemicellulose.

### 2.2.2.2 Solubility

The solubility of xylans is largely influenced by the degree of substitution and the pattern of distribution of substituents along the xylan backbone (Ebringerová & Heinze, 2000; Zhang, Smith & Li, 2014a). In a study, four different xylan samples i.e. DMSO-extracted beech, steam-

extracted wheat straw, alkaline extracted beech and oat spelt xylan, were investigated for their solubility properties in two aqueous solutions with different DMSO: water ratios. DMSO-extracted beech and steam-extracted wheat straw xylan showed a high DS and hence higher solubility in the aqueous systems, whereas less substituted alkaline extracted beech and oat spelt xylan showed more aggregation and the lowest solubility. This is because the latter had no acetyl groups, which enhance the solubility of the polymers (Saake, Kruse & Puls, 2001). Therefore, alkaline extracted xylans will be preferable for the production of insoluble entrapment matrices as acetyl substituents are lost. A high degree of substitution (DS) results in increased solubility of xylans in water and low solubility of the less substituted xylans is due to the association of unsubstituted regions of the xylan by intermolecular hydrogen bonds consequently increasing the viscosity of xylan solution and precipitation of xylans (Izydorczyk & Biliaderis, 1995; Zhang *et al.*, 2014a). The presence of arabinose along xylan backbone causes steric hindrances that prevent the aggregation of xylans and thus the removal of arabinose and other side groups from xylan backbone reduces their solubility (Izydorczyk & Biliaderis, 1995) leading to formation of insoluble entrapment materials.

### **2.2.2.3 Biological activity**

Xylans have been shown to have different therapeutic activities. GAX extracted from wheat bran was shown to suppress citric-acid induced cough in adult guinea pigs to a level comparable with centrally acting codeine (Prisenžňáková, Nosáľová, Hromádková & Ebringerová, 2010). Barbat and co-workers showed that 4-*O*-methylglucuronoxylan inhibited the cell proliferation, migration, and invasion ability of A431 human epidermoid carcinoma cells. Furthermore, this biological activity was found to be dependent on the distribution of MeGlcA side groups along the xylose backbone as well as the DP (Barbat, Gloaguen, Moine, Sainte-catherine, Kraemer, Ropartz & Krausz, 2008). In vitro studies also indicated that AGX from corncobs had immunostimulatory activity when investigated for mitogenic and co-mitogenic activity using the co-mitogenic thymocyte test (Ebringerová & Hromádková, 1995). The above mentioned biological activities make xylan biopolymer advantageous for the development of insoluble entrapment matrices especially applied in the biomedical field such as drug delivery devices.

### 2.2.3 Applications of Xylans

Xylans have a wide variety of industrial and non-industrial applications that include the production of xylitol which is an artificial sweetener, wet-end additives in paper making, chemicals such as propionic acid, lactic acid, furfural and ethanol (Ramsay, Hassan & Ramsay, 1998; Garde, Jonsson, Schmidt & Ahring, 2002; Nigam, 2002; Lima, Oliveira & Buckeridge, 2003; Liu, Okuyama, Tamura, Nakagawa, Imai & Tomishige, 2015; Wang, Ren, Li, Deng & Sun, 2015). Furthermore, xylans from cereals and agro-residues such as wheat bran were found to have health benefits such as stimulating the innate and acquired immune responses, improving digestion, reducing high cholesterol levels (Lopez, Levrat, Guy, Messenger, Demigné & Rémésy, 1999; Zhou, Liu, Guo, Wang, Peng & Cao, 2010; Hu & Yu, 2013). One of the growing research areas is the application of xylans in the production of packaging films and insoluble three-dimensional hydrogels that can be used in drug delivery, tissue engineering and cosmetics amongst other industries (Deutschmann & Dekker, 2012). However, the application of xylans as hydrogels for entrapment of substances is limited by their high solubility in water and low molecular weight. The solubility of xylans is influenced by the intra- and inter molecular bonds present prior to extraction or created by the isolation of xylan from biomass and the drying process (Ebringerová *et al.*, 2005). Therefore, the selection of extraction and drying process of xylan is important in obtaining the properties of xylan suitable for application as hydrogels. In addition, the presence of substituents such as acetyl and arabinose groups along the xylose backbone as well as the pattern of substitution affect the solubility of xylans in aqueous solution. Xylan hydrophilicity can be physically, chemically or biologically modified to form insoluble hydrogels that can be used for the controlled release of substances (Garcia, Nagashima Jr, Praxedes, Raffin, Moura & Egito, 2001; Chimphango, van Zyl, *et al.*, 2012b; Gomes *et al.*, 2015).

### 2.3 Isolation of xylans

In the recent years, there has been increase of interest in the isolation of hemicellulose from biomass. An important factor in the effective utilization of biomass is the fractionation of the main lignocellulosic components, which are polysaccharides and lignin at relatively mild extraction conditions, with minimal polymer degradation and while achieving maximal extraction efficiency (García, Díaz, Garcia, Feria, Gómez & López, 2013). Different extraction methods of hemicellulose have been investigated and these include alkaline, hydrothermal,

organic solvent, and mechanical thermal treatments. The interest of researchers has been to develop suitable fractionation and purification methods to obtain both high yield and purity of hemicellulose (Peng, Peng, *et al.*, 2012). The structure of lignocellulosic material is very complex and the hemicellulose forms hydrogen bonds and covalent ether linkages with cellulose and lignin, respectively. This complex structure of lignocellulose biomass, specifically the lignin network as well as ester and ether lignin-carbohydrate linkages, restrict the fractionation of hemicellulose from the lignocellulosic biomass (Ebringerová & Heinze, 2000; Aguedo, Fougny, Dermience & Richel, 2014). Therefore, isolation of hemicellulose requires strict conditions that are able to disrupt the complex network of lignocellulose (Zhang *et al.*, 2014a). However, extraction conditions should be carefully considered so as to maintain the critical functional properties of hemicellulose required for the functioning of entrapment matrices. The isolation of hemicellulose involves pre-treatment of biomass, extraction of hemicellulose from biomass as well as fractionation and purification from mixture with other components of lignocellulose biomass (Peng, Peng, *et al.*, 2012).

### **2.3.1 Pre-treatment of biomass**

Hemicellulose extraction may be done directly from biomass or from pre-treated biomass. Pre-treatment may involve the removal of lignin resulting in hollocellulose or the removal of protein and starch using different enzymes or dewaxing (Glasser, Kaar, Jain & Sealey, 2000; Höjje *et al.*, 2005) to improve the extractability and purity of hemicellulose. The presence of lignin networks together with ester and ether lignin-carbohydrate linkages restrict the extraction of hemicellulose from biomass (Ebringerová & Heinze, 2000), therefore the removal of lignin may aid the liberation of xylan. Furthermore, the removal of proteins and starch enhances the isolation of hemicellulose from flours, bran, hulls and husks (Ebringerová *et al.*, 2005). A study compared the alkali extraction of hemicellulose from barley husks pre-treated with HCL or enzymes, and delignified with chlorite or organosolv. The extraction sample with highest yield and purity was obtained with HCL pre-treatment and sodium chlorite delignification. The lignin content was reduced to 2.5% with sodium chlorite delignification and to 3.9% by organosolv delignification from 11.5%. The HCL treatment was shown to remove starch, proteins and fats. The same study revealed that sodium chlorite also removes starch and protein in addition to delignification, therefore the HCL pre-treatment may be unnecessary when using the chlorite delignification (Höjje *et al.*, 2005). However,

sodium chlorite is a hazardous and expensive substance (Ebringerová & Heinze, 2000). Furthermore, delignification results in degradation of hemicellulose, which may be unsuitable for formation of entrapment matrices. Wheat bran has a relatively low lignin content (Table 2.1) and it will be favourable to eliminate the delignification step to avoid the degradation of hemicellulose. Wheat bran also has a high starch content (Brillouet & Mercier, 1981), which may be necessary to remove so as to increase the extractability of hemicellulose for the formation of entrapment matrices.

### **2.3.2 Extraction of hemicellulose**

There are many hemicellulose extraction methods reported in literature but only a few are discussed in this review. An extraction method that would result in high yield and purity of hemicellulose whilst minimising the degradation of polymer would be suitable for the formation of strong entrapment matrices with high capacity to entrap substances for the controlled release.

#### **2.3.2.1 Alkaline extraction**

The alkaline method has been shown to effectively extract polymeric hemicellulose from lignocellulosic biomass (Glasser *et al.*, 2000), which would be suitable for formation of entrapment matrices. The hydroxyl ions result in the hydrolysis of ester and ether linkages between hemicellulose and lignin, and disruption of intermolecular hydrogen bonds between hemicellulose and cellulose thereby disrupting the complex structural network of the cell matrix. This results in a certain fraction of hemicellulose being brought to solution (Xu, Sun, Liu & Sun, 2006; Peng, Peng, *et al.*, 2012). Furthermore, the solubilisation of hemicellulose is increased by the uronic acid residues, which are converted to negatively charged forms in the presence of alkaline media (Zhang *et al.*, 2014a). Alkaline treatment also results in neutralisation of acetyl groups of the hemicellulose (Gabrieli, Gatenholm, Glasser, Jain & Kenne, 2000; Peng, Peng, *et al.*, 2012; García *et al.*, 2013; Egüés, Stepan, Eceiza, Toriz, Gatenholm & Labidi, 2014), which results in reduced degree of substitution. The removal of acetyl groups and reduction in the degree of substitution would be beneficial in the formation of insoluble entrapment matrices. However other substituents of hemicellulose such as arabinose remain present along the xylan backbone after mild alkaline extraction. The type of alkaline solution used affects the yield of hemicellulose and these chemicals include sodium hydroxide, potassium hydroxide, calcium hydroxide, barium hydroxide and ammonium

hydroxide. The most commonly used alkaline solutions are sodium and potassium hydroxide because they have been shown to produce high xylan yields. In addition to alkali concentration, extraction time and temperature also impact the extraction yields of hemicellulose (Wang & Zhang, 2006; Zhang *et al.*, 2011; Peng, Peng, *et al.*, 2012). In a study conducted by Xu and colleagues, the extraction of bagasse performed with 1M NaOH for 18h at 20, 25, 30, 35, and 40°C, released 55.5%, 57.3%, 59.1%, 60.9%, and 62.1% of hemicelluloses, respectively (Xu *et al.* 2006). The results indicate that the maximum hemicellulose yield was achieved at 40°C. However, another study revealed that temperature did not significantly affect the hemicellulose yield, but time influenced the extraction efficiency. The longer extraction time would result in high hemicellulose yields since hemicellulose yield increases with time. However, changes in molecular structure and degradation of hemicellulose may result from extraction times longer than 10 hrs (Brienzo, Siqueira & Milagres, 2009).

The alkaline extraction can also be performed with hydrogen peroxide in a two-stage treatment process. Rice straw was extracted with 1% NaOH at 55°C for 2 hrs and then followed by the treatment with 0.0 to 5.0% hydrogen peroxide at 45°C for 12hrs at pH 11.5 and resulted in 67.2 to 88.5% of the originally present hemicelluloses being solubilized (Sun, Tomkinson, Ma & Liang, 2000). The two-stage treatment also showed increasing dissolution of lignin with increase of hydrogen peroxide. In addition, treatment with alkaline peroxide enhanced the isolation of hemicelluloses with larger molecular size compared with alkaline treatment in the absence of hydrogen peroxide. In another study, the increasing trend in the yield of hemicellulose from 2 to 6% hydrogen peroxide was also observed (Brienzo *et al.*, 2009). These results show that alkaline peroxide treatment is an effective method of solubilizing hemicellulose from lignocellulose materials. The H<sub>2</sub>O<sub>2</sub> produces radicals that oxidise lignin structures, which leads to the introduction of hydrophilic groups (carboxyl) and cleavage of some bonds, and subsequently the release of lignin and hemicellulose (Sun *et al.*, 2000; Peng, Peng, *et al.*, 2012). However, the treatment with alkaline peroxide at concentration higher than 1% causes degradation of hemicellulose (Sun *et al.*, 2000). Furthermore, the use of hydrogen peroxide may be harmful to the environment. Therefore, the extraction of soluble hemicellulose with alkaline solution that has no hydrogen peroxide would result in polymeric hemicellulose suitable for the formation of insoluble entrapment matrices.

Alkaline extraction of hemicellulose from wheat bran has been studied since wheat bran is a rich source of arabinoxylans. Table 2.2 shows the pre-treatment, extraction conditions and purification of hemicellulose from wheat bran and corn bran using alkaline method. From Table 2.2, it can be noted that the highest extraction yield achieved was 23.8% (Maes & Delcour, 2002) and the highest molecular weight was 670 kDa (Aguedo *et al.*, 2014). However, some of the studies did not report the MW of the isolated hemicellulose. The effect of sodium hydroxide concentration and extraction temperature on wheat bran was investigated in one study. The time was kept constant at 6 hrs whilst NaOH concentration was varied between 0.1 – 1M at temperatures 20, 40, 60 and 80°C. Experimental results showed that a low yield of 16% and 13% was obtained at laboratory and pilot scale respectively and a high purity of 75% in both scenarios. The studies also showed that temperature had a low impact on extraction efficiency at NaOH concentrations below 0.5 M. However, the use of alkaline solutions in extraction of hemicellulose compromises the purity of hemicellulose as other components of lignocellulose such as lignin are co-released (Zhang, Smith & Li, 2014b). A yield higher than 23.8% obtained by Maes and Delcour (2002) whilst maintain a purity within a the range 45.1 to 86.4% reported in literature Table 2.2.

Table 2.2: Alkali extraction of arabinoxylan from wheat bran and corn bran

Feedstock	Pretreatment	Extraction conditions (Solvent, temp, time, S/L)	Yield, Purity, MW	Purification method	Reference
Wheat bran	Destarching	0.44M [NaOH], 80°C, 15 hrs, 11%	20.8%, 57.5%, 670 kDa	Ultrafiltration	(Aguedo <i>et al.</i> , 2014)
Wheat bran	Destarching Delignification	0.5M [NaOH], 40°C, 6 hrs, 5%	16.4%, 76.2%, -	Microfiltration	(Bataillon, Mathaly, Nunes Cardinali & Duchiron, 1998)
Wheat bran	Destarching	24% [KOH], rtp, 3hrs, 6%	2%, 72%, 218 kDa	Enzyme treatment, Ultrafiltration	(Zhang <i>et al.</i> , 2011)
Wheat bran	Destarching	1M [KOH], 65°C, 2hrs, 10%	19.9%, 45.1%, -	Ethanol precipitation	(Chauvelon, Renard, Saulnier, Buleon, Granet, Krausz & Benhaddou, 1997)
Wheat bran	Destarching	2% [alkaline H <sub>2</sub> O <sub>2</sub> ], 60°C, 4 hrs, 10%	23.8%, 64.8%,	Ethanol precipitation	(Maes & Delcour, 2002)
Wheat bran	Destarching	2M [NaOH], 80°C, 1.5 hrs, 20%	7.6%, 86.4%, 300 – 350kDa	ultrafiltration and column chromatography	(Shiiba, Yamada, Hara, Okada & Nagao, 1993)
Wheat bran	Destarching Delignification	[saturated Ba(OH) <sub>2</sub> ], 80°C, 1.5hrs, 20%	19.4%, 83%, 390kDa	Enzymatic treatment and washing	(Bergmans <i>et al.</i> , 1996a)
Corn bran	-	5% [NaOH], rtp, 2 hrs, 10%	19.3%, 62.93%, 127.5kDa	Ethanol precipitation	(Kale, Pai, Hamaker & Campanella, 2010)
Maize bran	Destarching	0.5M [NaOH], 30°C, 2 hrs, 5%	19.1%, 73.04%, 270kDa	Ethanol precipitation and ultrafiltration	(Saulnier, Marot, Chanliaud & Thibault, 1995)

### 2.3.2.2 Hydrothermal extraction

Hydrothermal treatment is another procedure used to extract hemicellulose and the process entails heating biomass to high temperatures in heating reactors and may include steam explosion or microwave radiation (Peng, Peng, *et al.*, 2012; Aguedo *et al.*, 2014). A xylan yield of 19.5% was achieved when thermal treatment was applied on wheat bran at 180°C for 3 minutes at pH 5. However, the thermal treatment released xylans with lower molecular weight and Ara/xyl ratio as compared to xylans extracted by alkaline treatments (Aguedo *et al.*, 2014). This is because the high temperatures depolymerise the hemicelluloses yielding oligomeric products with low molecular weight and low degree of polymerisation (Ebringerová & Heinze, 2000; Glasser *et al.*, 2000), which are not suitable for the formation of strong entrapment matrices. Furthermore, the hydrothermal treatment of xylans yields undesirable by-products such as furfural acid (Aguedo *et al.*, 2014) that could contaminate the hemicellulose to be modified into entrapment matrices. Moreover, the use of organic solvent (Dimethylformamide, DMF) together with hydrothermal treatment was shown to increase the molecular weight of extracted hemicellulose (Ma, Jia, Zhu, Li, Peng & Sun, 2012).

Microwave-assisted extraction has been used to extract hemicellulose by directly applying electromagnetic radiation on plant biomass, which absorbs microwaves and converts them into heat (Peng, Peng, *et al.*, 2012). Microwave irradiation followed by steam pre-treatment was used to extract arabinoxylan from barley husks and the resulting hemicellulose had a molecular weight of 40 000 Da and a yield of 9%. Moreover, the acetyl group remain present after extraction as compared to alkaline method (Roos, Persson, Krawczyk, Zacchi & Ståhlbrand, 2009). This extraction method has advantages such as high extraction efficiency, less solvent requirement, shorter extraction time. However, it is difficult to obtain a good yield without extensive depolymerisation of the hemicellulose and contamination with other lignocellulosic components (Peng, Peng, *et al.*, 2012). The contamination with undesirable by-products, degradation of hemicellulose and the presence of acetyl substituents after extraction make hydrothermal treatment not suitable for extraction of hemicellulose that will be applied as entrapment matrices for the delivery of chemicals or biological substances, especially for scenarios where purity is of essence.

### 2.3.2.3 Organic solvent extraction

Organic solvents are also used to extract xylan and dimethylsulfoxide (DMSO) is the most commonly used solvent sometimes in combination with water (Sun, Wen, Xu & Sun, 2011). However, the use of DMSO presents some challenges, especially in the scale-up extraction of hemicellulose. The drawbacks of using DMSO include difficulty in separation from extracted hemicellulose fractions due to its low vapour pressure, environmentally unfriendly, costly and it might cause oxidation when mixed with other agents, such as certain alkylating or acylating agents (Haimer, Wendland, Potthast, Henniges, Rosenau & Liebner, 2010). In addition, the acetyl groups are retained with this method of extraction (Peng, Peng, *et al.*, 2012). Therefore, this method of extraction would not be favourable in obtaining hemicellulose that will be modified into entrapment matrices.

### 2.3.3 Purification

The presence of other lignocellulosic components such as proteins, phenolics and other polysaccharides such as starch and pectin in the seed endosperm (flours) and seed coats (bran) complicates the isolation of xylan. As such, these other components of the lignocellulose material are solubilised as well during the extraction of xylan from wheat bran (Ebringerová & Heinze, 2000; Zhang *et al.*, 2011). There are several methods of purifying extracted hemicellulose from other co-extracted polysaccharides and components after extraction, which include alcohol precipitation, enzymatic treatment, H<sub>2</sub>O<sub>2</sub> treatment, ultrafiltration, ammonium sulphate precipitation and column chromatography (Shiiba *et al.*, 1993; Gabriellii *et al.*, 2000; Zhang *et al.*, 2011; Peng & She, 2014). Ethanol precipitation is the most commonly used method of fractionation. A purity of 83% and 72% for hemicellulose was achieved (Table 2.2) after hemicellulose extracts were treated with enzymes alpha-amylase and a combination of lichenase and  $\beta$ -glucosidase, respectively (Bergmans *et al.*, 1996a; Zhang *et al.*, 2011). Table 2.2 also shows that a high purity of 86.4% for extracted hemicellulose was obtained after the water soluble component was dialysed by ultrafiltration and then passed through a diethylaminoethyl (DEAE)-Sepharose CL-6B column (Shiiba *et al.*, 1993). However, ion exchange method has been found to cause irreversible adsorption on the surface of the resin particles (Gabriellii *et al.*, 2000). Glasser *et al.*, (2000) compared four different types of purification methods which, are alcohol precipitation, H<sub>2</sub>O<sub>2</sub> treatment followed by ultrafiltration, ion-exchange and carbon treatment. The findings of their study showed that

precipitation of hemicellulose in a non-solvent, such as methanol, and ultrafiltration were effective in separating the polysaccharide from salts and other impurities. Nevertheless, ultrafiltration has the advantage of being easier to engineer at large scale operations. On the other hand, carbon treatment and ion-exchange were not ideal techniques for isolating polymeric hemicellulose as they resulted in loss of hemicellulose (Glasser *et al.*, 2000; Aguedo *et al.*, 2014). For this study, dialysis was used for purification of hemicellulose from other lignocellulose components after extraction so as to remove impurities and obtain hemicellulose suitable for the formation of insoluble entrapment matrices.

## 2.4 Hydrogels

Hydrogels are insoluble three-dimensional matrices formed by crosslinking of hydrophilic polymeric chains and can absorb large quantities of fluids (Soppimath *et al.*, 2002). The hydrophilic groups of the cross-linked polymer chains allow the hydrogels to absorb water up to a thousand times of their original weight (Laftah *et al.*, 2011). Hydrogels can also be referred to as macroparticles, microparticles or nanoparticles depending on their size. Nanoparticles also known as nanocapsules or nanospheres or nanoliposomes have a particle size below 1 000 nm. Microparticles, microspheres, microcapsules have a particle size between 3 – 800  $\mu\text{m}$  and particles larger than 1 mm are called macroparticles. The use of hydrogels as substance carriers has the advantages of protecting sensitive or unstable substances from harsh external environment, tailoring the release of substance, masking unpleasant taste, odour, colour of substances, improving bioavailability and avoiding adverse effects of enclosed substances (Ravi Kumar, 2000; Dubey, Shami & Bhasker Rao, 2009; Jyothi, Prasanna, Sakarkar, Prabha, Ramaiah & Srawan, 2010). For the purpose of this study, the term hydrogels may also be used to refer to microparticles and nanoparticles as well.

Apart from being abundant and renewable, the benefit of using hemicellulose for production of hydrogels is that it is a natural polymer, which is biodegradable, biocompatible and has low toxicity (Peppas, Hilt, Khademhosseini & Langer, 2006; Marcelino, da Silva, Gomes, Oliveira, Nagashima-Junior, Pinheiro, da Silva, Timoteo, Agnez-Lima, Ayala, Oliveira & do Egito, 2015). However, the application of hemicellulose as insoluble entrapment materials is limited by its high solubility due to its low molecular weight and side group substitution along the backbone (Ebringerova *et al.*, 2005; Zhu Ryberg, Edlund & Albertsson, 2011). There are different techniques of hydrogel formation from water soluble polymeric chains, which include

physical, chemical and biological methods (Chimphango, van Zyl, *et al.*, 2012b; Martínez-López, Carvajal-Millan, Miki-Yoshida, Alvarez-Contreras, Rascón-Chu, Lizardi-Mendoza & López-Franco, 2013; Ahmed, 2015). The structural varieties of hemicelluloses allow for chemical, physical, and enzymatic modification to form hydrogels for the delivery of chemical and bioactive substance in different applications. The variability in sugar constituents, glycosidic linkages and structure of glycosyl side chains as well as two reactive hydroxyl groups at the xylose repeating unit of the main hemicellulose allow for the various modifications to occur (Ebringerová & Heinze, 2000; Ebringerová *et al.*, 2005). Various modification methods of the biopolymers have led to the use of hemicellulose based or derived materials as coatings, encapsulation materials, adhesives, additives, thickeners, emulsifiers, films, and chemicals among many other functions. These valuable materials find many applications across the medical, pharmaceutical, chemical, fuel, and paper industry (Nigam, 2001; Garde *et al.*, 2002; Lima *et al.*, 2003; Melandri, De Angelis, Orioli, Ponzielli, Lualdi, Giarratana & Reiner, 2006; Tatar, Tunç, Dervisoglu, Cekmecelioglu & Kahyaoglu, 2014; Hu, Wang, Xie, Hu, Ma & Zeng, 2015; Silva, Oliveira, Neto, Pimentel & Santos, 2015). Of particular interest is the film forming property of hemicellulose that enables it to be used as a delivery system of drugs, chemicals or bioactive substances for controlled or targeted release.

#### **2.4.1 Structure of hydrogels**

Hydrophilic linear polymers form a network via physical and chemical crosslinks to form three-dimensional hydrogel structure. The crosslinking of the polymeric chains is facilitated by covalent bonding, ionic interactions, hydrogen bonding van der Waal forces, entanglements and crystallite formation (Laftah *et al.*, 2011; Ullah *et al.*, 2015). Chemical crosslinks occur by hydrogen bonding enabled by crosslinking agent whilst the physical crosslinks are facilitated by physical junctions facilitated by entanglements, hydrogen and ionic linkages. According Soppimath and co-workers, van der Waal forces are also involved in the maintenance of the three-dimensional structure of hydrogels (Soppimath *et al.*, 2002). The hydrophilic character of hydrogels allows them to absorb polar substances whilst physical and chemical crosslinking keep them from fragmenting during swollen state (Ahmed, 2015).

The structure of hydrogels is mainly characterised by the polymer volume fraction in the inflated state, molecular weight of the polymer chain between two consecutive crosslinking points ( $M_c$ ), and the corresponding mesh size (Figure 2.2).

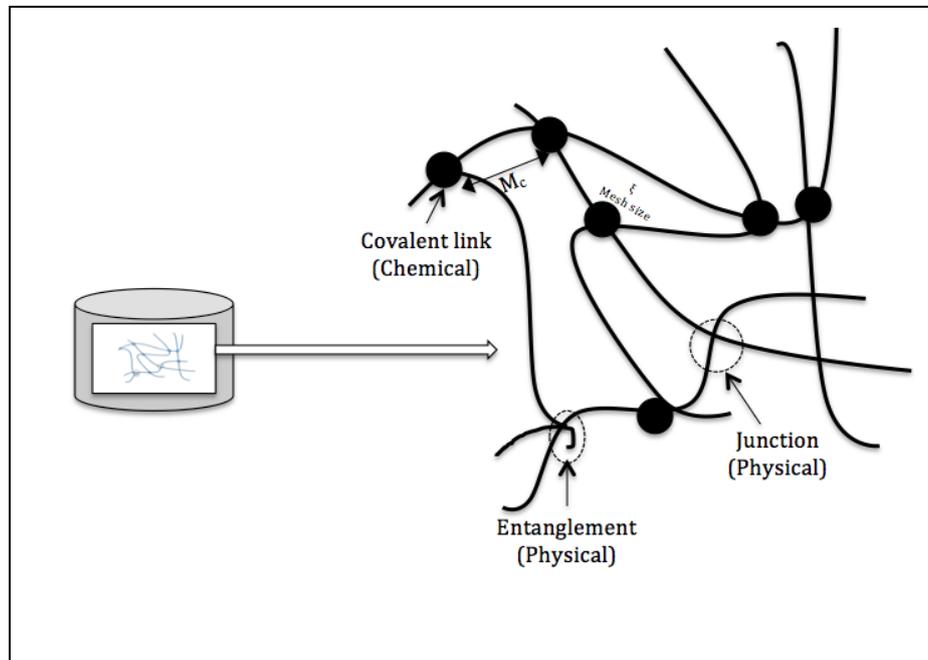


Figure 2.2: Structure of hydrogel illustrating crosslinking (redrawn from Ullah *et al.*, 2015).

The polymer volume fraction in the inflated state refers to the measure of amount of substance absorbed by the hydrogel. The molecular weight between consecutive crosslinks is the measure of the degree of cross linking of the polymer. The mesh size is the measure of spaces between the polymeric chains. The mesh size and the molecular weight between two consecutive crosslinks are reported as average values (Peppas, 2000).

#### 2.4.2 Classification of hydrogels

The classification of hydrogels can be based on various properties such as source, preparation method, physical structure, nature of cross linking, ionic charge, response to stimuli and mechanical structure (Ahmed, 2015; Ullah *et al.*, 2015). Figure 2.3 shows the different classification of hydrogels based on their properties.

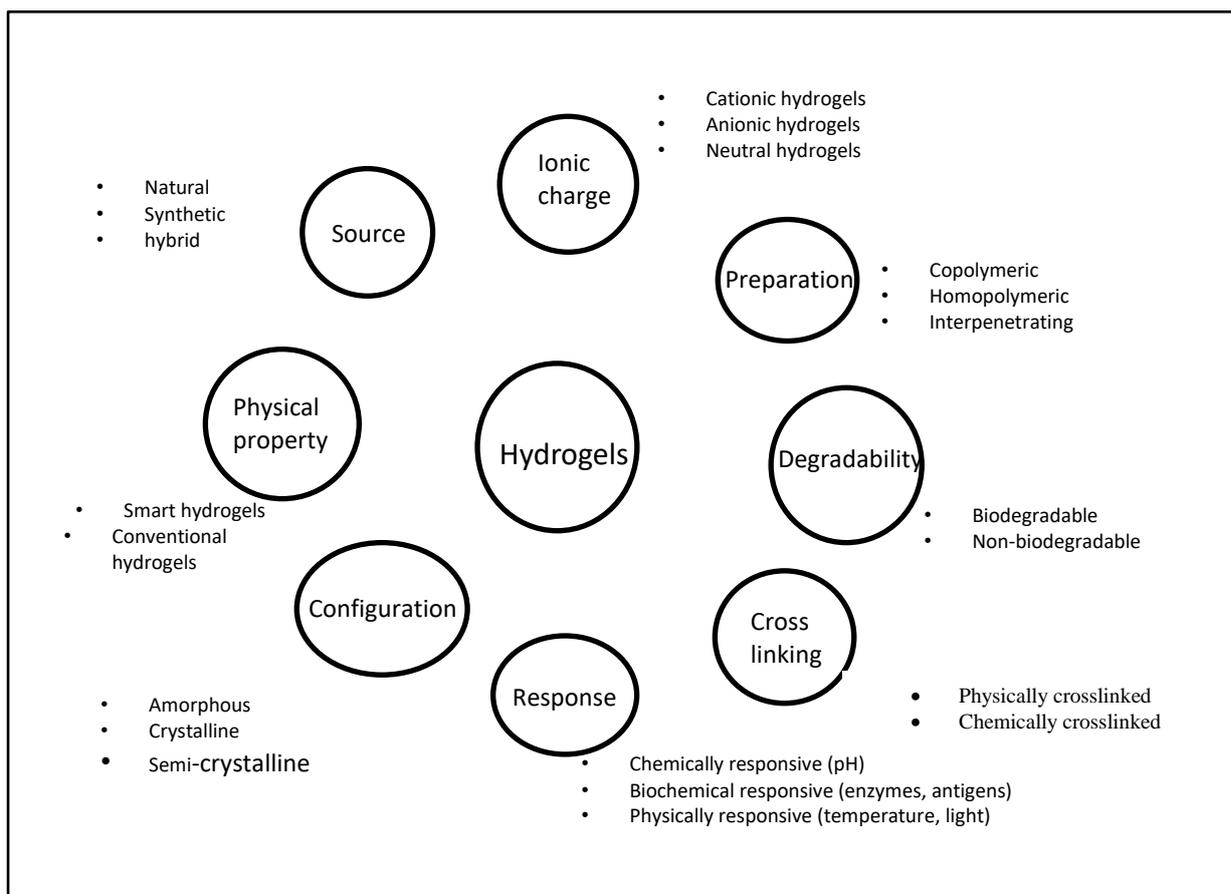


Figure 2.3: Hydrogel classification based on based on various properties (redrawn from Ullah *et al.*, 2015).

## 2.4.3 Properties of hydrogels

### 2.4.3.1 Swelling and absorption

The presence of hydrophilic functional groups along the polymer chains give the hydrogel the capacity to absorb polar substances and swell up to 1000 times their dry weight (Laftah *et al.*, 2011; Ahmed, 2015). Water exists in swollen hydrogels as primary bound water, secondary bound water and free water. During swelling, water initially interacts with the hydrophilic functional groups of the polymer network such as  $-\text{OH}$ ,  $-\text{CONH}$  and  $-\text{COOH}$  and it is known as primary bound water. After interacting with the polar groups of the matrix, water interacts with the hydrophobic groups resulting in secondary bound water also known as hydrophobically bound water. Lastly water is absorbed by the hydrogels up to equilibrium state by entering the spaces in between network chains and it is referred to as free or bulk

water (Gibas & Janik, 2010). The equilibrium absorption capacity is used to calculate the swelling capacity of hydrogels according to the following equation:

$$Q_{eq} = \frac{W_2 - W_1}{W_1} \quad (2.1)$$

Where  $Q_{eq}$  is the equilibrium absorption capacity expressed as a gram of water per gram of sample,  $W_1$  and  $W_2$  are equal to the mass of hydrogel sample before and after swelling, respectively (Guan, Bian, Peng, Zhang & Sun, 2014). The equilibrium absorption capacity is also referred to as the equilibrium swelling ratio (Karaaslan, Tshabalala & Buschle-Diller, 2010).

Factors affecting swelling or absorption capacity include chemical composition, cross-linking density, network structure, specific stimuli, concentration and quality of solvent (Peppas, 2000; Laftah *et al.*, 2011). A study conducted, revealed that an increase in the amount cross linker resulted in increased crosslinking density, which in turn decreased the water absorption capacity. This is because a higher degree of crosslinking results in tighter structure and reduced distances between crosslinking points, thereby reducing the amount of water that can occupy the spaces in the network structure (Sun, Wang, Jing & Mohanathas, 2013). Hydrogels with hydrophilic groups have a higher absorption capacity as compared to those with hydrophobic groups. The hydrophobic groups on polymer networks collapse in the presence of water and reduce their interaction with water and lower absorption capacity. Also hydrogels may swell in response to environmental stimuli such as pH, temperature, and presence of enzyme (Peppas, 2000; Laftah *et al.*, 2011).

The ability of hydrogels to absorb and retain water allow them to be used as delivery systems for chemical and bioactive substances (Sun *et al.*, 2013; Guan *et al.*, 2014). The chemical and bioactive substances can be incorporated into hydrogel matrix by entrapment, encapsulation, and adsorption (Hans & Lowman, 2002). The incorporation of chemical or bioactive substance can occur simultaneously with the formation of hydrogel or after hydrogel matrix is formed (Soppimath, Aminabhavi, Kulkarni & Rudzinski, 2001). However, some chemical methods of hydrogel formation may be harsh for sensitive substances such as proteins and it would be ideal to incorporate the substance after hydrogel formation.

#### 2.4.4 Substance release from hydrogels

The release properties of hydrogels are an important factor to be considered in the delivery of chemical or bioactive substances. One of the goals in formulation of delivery systems is the controlled release of substances to suit a desired requirement (Soppimath *et al.*, 2001). However, the challenge encountered with substance delivery from hydrogels is the initial burst release of substance. Initial burst release is probably due to poorly entrapped substance or adsorption of substance on the outside of matrix (Soppimath *et al.*, 2001; Hans & Lowman, 2002).

There are several factors that affect the release properties of entrapped substance such as size of entrapped substance, chemical composition of hydrogel, network structure of hydrogels, method of substance loading and amount of drug incorporated (Soppimath *et al.*, 2001; Hans & Lowman, 2002; Edlund & Albertsson, 2008). The effect of size of the entrapped substance on the release rate from hydrogels was investigated. Hydrogel microspheres were produced from soft wood hemicellulose and 2-hydroxyethyl methacrylate (HEMA) by water-in-oil emulsion polymerisation and macromolecular bovine serum albumin (BSA) and small hydrophilic caffeine molecules were incorporated into the hydrogels. Caffeine was found to have a large initial burst release with all of it being released in 60 mins whereas BSA had a prolonged release rate of greater than 200 mins (Edlund & Albertsson, 2008). These results showed that larger incorporated substances have a small initial burst release and longer sustained release as compared to smaller substances (Hans & Lowman, 2002; Edlund & Albertsson, 2008). Furthermore, the choice to incorporate substance before or after hydrogel formation may influence the release patterns. In a study where xylan hydrogels were formed by enzymatic hydrolysis of xylan side groups, horseradish peroxidase (HRP) was more readily released from hydrogels when it was incorporated after hydrogel formation than when it was added during formation of hydrogel (Chimphango, van Zyl, *et al.*, 2012b).

The release of entrapped substances from hydrogels occurs by diffusion or biodegradation of the hydrogel. Substance release occurs by diffusion when the rate of diffusion of substance is greater than the erosion of matrix or in the absence of matrix degrading enzymes, or else the release is governed by degradation (Soppimath *et al.*, 2001). The cumulative release ( $M_t/M_0$ , %) is determined as the ratio of the amount of substance released ( $M_t$ ) at time  $t$  to the initial amount of substance incorporated ( $M_0$ ) (Karaaslan *et al.*, 2010).

The mechanism of substance release can be defined by fickian (diffusion-controlled) or non-fickian (relaxation controlled) transport mechanism (Ritger & Peppas, 1987; Peppas, 2000). The substance release behaviour from polymeric delivery systems can be described using the following exponential relation:

$$\frac{M_t}{M_\infty} = kt^n \quad (2.2)$$

Where  $M_t/M_\infty$  is the fractional release of substance,  $t$  is the release time,  $k$  is a constant,  $n$  is the diffusional exponent characteristic of release mechanism,  $M_t$  is the mass of substance released at time  $t$  and  $M_\infty$  is the mass of substance release as time approaches infinity. The diffusional exponent  $n$  is used to determine the release mechanism of substance from the hydrogel. Fickian diffusion is defined by  $n$  equal to 0.50 and non-fickian by  $n$  greater than 0.50 (Korsmeyer, Gurny, Doelker, Buri & Peppas, 1983; Ritger & Peppas, 1987). The equation is applicable for the initial 60% fractional release from hydrogels. However, the release behaviour of substances can follow a combination of both fickian and non-fickian mechanism (Ritger & Peppas, 1987; Karaaslan *et al.*, 2010).

#### **2.4.4.1 Mechanical properties**

The mechanical properties of hydrogels are important in the controlled delivery of chemical and bioactive substances. Some substances are highly sensitive to the environment such as proteins or toxic, and an ideal hydrogel will be one that can protect the functional properties of the encapsulated substance. Thus, the integrity of the hydrogel during the lifetime application is imperative. The crosslinking density of hydrogel is central to the mechanical stability of the latter. A highly cross-linked hydrogel results in a stronger network matrix however, reducing the crosslinking density makes the structure more brittle. Therefore, the crosslinking density can be adjusted to obtain the desired mechanical strength to suit application (Peppas, 2000).

#### **2.4.4.2 Stimuli responsive**

Hydrogels with ability to perceive and respond to environmental changes are called 'smart' or 'intelligent' hydrogels. The environmental stimuli may be chemical or physical and that includes changes in temperature, ionic strength, pH, magnetic field, light, radiation, glucose and antibody concentration (Figure 2.4). The stimuli could trigger drastic changes in hydrogel

swelling performance, network structure, mechanical properties, and permeability (Peppas, 2000; Gupta, Vermani & Garg, 2002; Ahmed, 2015). The ability of hydrogels to respond to environmental stimuli makes them favourable as delivery systems for controlled release of substances, especially in pharmaceutical and biomedical field. An ideal substance delivery system should perceive stimuli and alter substance absorption or release accordingly (Gupta *et al.*, 2002).

Hemicellulose based hydrogels produced by grafting acrylic acid into hemicellulose by redox initiation were found to be pH responsive. The hydrogels had carboxylic groups that are ionisable, and the latter influenced the swelling ratio at various pH. In addition to ionised pendant groups, fixed charges on the polymeric network and electrostatic repulsive forces inside the hydrogel affected the swelling properties of the hydrogel at different pH. At low pH (pH 1 – 3), the hydrogels did not swell much because the carboxylic group remained unionised, which resulted in minimum electrostatic repulsion and low swelling. Between pH 3 and 4, the carboxylic group dissociated resulting in sharp increase in swelling ratio. This is because the pH of medium was higher than pKa of the hydrogel and this caused ionisation of the carboxylic group and subsequent increase in electrostatic repulsion followed by increase in swelling ratio. However, in alkaline conditions (pH 8 – 10), the swelling ratio of hydrogels decreased due to sodium ions that weakened the electrostatic repulsion in the polymer network. These pH sensitive hydrogels can be used for controlled release of oral drugs in the small intestines where the environment is alkaline (Sun *et al.*, 2013).

#### **2.4.5 Applications of hydrogels**

Hydrogels are used in different industries, which include agriculture, cosmetics, water treatment, biomedical and food. In the biomedical industry, hydrogels are being exploited to target delivery of drug or protein to a specific location in the body where needed and also to regulate the rate of release to suit a particular need e.g. release insulin when required. Furthermore, they are also used as contact lenses, materials for biosensors, artificial hearts and skins. Hydrogels are suitable for these biomedical applications because they resemble natural living tissue due to their high-water contents and soft consistency. In this study the hydrogels will be applied as entrapment materials for the controlled release of gallic acid.

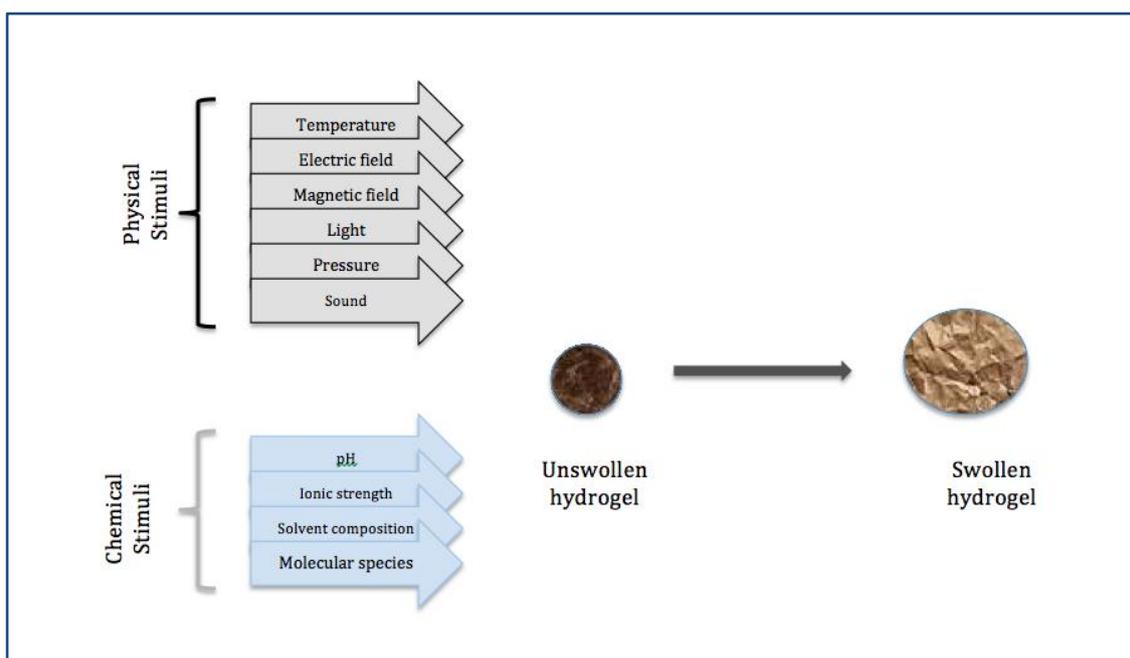


Figure 2.4: Stimuli responsive swelling of hydrogel (redrawn from Ahmed, 2015).

## 2.5 Modification of hemicellulose into hydrogels

### 2.5.1 Chemical methods

Many different techniques have been employed to modify hemicellulose into hydrogels using chemicals and these include suspension polymerisation, emulsion polymerisation, solution polymerisation, interfacial cross-linking, coacervation and sol-gel encapsulation. Some of the chemical methods used to produce hydrogels from both synthetic and natural polymers have been studied and reviewed by several researchers (Ghosh, 2006; Dubey *et al.*, 2009; Jyothi *et al.*, 2010; Laftah *et al.*, 2011; Ahmed, 2015).

#### 2.5.1.1 Interfacial cross-linking

Interfacial cross-linking (ICL) involves the emulsification and cross-linking reaction steps (Eduardo da Silva *et al.*, 2012; Marcelino *et al.*, 2015). In the first step, an aqueous phase containing dissolved polymer is mixed with organic phase to produce an emulsion. This is followed by addition of crosslinking agent resulting in microparticle formation as shown in Figure 2.5 (Jyothi *et al.*, 2010; Eduardo da Silva *et al.*, 2012).

Microparticles were produced by ICL method and terephthaloyl chloride (TC) was used as cross-linking agent. An emulsion was formed by adding an alkaline xylan solution to

chloroform:cyclohexane mixture before addition of TC. The use of TC improved the thermal and mechanical properties of xylan microparticles. However, xylan microparticles were found to be more toxic than xylan when their biocompatibility was evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT) assay. Xylan microparticles and xylan showed cell viability of 70% at concentrations 0.372 mg/ml and 4.1 – 12.4 mg/ml respectively. The cytotoxic effect of xylan microparticles was probably due to free unreacted radicals from TC, which oxidise main cell components. Therefore, this method maybe unfavourable for hydrogel formation, where biosafety is a concern as residual chemicals may cause toxicity.

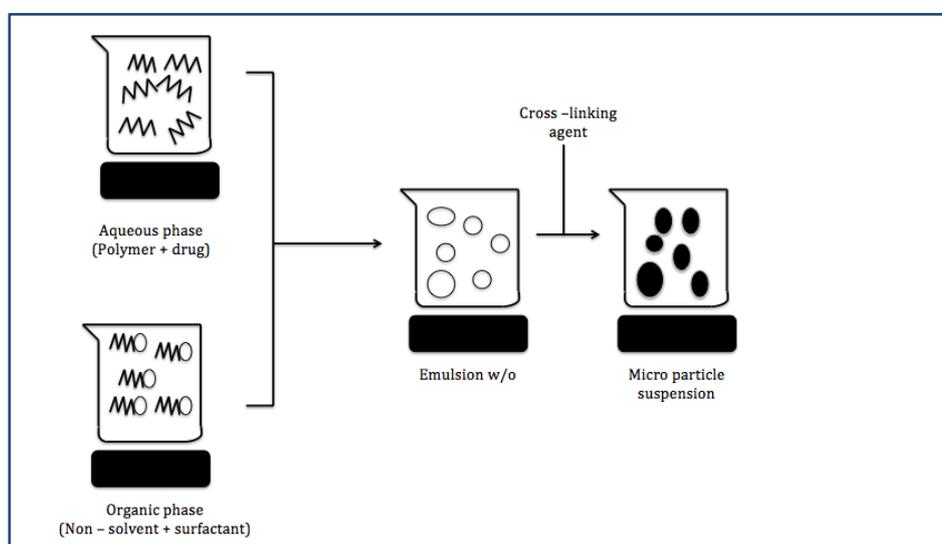


Figure 2.5: Scheme showing interfacial cross-linking formation of microparticles (redrawn from Eduardo da Silva *et al.*, 2012).

### 2.5.1.2 Coacervation

The preparation of microparticles by coacervation occurs by partially dissolving a homogeneous polymer solution into a polymer-rich phase (coacervate) and a poor polymer phase (coacervation medium). Coacervation can be divided into simple or complex, where simple coacervation entails addition of desolvation agent for phase separation whilst complex coacervation involves the mixture of two oppositely charged polymers. In complex coacervation there are three steps involved, which include, the formation of three immiscible phases, deposition of the coating and rigidization of the coating (Jyothi *et al.*, 2010).

Garcia and colleagues described a simple coacervation method for the preparation of micro and nanoparticles from xylan extracted from corncobs based on the neutralisation of base with HCL or acetic acid in the presence of a surfactant. The microparticles formed spontaneously when alkaline xylan was neutralised with either of the acids and polyoxyethylene (20) sorbitan monolaurate (Tween®20) was added as a surfactant. The xylan concentration had a direct effect on the particle size, as samples with polymer concentration lesser than 32 mg/ml formed nanoparticles (100 – 900 nm) and those greater than 32 mg/ml formed microparticles (> 10 µm). Complete neutralisation was found to be imperative in the particle formation as excess acid was believed to degrade the polymer forming a dispersible flocculate. The addition of surfactant to an optimal value of 1.5%<sub>(v/v)</sub> resulted in the stability of both particle size and morphology (Garcia *et al.*, 2001). Since the hydrogels form when base is neutralised, this may be a safer method for hydrogel formation as compared to other chemical methods.

### **2.5.1.3 Radical polymerisation**

Hydrogels formed by radical polymerisation involve the chemical crosslinking of water-soluble polymers derivatised with polymerizable groups via use of a cross linker. The swelling behaviour of hydrogels can be controlled by the amount of crosslinking agent. However this method is unfavourable due the toxicity of crosslinking agents, which may affect the integrity of enclosed substance (Ullah *et al.*, 2015). In a study, xylans were isolated from *Eucalyptus urograndis* using 24% KOH at room temperature (rtp) for 24 hrs and DMSO at 50°C for 12 hrs to produce non-acetylated and acetylated xylans respectively. Both xylan samples were modified by radical polymerisation of the chains using 2-hydroxyethyl methacrylate (HEMA) as crosslinking agent to produce hydroxyethyl methacrylate derivatised xylan (xylan-HEMA). The proportion of xylan:HEMA was varied (40:60 and 60:40). The increase in attached HEMA resulted in an increase in crosslinking density and subsequently reduced swelling behaviour. Hydrogels produced from acetylated xylans with DS of 0.10 and xylan:HEMA ratio of 60:40 had 51% bounded water and 66% for non-acetylated xylan hydrogels. These results indicated that hydrogels from non-acetylated xylans had higher swelling capacity as compared to those from acetylated xylans. This is because the presence of acetyl groups repels water molecules resulting in reduced water absorption whilst their absence confers the structure of a hydrophilic character. Furthermore, the presence of acetyl groups enhanced the release of

the representative drug doxorubicin as non-acetylated and acetylated samples levelled off at about 50% and 80% at pH 2.5 of release medium. It can be concluded that alkali extracted xylans are more suitable delivery systems for slow release of substances than DMSO extracted xylans. Other crosslinking agents include N,N-methylene-bis-(arylamide). Therefore, radical polymerisation is not suitable for hydrogel formation due to toxic substance used as crosslinking agents.

#### **2.5.1.4 Interpenetrating and semi-interpenetrating networking**

Interpenetrating networks are defined as blend of two or more cross-linked polymeric chains in the presence of the other. When one of the polymers is linear the structure is said to be interpenetrating. The purpose of blending is usually to enhance the attributes of one polymer while maintaining the key properties of the other (Myung, Waters & Wiseman, 2008; Karaaslan *et al.*, 2010; Ullah *et al.*, 2015).

In one study, hemicellulose was isolated from aspen by alkaline extraction method and subsequently used to form semi-interpenetrating hydrogels with chitosan using glutaraldehyde as cross-linking agent. To isolate hemicellulose, milled aspen was treated with 0.05 M of HCL at 70°C for 2 hrs to remove pectins, starch and fats followed by delignification with 0.025 M NaOH in 70% ethanol at 75°C for 2 hrs, and extraction with 0.1 M NaOH at room temp for 16 hrs. The extracted hemicellulose was precipitated with 95 % ethanol and freeze dried. To prepare semi-interpenetrating hydrogels, chitosan was dissolved in acetic acid and hemicellulose in deionized water. The two polymer solutions were stirred for 8hrs in weight ratios 30:70 and 70:30 and glutaraldehyde was added after stirring resulting cross-linked chitosan in the presence of hemicellulose. It was observed that increasing hemicellulose content increased the swelling ratios of the hydrogels due to crystallites introduced. The semi-interpenetrating hydrogels were also shown to be suitable pH-sensitive controlled drug delivery vehicles as the release of riboflavin model drug was higher and faster at pH 2.2 than at 7.4 (Karaaslan *et al.*, 2010). However, this method of preparing hydrogels uses crosslinking agents, which may be toxic.

#### **2.5.2 Biological modification of hemicellulose into hydrogels**

The biological modification of hemicellulose involves the use of debranching enzymes, which have advantages of being environmentally friendly, mild and specific. The enzymes that

hydrolyse xylan are divided into two groups, which are those that cleave the main xylan backbone and those that break the side groups on the main xylan chain also called accessory enzymes. The enzymes that degrade the main chains of xylans include endo-xylanase, exo-xylanase and  $\beta$ -xylosidase. The accessory enzymes include  $\alpha$ -L-arabinofuranosidase,  $\alpha$ -D-glucuronidase, acetyl xylan esterase, ferulic acid esterase,  $p$ -coumaric acid esterase (Saha, 2003). Accessory enzymes  $\alpha$ -L-arabinofuranosidase and  $\alpha$ -D-glucuronidase have been shown to alter the solubility of xylans precipitation into micro and nanohydrogels by hydrolysing the respective side groups and modifying the side chain distribution pattern (Chimphango, van Zyl, *et al.*, 2012a; Gomes *et al.*, 2015).

### 2.5.2.1 $\alpha$ -L-Arabinofuranosidase

The  $\alpha$ -L-arabinofuranosidase (EC 3.2.1.55) is a side chain removing enzyme that hydrolyses the terminal non-reducing  $\alpha$ -linked L-arabinofuranose residues of both oligosaccharide and polysaccharide chains (Saha, 2003; Shinozaki, Kawakami, Hosokawa & Sakamoto, 2014). The  $\alpha$ -L-arabinofuranosidase are classified based on their substrate specificity or their amino acid sequence. They are classified into five glycoside hydrolase (GH) families (GH3, 43, 51, 54 and 62) based on difference in amino acid sequence. According to substrate specificity they are classified into 3 types, type A degrade arabinooligosaccharides but not the polymeric forms. Type B degrade both oligosaccharides and polymers and type C specifically acts towards arabinosidic bonds in arabinoxylans. Both type A and B act on synthetic  $p$ -nitrophenyl  $\alpha$ -L-arabinofuranosidase ( $p$ NPA) whilst type C is not active against the latter (Saha, 2000; Shinozaki *et al.*, 2014). These ABFs occur naturally and have been isolated and purified from microorganisms such as *Aspergillus niger*, *Bacillus subtilis*, and *Penicillium chrysogenum* (Kaneko, Sano & Kusakabe, 1994; Gielkens, Gonzalez-Candelas, Sanchez-Torres, van de Vondervoort, de Graaff, Visser & Ramon, 1999; Shinozaki *et al.*, 2014). However, the isolated  $\alpha$ -L-arabinofuranosidase are often contaminated with main chain degrading enzymes such as endo-xylanase. This contamination is unsuitable for xylan hydrogel formation because high degree of polymerisation is required for the precipitation of xylan into hydrogels. Recombinant  $\alpha$ -L-arabinofuranosidase (AbfB) free of main chain degrading enzymes and with activity against PNP was produced using (AbfB) gene of *A. niger* (Chimphango, Rose, van Zyl & Gørgens, 2012). In a study, oat spelt xylan was hydrolysed with the recombinant  $\alpha$ -L-arabinofuranosidase by selective cleavage of arabinose side groups leading to formation of

hydrogels with mean particle size 21 – 10 000 nm. The effect of xylan concentration, hydrolysis time and plasticiser polyethylene glycol (PEG) 1000 on the arabinose release, mean particle size and zeta potential was investigated. Recombinant  $\alpha$ -L-arabinofuranosidase was successfully used for the *in situ* and *ex situ* formation of xylan hydrogels and encapsulation of horse radish peroxidase. The rate of release was found to be dependent on whether the horseradish peroxidase was added after or before hydrogel formation (Chimphango et al. 2012a).

### 2.5.2.2 $\alpha$ -D-glucuronidase

$\alpha$ -D-glucuronidase is the accessory enzyme that cleaves the  $\alpha$ -1,2-linked D-glucuronic acid 4-O-methylglucuronic acid from xylooligomers or polymeric xylans (Shao, Obi, Puls, Wiegel & Puls, 1995; Biely, de Vries RP, Vrsanská & Visser, 2000; Chong, Battaglia, Coutinho, Henrissat, Tenkanen & De Vries, 2011). Based on their substrate specificity,  $\alpha$ -D-glucuronidases can be divided into two categories. The first group of enzymes cleaves the side groups from xylooligomers generated by main chain degrading enzymes and they belong to the GH67 family. Enzymes of second group hydrolyse side groups from polymeric xylan and belong to the G115 family (Kolenová, Ryabova, Vršanská & Biely, 2010).  $\alpha$ -D-glucuronidase have been isolated and purified from many microorganisms such as *Aspergillus fumigatus* and *Talaromyces emersonii*, (Shao et al., 1995; Biely et al., 2000; Heneghan, McLoughlin, Murray & Tuohy, 2007; Rosa, Ravanal, Mardones & Eyzaguirre, 2013). However,  $\alpha$ -D-glucuronidase acting specifically on polymeric xylan (EC 3.2.1.139) have been isolated and purified from fungus *Schizophyllum commune* and yeast *Scheffersomyces stipis* (Tenkanen & Siika-Aho, 2000; Ryabova, Vrsanská, Kaneko, van Zyl & Biely, 2009). Recombinant *S. stipis*  $\alpha$ -D-glucuronidase was successfully produced in *Saccharomyces cerevisiae* using fed-batch culture under the control of phosphoglycerate kinase promoter (Anane, van Rensburg & Görgens, 2013).  $\alpha$ -D-glucuronidase and  $\alpha$ -L-arabinofuranosidase were used together to precipitate polymeric xylan from sugar cane bagasse resulting in the formation of micro and nanoparticles. Precipitation of xylan was maximum at high lignin content and high DP (Gomes et al., 2015).

## Chapter 3: Problem statement, key questions and objectives

### 3.1 Problem statement

Hydrogels have found application in various fields as delivery devices for controlled or target release of chemicals and bioactive substances (Dube, Nicolazzo & Larson, 2010; Hiwale, Lampis, Conti, Caddeo, Murgia, Fadda & Monduzzi, 2011). However, most hydrogels are formed from synthetic polymers and other natural polymers such as cellulose, which have other applications. The application of hemicelluloses as hydrogels to be used as insoluble entrapment matrices is limited by its hydrophilicity (Zhu Ryberg *et al.*, 2011). Hemicelluloses are soluble due to low molecular weight and side chain substitution along the xylose backbone (Ebringerová *et al.*, 2005; Zhu Ryberg *et al.*, 2011). A high molecular weight or DP of xylans is favorable for their application as entrapment matrices for the delivery of chemical and biological substances (Gomes *et al.*, 2015). However, some isolation methods result in the depolymerization of polymeric hemicellulose forming oligosaccharides or production of undesirable by-products such as furfural acids, which are unfavorable for the formation of entrapment matrices (Aguedo *et al.*, 2014). Therefore, it is imperative to isolate xylans to be used as delivery systems from other cell wall components in polymeric form with high purity as well as yield. The degree of substitution and pattern of side group substitution can be modified by several physical, chemical and biological methods to produce insoluble entrapment materials or hydrogels for the controlled delivery of chemical and biological substances (Chimphango, van Zyl, *et al.*, 2012b; Gomes *et al.*, 2015). However, the production of hydrogels has limitations such as low solubility, high crystallinity, unfavorable mechanical and thermal properties, residual monomers, and toxic cross linkers (Ullah *et al.*, 2015). There is growing interest in the formation of hydrogels with ability to maintain integrity of incorporated chemical or bioactive substance using nontoxic crosslinking chemicals, as well as delivering the imbibed substance in a controlled manner. This study will seek to modify soluble xylan derived from agro-residue wheat bran into insoluble entrapment materials and hydrogels for the controlled release of chemical and bioactive substances.

## 3.2 Key Questions

This research project proposes to answer the following questions:

- What will be the optimum extraction conditions that will be required to maximize the yield and xylan content (purity) of polymeric hemicellulose extracted from wheat bran suitable for the formation of entrapment materials for controlled release of gallic acid? A yield higher than 23.8% obtained by Maes and Delcour (2002) whilst maintain a purity within a the range 45.1 to 86.4% reported in literature Table 2.2 will be desirable.
- How do entrapment matrices produced by coacervation method compare to those produced by side chain removing enzymes from wheat bran hemicellulose differ with regards to size, chemical structure, loading capacity and release of gallic acid?

## 3.3 Aims and objectives

The main objective of this study is to develop insoluble entrapment matrices from soluble hemicellulose isolated from wheat bran for the controlled release of chemical and bioactive substances. The specific objectives include:

- To identify the optimal conditions for the extraction of soluble polymeric hemicellulose, from wheat bran using alkali extraction method, for the application of substance delivery entrapment matrices.
- To evaluate the effects of process variables on the extraction yield of hemicellulose from wheat bran.
- To assess the morphology, size and stability of arabinoxylan hydrogels formed by coacervation method and side chain removing enzymes from isolated wheat bran hemicellulose.
- To assess the ability of water insoluble entrapment materials produced from wheat arabinoxylan by coacervation method and side chain removing enzymes to encapsulate and slowly release gallic acid.

## Chapter 4: Alkaline extraction of polymeric arabinoxylan from wheat bran

### 4.1 Abstract

Wheat bran is an abundant agro-residue, which is not a primary food source and is rich in hemicellulose that can be fractionated and used as an alternative to synthetic polymers. Alkaline method was used to extract soluble polymeric hemicellulose from destarched wheat bran. Fractional factorial design was used to screen for the significant effects on the yield of soluble arabinoxylan. Experimental results for screening showed that NaOH concentration, extraction time and temperature had a significant effect ( $p < 0.05$ ) on the yield of arabinoxylan whilst solid loading was found to be insignificant. Furthermore, solid loading had a negative effect on the yield indicating that as solid loading was increasing, the yield was reducing. Therefore, the solid loading was fixed at 5% and central composite design was used to further investigate effects of NaOH concentration (0.5 - 1.5 M), temperature (60 - 80°C) and time (3 - 5 hrs) on the yield and purity (arabinoxylan content) of hemicellulose extracted. These extraction conditions allowed for 63% of hemicellulose initially present in the destarched wheat bran to be extracted. The purity of extracted hemicellulose was up to 53% with low lignin content, which was below 3%. The extracted hemicellulose was in polymeric or oligomeric form as they were no monomers detected in the extract after alkaline extraction. According to the results, alkaline extraction is suitable for extraction polymeric arabinoxylan suitable for the application in hydrogel formation.

## 4.2 Introduction

Hemicelluloses have attracted much attention as a renewable source of biopolymers, which can be an alternative to synthetic polymers. Hemicellulose are the second most abundant polysaccharide in the lignocellulosic biomass with xylan being the major type of hemicellulose with an abundance of 20 – 35% (Saha, 2003; Ebringerová *et al.*, 2005). Xylans are heterogenous polymers composed of  $\beta$ -1,4-linked D-xylopyranose backbone chain, which is branched with arabinose, glucuronic acid or 4-O-methylglucuronic acid, ferulic, acetic, and p-coumaric acid side groups (Saha, 2003). Based on the type and arrangement of these substituents along the main backbone chain, xylans are subdivided into glucuronoxylans, arabinoxylans, (arabino)glucuronoxylans, (glucurono)arabinoxylans, homoxylans and heteroxylans (Ebringerová & Heinze, 2000; Ebringerová *et al.*, 2005).

Wheat bran a by-product of the milling process of wheat to produce flour is a cost effective and abundant source of xylans, with an estimated annual production of about 150 million tonnes (Bergmans *et al.*, 1996a; Aguedo *et al.*, 2014; Prückler *et al.*, 2014; Reisinger, Tirpanalan, Huber, Kneifel & Novalin, 2014). Wheat bran is mostly used as animal feed ingredient, however there is increase in the value-added applications of wheat bran or its components such as enhancing dietary fiber in certain food for human consumption. Nevertheless, due to the decreasing economic value of wheat bran as animal feed ingredient, wheat bran lignocellulosic components can be fractionated and used in the production of value-added materials (Bergmans *et al.*, 1996a; Reisinger *et al.*, 2014).

Wheat bran arabinoxylans have been investigated for the production of food packaging films and coatings (Zhang *et al.*, 2011). Water-soluble wheat arabinoxylans were found to have ability to form three-dimensional network gels with high water holding capacity by oxidative crosslinking. The results showed that high molecular weight arabinoxylans were found to form stronger network gels with high degree of crosslinking and consequently resulted in increased ability to entrap and hold water (Izydorczyk & Biliaderis, 1992).

Xylans are associated with lignin in the lignocellulosic structure by covalent bonding namely ester and ether linkages to form lignin-carbohydrate complexes (LCC). Furthermore, the individual polysaccharide chains in the lignocellulose structure are bound to each other through extensive hydrogen bonding. This complex network of xylan with other constituents

of the lignocellulosic biomass restrict the isolation of xylan with simple conditions from the network (Ebringerová & Heinze, 2000; Peng & She, 2014; Zhang *et al.*, 2014a). Different hemicellulose extraction methods from destarched wheat bran have been studied such as alkaline, hydrothermal, enzymatic, organic solvent, acid and mechanical treatments (Zeitoun, Pontalier, Marechal & Rigal, 2010; Aguedo *et al.*, 2014; Reisinger *et al.*, 2014). In addition to the complex bonding of the lignocellulosic structure, the extraction of xylan from wheat bran is further restricted by the presence of the other constituents such as proteins, phenolics and starch (Ebringerová & Heinze, 2000). Destarching of the wheat bran is undertaken to remove starch as the presence of starch complicate the isolation of arabinoxylan from lignocellulose matrix (Ebringerová & Heinze, 2000; Zhou *et al.*, 2010). The extraction yield, compositions and macromolecular properties of extracted xylans vary largely based on the extraction method used (Zhang *et al.*, 2011). Hydrothermal, mechanical, acid and enzymatic extraction of xylan leads to depolymerization of the xylans to yield low molecular weight chains (Peng, Peng, *et al.*, 2012; Aguedo *et al.*, 2014), which would not be suitable for hydrogel formation. Alkaline treatment of biomass has been found to be more efficient in the liberation of highly polymeric xylans (Gabriellii *et al.*, 2000; Peng, Peng, *et al.*, 2012). The hydroxide ions of the alkaline solution cleave the hydrogen bonds and covalent linkages that bind xylan to other components of the lignocellulosic biomass thereby releasing xylan (Peng, Peng, *et al.*, 2012; Zhang *et al.*, 2014a). However, alkaline treatment results in the loss of acetyl groups along the xylan backbone (Gabriellii *et al.*, 2000).

The aim of this study was to isolate polymeric water-soluble xylans from wheat bran that can be applied as insoluble entrapment materials. In the study, an alkaline method was used to extract water-soluble xylan from wheat bran and the effects of extraction conditions were investigate based on extraction yield, chemical composition, purity degree of polymerization and substitution of extracted xylans.

## **4.3 Materials and Methods**

### **4.3.1 Materials**

The wheat bran that was used in this study was obtained from Essential Grains, Pioneer Food (Paarl, South Africa). Sodium hydroxide pellets and glacial acetic acid were purchased from Sigma-Aldrich. The analytical grade sugars xylose, glucose and arabinose used as standards were purchased from Sigma-Aldrich. Sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) was obtained from Merck Millipore.

### **4.3.2 Wheat bran compositional characterization**

#### **4.3.2.1 Sample preparation**

Sample preparation for the compositional analysis of wheat bran was done according to NREL/TP-510-42620 method (Hames, Ruiz, Scarlata, Sluiter, Sluiter & Templeton, 2008). Wheat bran was sampled using quartering method. The wheat bran was mixed on the ground and divided into four parts after, which one part was removed and the remaining three parts were mixed and divided into four parts. One part was removed again, and the process was repeated until a sample of about 500 g was obtained. The feedstock was conditioned in a room with temperature of 23°C and relative humidity of 55% for 48 hrs before milling. The conditioned homogenous sample was milled in a Retsch ZM 200 mill (with 0.5 mm filter) to reduce particle size and the resulting particles were sieved with a Vibratory Shaker Retsch AS200 consisting of stackable sieves of 850 µm/20 mesh size, 425 µm/40 mesh size and 250 µm/60 mesh size. The particles collected from each sieve were stored in a conditioning room for at least 24 hours and placed in polyethylene bags. The samples with particle size of 425 µm and 625 µm were used for compositional analysis. The samples with particle size of 850 µm were used for hemicellulose extraction.

#### **4.3.2.2 Determination of moisture content**

The moisture content of wheat bran was determined according to the NREL/TP-510-42621 method (Sluiter, Hames, Hyman, Payne, Ruiz, Scarlata, Sluiter, Templeton & Wolfe, 2008). A sample of 2.0 g was weighed on a pre-weighed empty vessel and placed in an oven of 105 ± 3°C for 24 hrs. The dry sample was weighed after cooling in a desiccator. The moisture content was determined according to the following equation:

$$\% \text{ Moisture} = \frac{M_w - W_D}{W_D} \times 100 \quad (4.1)$$

where  $M_w$  and  $M_D$  is the mass of sample before and after drying respectively.

#### 4.3.2.3 Determination of Ash content

The ash content of wheat bran was analysed according the NREL/TP-510-42622 method (Sluiter, Hames, Ruiz, Scarlata, Sluiter & Templeton, 2008a). A mass of 1.5 g of biomass was weighed in crucible of known mass and placed in muffle furnace set to a temperature of  $575 \pm 25^\circ\text{C}$  for 4 hrs, after which the crucible with sample was allowed to cool in desiccator and weighed. The ash content was determined using the following equation:

$$\% \text{ Ash} = \frac{W_{CA} - W_C}{W_S} \times 100 \quad (4.2)$$

where  $W_{CA}$ ,  $W_C$  and  $W_S$  is the weight of crucible plus ash, weight of crucible and weight of sample respectively.

#### 4.3.2.4 Determination of extractives

The extractives in wheat bran were quantified using two-step extraction process following the NREL/TP-510-42619 method (Sluiter, Hames, Ruiz, Scarlata, Sluiter & Templeton, 2008c). Firstly, water extractives were removed using 200 mL of distilled water by placing a tared extraction thimble with a sample of 5 g of biomass in soxhlet apparatus for 24 hrs. The water was then boiled off and the receiving flask of the soxhlet apparatus was dried and weighed. The alcohol extractives were removed in the second step using ethanol in the same manner as water extractives. The extractives content was determined using the following equation:

$$\% \text{ Extractives} = \frac{W_{FE} - W_F}{W_S} \times 100 \quad (4.3)$$

where  $W_{FE}$ ,  $W_F$  and  $W_S$  is the weight of flask plus extractives, weight of flask and weight of sample respectively.

#### **4.3.2.5 Determination of structural carbohydrates and lignin**

The extractives free wheat bran sample was used to determine the carbohydrates and lignin according to the NREL/TP-510-42618 procedure (Sluiter, Hames, Ruiz, Scarlata, Sluiter, Templeton & Nrel, 2010). A sample of 0.3 g was hydrolysed with 3 mL of 72% (w/w) H<sub>2</sub>SO<sub>4</sub> by placing in a water bath at 30 °C for 1 hr while stirring every 10 minutes. The sample was diluted to a concentration of 4% (w/w) using 84 mL of distilled water followed by autoclaving for 1 hr at 121°C. The autoclaved hydrolysis solution was filtered through pre-weighed crucibles. The crucible was dried at 105 ± 3°C for 24 hrs and weighed, after which the crucible was placed in muffle furnace at 75 ± 25°C for 4 hrs and weighed. The filtrate of the hydrolysis solution was captured in a flask and 50 mL were used for acid soluble lignin determination using UV-Visible spectrophotometer at wavelength 320 nm and 10 mL were used for HPLC analysis by adjusting pH between 3 and 7 using 7 M KOH and filtering through 0.22 µm nylon syringe filter. The HPLC system consisted of TSP Spectra System, equipped with a Shodex R I101 refractive index detector operated at 45°C, and with an Aminex HPX-87H Ion Exclusion Column of flow rate of 0.6ml/min. The column temp set to 65°C with isocratic mobile phase of 5 mM H<sub>2</sub>SO.

#### **4.3.2.6 Determination of protein content.**

The protein content of wheat bran and destarched wheat was determined according to AOAC, method 960.52 using the Kjeldahl procedure (VELP SCINTIFICA, Italy). A wheat bran sample of 1 g was digested with 12 mL of concentrated sulphuric acid (98%) in two stages, firstly at 300°C for 40 mins followed by 420°C for 90 mins in digestion tubes. The digestion tubes were cooled to about 50°C. The cooling was followed by automated distillation program using sodium hydroxide solution and received onto boric acid solution. The nitrogen content was determined by titration with 0.2 N of HCL. The nitrogen was converted to crude protein by a factor of 6.25.

#### **4.3.2.7 Determination of starch content**

The starch content of wheat bran was determined using Megazyme total starch assay kit (McCleary method). The starch in wheat bran was hydrolyzed using α-amylase enzyme at 100°C to maltodextrins. The maltodextrins were then hydrolyzed by amyloglucosidase enzyme at 50°C to D-glucose, which was quantified using a colourimetric reaction employing peroxidase.

### **4.3.3 Isolation of hemicellulose from wheat bran**

#### **4.3.3.1 Pre-treatment of wheat bran**

The wheat bran (Essential Grains, Pioneer Foods, Paarl, South Africa) was destarched according to method adapted from Jacquemin et al. (2012) by mixing 40g bran in 400mL of water in 500 mL Schott bottles placed in water bath at 40°C for 15mins with shaking. The wheat bran was separated from starchy water using a sieve (500 µm pores) and washed with three volumes of water. The wet destarched wheat bran was placed in aluminium foil trays and dried at 45°C in oven (Lasec) for 24 hours. The composition of destarched wheat bran measured following Section 4.3.2.

#### **4.3.3.2 Alkaline extraction of hemicellulose**

Alkaline extraction protocol was used to obtain hemicellulose from destarched wheat bran using a method adapted from Aguedo et al. (2014). Figure 4.1 shows the flow diagram for the hemicellulose extraction. The extraction was performed with 10 g of wheat bran and 200 mL of NaOH in 500 mL Schott bottles placed in a shaking water bath. After extraction, the supernatant was separated from the solid residue by centrifugation (10 000 *g* for 15 mins). The solid residue was washed thoroughly in a sieve (500 µm pores) using tap water to remove residual NaOH and dried at room temperature. The supernatant pH was adjusted to 5 with glacial acetic acid and then dialyzed (seamless cellulose, retention 99.99%, MWCO 12400) with distilled water for 3 days to remove salts. The liquid extract was concentrated after dialysis using a rotary evaporator at 60°C and pressure of 700 mmHg until a third of the volume remained. The concentrated liquid extract was freeze dried and milled (Retsch ZM 200 mill) to obtain powdered hemicellulose.

#### **4.3.3.3 Experimental design for the extraction of hemicellulose**

A two-level fractional factorial design was used to study the effect of extraction conditions (extraction time, temperature, NaOH concentration and solid loading) on the yield of hemicellulose. Table 4.1 shows the input variables and levels for the fractional factorial design that were selected based on Koegelenberg's work (Koegelenberg, 2016). The experimental set was composed of 12 experimental runs that included 4 center points to estimate random error. The independent variables were coded according to the following equation:

$$x_i = (X_i - X_0)/\Delta X_i \quad (4.4)$$

where,  $x_i$  is the coded value of the independent variable,  $X_i$ , the real value,  $X_0$ , the real value at the center point and  $\Delta X_i$ , the step change value.

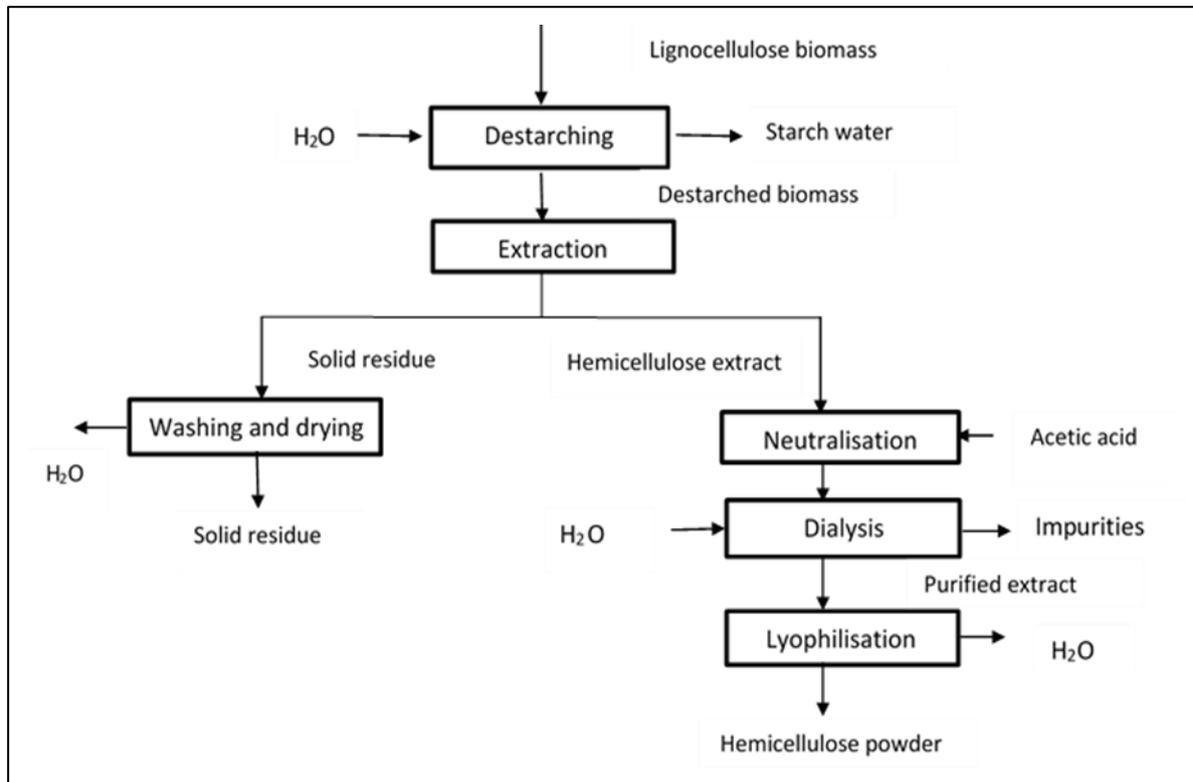


Figure 4.1: Hemicellulose extraction process flow

Following screening of factors using fractional factorial design, the central composite design was used to study the alkaline extraction. The conditions of extraction that were investigated using Central composite design (CCD) were sodium hydroxide concentration (0.5 – 1.5 M), temperature (60 - 80°C), and time (3 – 5hrs). The experimental set up included 16 runs and 3 replications of the centre point. The experimental data was fitted to the following second order model:

$$y_i = \beta_0 + \sum_{i=1}^n \beta_i x_i + \sum_{i=1}^n \beta_{ii} x_i^2 + \sum_{i < j} \beta_{ij} x_i x_j + \epsilon \quad (4.5)$$

where  $y_i$  is the  $i^{\text{th}}$  response variable,  $x_i$  is the  $i^{\text{th}}$  input parameter,  $n$  is the number of input parameters and  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$ ,  $\beta_{ij}$  are the fixed response, linear, quadratic and cross products coefficients, respectively. The statistical significance was checked with ANOVA at a probability of ( $p$ ) of 0.05. The output responses measured were yield, purity. The desirability function was performed at mean values and used in the validation of extraction. STATISTICA version 13. 2 (Statsoft, USA) was used to design the experiment as well as to statistically analyse the data. The yield was calculated as the percentage of hemicellulose present in extract relative to the available hemicellulose in wheat bran and the purity was measured as weight of hemicellulose relative to the dry weight of extract (Bataillon *et al.*, 1998) as shown with the following equations:

$$\text{Yield \%} = \frac{\text{Arabinoxylan present in extract (g)}}{\text{Arabinoxylan present in wheat bran (g)}} \times 100 \quad (4.6)$$

$$\text{Purity \%} = \frac{\text{Arabinoxylan weight (g)}}{\text{dry weight of extract (g)}} \times 100 \quad (4.7)$$

Table 4.1: Factors and levels used in the fractional factorial design for hemicellulose extraction from wheat bran

Factor	Low (-1)	Center (0)	High (+1)
NaOH concentration (M)	0.5	1	1.5
Extraction time (hrs)	2	3.5	5
Extraction temperature (°C)	40	60	80
Solid loading (SL)	5	6.5	8

#### 4.3.4 Analysis of liquid extract for sugars, degraded products and solubilised lignin

The liquid extract after dialysis was analysed for any monomeric sugars that could result from xylan degradation by filtering through 0.22  $\mu\text{m}$  nylon syringe filters and analysing by HPLC according to NREL/TP-510-42623 method (Sluiter, Hames, Ruiz, Scarlata, Sluiter & Templeton, 2008b). The hemicellulose liquid extract was hydrolysed in 1 M  $\text{H}_2\text{SO}_4$  at 100°C for 90 mins

(Zhou *et al.*, 2010) in tightly capped test tubes, cooled to room temperature and the pH was then adjusted to between pH 3 and pH 7 using 7 M KOH and 3.5% PCA. The hydrolysed liquid extract was then filtered through 0.22 µm nylon syringe filters into vials and analysed by HPLC. The non-hydrolysed liquid extract was also filtered through 0.22 µm nylon syringe filters into vials and analysed by HPLC. The HPLC system consisted of TSP Spectra System, equipped with a Shodex R I101 refractive index detector operated at 45°C, and with an Aminex HPX-87H Ion Exclusion Column of flow rate of 0.6 mL/min. The column temp set to 65°C with isocratic mobile phase of 5 mM H<sub>2</sub>SO<sub>4</sub>. The acid soluble lignin in the hydrolysed liquid extract was determined using UV-visible spectrophotometer at wavelength 320 nm based on NREL/TP-510-42618 method (Sluiter *et al.*, 2010).

#### **4.3.5 Analysis of solid residue for carbohydrates, lignin, ash and moisture content**

The dried solid residues were conditioned in room where temperature was set at 23°C and relative humidity at 55% for 48 hrs and then milled with Retsch ZM 200 mill to approximately 425 µm particle size. The solid residues were then analysed for moisture, ash, carbohydrates and lignin following the same methods as the raw material. The extraction mass balance (EMB) was determined according to the following equation:

$$EMB = \frac{\sum C_{Li} + \sum C_{Si}}{\sum C_{Ri}} \quad (4.8)$$

Where  $C_i$  is the mass of each component (e.g. xylose or arabinose) and the subscripts L, S and R refer to the liquid extract, solid residue and raw material (Um & van Walsum, 2010).

## 4.4 Results and discussion

### 4.4.1 Composition original wheat bran and destarched wheat bran

The composition of the original wheat bran (WB) and destarched wheat bran (DWB) were analysed and the results of this study together with the compositions recorded in literature are shown in Table 4.2. The starch content in the wheat bran used in this study was 13.44% and relatively low compared to values from literature (Table 4.2). Brillouet and Mercier, (1981) have explained this variation to be due to the different milling conditions of wheat used in industry.

Table 4.2: Composition of wheat bran (WB) and destarched wheat bran (DWB) from this study and other authors

Component (%)	This study <sup>c</sup>		(Bataillon <i>et al.</i> , 1998)		(Brillouet & Mercier, 1981)		(Koegelenberg & Chimphango, 2017)	
	WB	DWB	WB	DWB	WB	DWB	WB	DWB
<b>Starch</b>	13.44 ± 0.01	1.29 ± 0.00	29	1	29.4	0.8	6.8	0.8
<b>Protein</b>	19.60 ± 1.35	16.05 ± 0.64	14	9	14.2	25.4	18.9	22.4
<b>Ash</b>	7.30 ± 0.04	4.62 ± 0.13	4	1	4.3	1.7	6.9	4.8
<b>Lignin</b>	5.83 ± 0.25	11.43 ± 0.42	3	6	3	6.6	8.9	9.5
<b>Glucose</b>	18.06 ± 0.71	18.84 ± 0.18	11 <sup>a</sup>	25 <sup>a</sup>	22.1	22.4	29.0	21.9
<b>Xylose</b>	18.58 ± 0.32	22.86 ± 0.28	12	26	12.3	24.2	22.1	23.2
<b>Arabinose</b>	8.67 ± 0.67	14.11 ± 0.80	7	19	7.3	13.6	10.7	13.2
<b>Arabinoxylan</b>	23.98 ± 0.55 <sup>b</sup>	32.53 ± 0.73 <sup>b</sup>	19	45	17.2 <sup>b</sup>	33.3 <sup>b</sup>	26.2 <sup>b</sup>	29.1 <sup>b</sup>

<sup>a</sup>Expressed as cellulose content

<sup>b</sup>Arabinoxylan expressed as 0.88 (Xylose + Arabinose)

<sup>c</sup>Average values with standard deviations

The destarching process of wheat bran resulted in starch content being reduced to 1.29% and a reduction in the protein content from 19.60 to 16.05%. The reduction in the starch and protein enhances arabinoxylan extractability (Bataillon *et al.*, 1998). The ash content also reduced during the destarching process thereby reducing impurities and improving extractability of arabinoxylan. The ash and lignin content in the wheat bran and destarched wheat bran was comparable to that in literature Table 4.2. The arabinoxylan content in wheat

bran and destarched wheat bran was 23.98 and 32.53% with an arabinose/xylose ratio of 0.46 and 0.62, respectively. The arabinose/xylose ratio wheat bran and destarched wheat bran was comparable to that obtained by Bataillon *et al.*, (1998) with a ratio of 0.58 and 0.73, respectively. Aguedo *et al.*, (2014) obtained an arabinose/xylose ratio of 0.54 after destarching. The effectiveness of starch removal by washing and wet-sieving is attributed to the small size of starch granule compared to that of bran (Zhou *et al.*, 2010). The destarched wheat bran was subsequently used for the extraction of arabinoxylan.

#### 4.4.2 Influence of extraction parameters on hemicellulose yield using fractional factorial design (FFD)

The effects of sodium hydroxide concentration, extraction time, extraction temperature and solid loading on the yield of arabinoxylan during alkaline extraction were investigated using a FFD (Table 4.3). The xylan extraction yields were in the range of 33.32-64.1% and the lowest yield was observed at the lowest level of the design (Table 4.3).

Table 4.3: The arabinoxylan (AX) yield of liquid extract after alkaline extraction using fractional factorial design

Run	NaOH concentration (M)	Time (hrs)	Temperature (°C)	Solid loading (% w/v)	AX Yield %
1	0.5	2.0	40	5.0	33.32
2	1.5	2.0	40	8.0	43.04
3	0.5	5.0	40	8.0	34.87
4	1.5	5.0	40	5.0	46.24
5	0.5	2.0	80	8.0	57.96
6	1.5	2.0	80	5.0	61.44
7	0.5	5.0	80	5.0	64.31
8	1.5	5.0	80	8.0	60.54
C	1.0	3.5	60	6.5	60.71
C	1.0	3.5	60	6.5	62.09
C	1.0	3.5	60	6.5	59.88
C	1.0	3.5	60	6.5	58.04

The pareto chart (Figure 4.2) shows the effect of these variables on arabinoxylan yield according to the order of their significance ( $p < 0.05$ ). Temperature ( $p = 0.05$ ) was the most

significant followed by sodium hydroxide concentration ( $p=0.05$ ) and extraction time ( $p=0.05$ ). Solid loading was the least significant factor ( $p=0.05$ ). The temperature, time and sodium hydroxide had a positive effect in the yield. In contrast, the solids loading negatively affected the yield (Figure 4.2). As the solid loading of destarched wheat bran increased, the yield of arabinoxylan decreased and this could be attributed to the rheological properties of wheat bran. The wheat bran lignocellulosic suspension is highly viscous and the increase in solid loading causes a challenge in the mixing as well as mass transfer during arabinoxylan extraction (Koppram, Tomás-Pejó, Xiros & Olsson, 2014). The interaction of sodium hydroxide and extraction time was the only significant interaction and had a negative effect.

The analysis of variance (ANOVA) showed that the check for curvature and lack of fit were significant indicating that a first order model was not adequate and therefore a face-centered central composite design was used to optimise the significant factors (sodium hydroxide concentration, extraction time and temperature) for xylan extraction. The solid loading was fixed at 5% as higher solid loadings resulted in reduced yields and difficulty in the mixing.

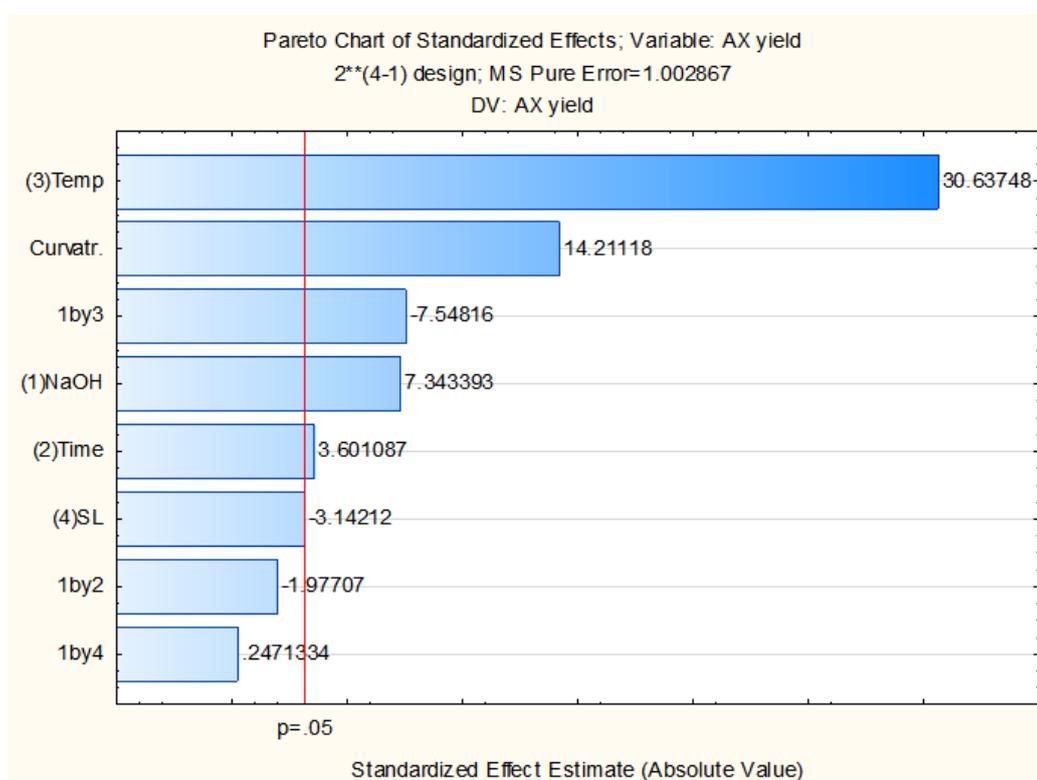


Figure 4.2: Pareto chart of the standardized effects of Arabinoxylan yield. The solid line indicates significance ( $p=0.05$ ). SL represents solid loading.

#### **4.4.3 Optimisation of hemicellulose extraction from wheat bran in a central composite designed experiment**

The experimental soluble arabinoxylan yields were observed in the range of 22.49 to 63.34% (Table 4.4). The highest xylan yield was observed at the sodium hydroxide concentration of 1.5 M, temperature of 80°C and 5 hrs. The analysis of the composition of liquid extract after alkaline extraction at different conditions showed that there were no monomeric sugars detected. This means that there was no or negligible degradation of arabinoxylan polymer into monomers and thus the extracted arabinoxylan was in polymeric or oligomeric form. Polymeric arabinoxylan is more suitable for the application of forming hydrogels that can be used as entrapment matrices for controlled release of substances. This is because polymeric arabinoxylans have been shown to form hydrogels with increased ability to entrap substances (Izydorczyk & Biliaderis, 1992).

Alkaline extraction has been shown to also result in the solubilisation of other components of the lignocellulosic matrix (Ebringerová & Heinze, 2000; Zhang et al., 2011). The contaminants of extracted hemicellulose such as lignin reduce the purity of hemicellulose and may affect the effectiveness of enzyme during modification of arabinoxylan to produce hydrogels. The extracted arabinoxylan in this study had lignin content of less than 3% (Table 4.4). The low lignin content in hemicellulose extracted from wheat bran has been recorded in literature. Zhang et al., (2011) reported a lignin content of up to 2% in the hemicellulose extracted from wheat bran. Condition 8 of the central composite design was used for the formation of hydrogels because of its high yield and purity and a low lignin content of 1.67%. The characterization of liquid extract and solid residue after arabinoxylan alkaline extraction was used for mass balance closure. The mass balance of arabinoxylan for central composite was between 90 and 100% (Table 4.5). The experimental data (Table 4.4) was used to fit second order models to describe the yield and purity of arabinoxylan. The analysis of variance was used to determine the model adequacy. The regression analysis and ANOVA data from arabinoxylan yield and purity are shown on Table 4.6 and Table 4.7 respectively.

Table 4.4: The experimental results of arabinoxylan (AX) extraction conditions using CCD

Run	NaOH concentration (M)	Time (hrs)	Temp (°C)	Glucose (%)	Xylose (%)	Arabinose (%)	AX (%) <sup>a</sup>	Lignin (%)	AX yield (%) <sup>b</sup>
1	0.50	3.00	60.00	5.46	27.29	18.53	40.32	1.39	37.32
2	0.50	3.00	80.00	3.67	23.50	17.03	35.67	2.54	45.86
3	0.50	5.00	60.00	5.39	26.06	17.96	38.74	1.78	34.93
4	0.50	5.00	80.00	3.82	26.00	19.24	39.81	2.74	42.70
5	1.50	3.00	60.00	5.68	33.57	18.00	45.38	1.99	46.51
6	1.50	3.00	80.00	4.77	31.67	17.12	42.93	2.16	60.01
7	1.50	5.00	60.00	6.15	36.32	19.46	49.09	2.21	53.93
8	1.50	5.00	80.00	5.75	38.31	22.08	53.14	1.67	63.34
9	0.16	4.00	70.00	5.28	25.49	20.52	40.49	2.81	22.49
10	1.84	4.00	70.00	5.62	35.09	21.35	49.66	2.94	57.93
11	1.00	2.32	70.00	5.14	33.28	18.29	45.38	2.06	46.97
12	1.00	5.68	70.00	3.94	26.81	14.93	36.73	2.14	51.11
13	1.00	4.00	53.18	5.17	29.14	14.29	38.22	1.77	47.66
14	1.00	4.00	86.81	5.05	35.82	21.48	50.42	2.19	57.38
15 (C)	1.00	4.00	70.00	4.91	30.25	17.57	42.09	1.68	54.36
16 (C)	1.00	4.00	70.00	6.26	37.73	21.82	52.40	1.84	54.93
17 (C)	1.00	4.00	70.00	5.76	34.57	19.57	47.64	1.96	55.76
18 (C)	1.00	4.00	70.00	5.93	34.90	19.23	47.64	1.82	59.18

<sup>a</sup>AX (Arabinoxylan) content = 0.88(xylose + Arabinose)

<sup>b</sup>Yield = weight of arabinoxylan relative to weight of destarched wheat bran

The regression coefficients were shown to be significantly larger compared to the standard error thus confirming that a second-order model was adequate to fit the data. ANOVA further confirmed that a second-order model was adequate to describe arabinoxylan yield and purity (Table 4.6 and Table 4.7). The R<sup>2</sup> was 0.96734 and 0.64109 for the model of arabinoxylan yield and purity respectively, thus confirming that 96.73 and 64.11% variability in the arabinoxylan

yields and purity was attributed to second-order relation to sodium hydroxide concentration, extraction temperature and time. The lack of fit was insignificant.

Table 4.5: Extraction mass balance (EMB) of arabinoxylan

Run	Liquid extract (%)	Solid residue (%)	EMB (%)
1	37.32	56.47	93.79
2	45.86	55.05	100.90
3	34.93	65.54	100.47
4	42.70	47.65	90.35
5	46.51	45.10	91.61
6	60.01	30.59	90.60
7	53.93	39.49	93.42
8	63.34	33.58	96.92
9	22.49	70.95	93.44
10	57.93	36.46	94.39
11	46.97	46.12	93.09
12	51.11	41.88	92.99
13	47.66	45.88	93.54
14	57.38	36.21	93.60
15C	54.36	39.57	93.92
16C	54.93	41.33	96.27
17C	55.76	37.70	93.46
18C	59.18	39.57	98.74

On the pareto chart, L and Q represent the linear first order and quadratic second order terms, respectively. The sodium hydroxide concentration had a significant ( $p < 0.05$ ) positive linear effect and negative quadratic effect on solubilizing hemicellulose as shown on the pareto chart on Figure 4.3. As the sodium hydroxide concentration increased, the arabinoxylan extraction yield increased. The increase of xylan extraction yields due to increase in sodium hydroxide concentration is in agreement with literature and the high yields are due to high concentration of sodium hydroxide releasing arabinoxylan strongly embedded in the complex cell wall matrix (Jayapal, Samanta, Kolte, Senani, Sridhar, Suresh & Sampath, 2013). The hydroxyl ions of the alkaline cleaved the extensive bonding that binds the xylan to the other components of wheat bran lignocellulose (Xu *et al.*, 2006; Peng, Peng, *et al.*, 2012). However, further increase of sodium hydroxide concentration causes reduction in the yield due to polymer degradation. The linear term of extraction temperature and the quadratic term

extraction time were also significant within 95% confidence interval. The increase in time allows for more arabinoxylan to be liberated from the wheat bran lignocellulose structure, however longer extraction times could degrade or alter the structure of arabinoxylan (Brienzo *et al.*, 2009). Therefore, longer extraction time will not be suitable for obtaining arabinoxylans for the formation of entrapment materials. However, the quadratic term of extraction temperature and linear term of extraction time did not have a significant effect on the extraction yield of arabinoxylan. The interactions of sodium hydroxide concentration with extraction time and sodium hydroxide with extraction temperature were not significant at 95% confidence.

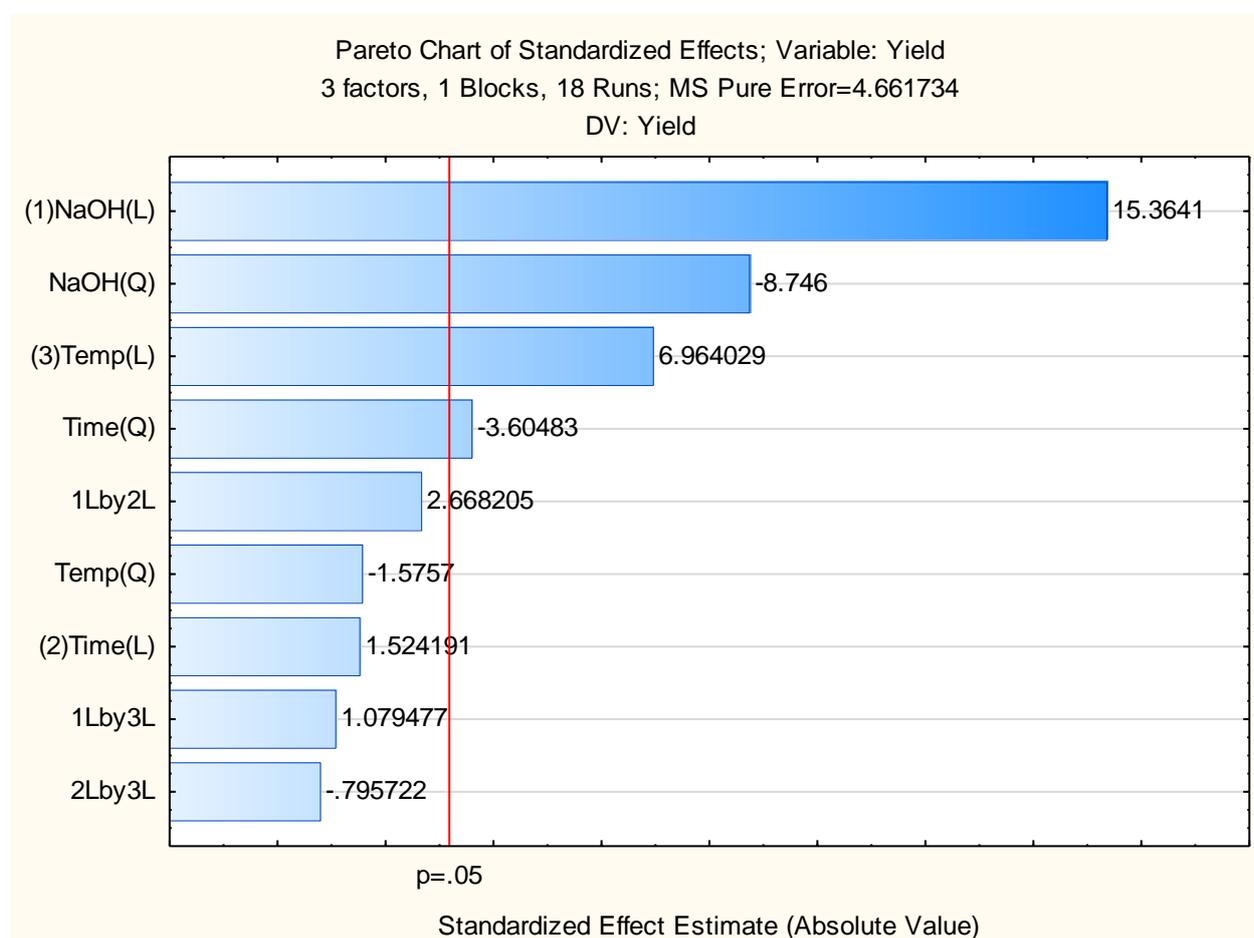


Figure 4.3: Standardized Pareto chart estimating the significance of the variables sodium hydroxide, extraction time and temperature on arabinoxylan yield from destarched wheat bran.

The purity of hemicellulose was also only significantly affected by the linear term of sodium hydroxide concentration at 95% confidence level as shown by the pareto chart (Figure 4.4).

The purity obtained in the central composite design study ranged from 35.67 to 53.14%. The interactions of all three factors were not significant on the purity of arabinoxylan. The experimental data was used to employ multiple regression analysis for the yield of arabinoxylan and the following equation was obtained:

$$y = 55.99 + 17.95x_1 - 10.62x_1^2 + 1.78x_2 - 4.38x_2^2 + 8.14x_3 - 1.91x_3^2 \quad (4.9) \\ + 4.07x_1x_2 + 1.65x_1x_3 - 1.21x_2x_3$$

And, the estimated regression model for purity of arabinoxylan is represented below by equation:

$$y = 47.45 + 7.53x_1 - 1.72x_1^2 + 0.28x_2 - 4.56x_2^2 + 2.72x_3 - 2.26x_3^2 \quad (4.10) \\ + 2.84x_1x_2 + 1.29x_1x_3 + 3.06x_2x_3$$

Where  $x_1$ ,  $x_2$ , and  $x_3$  are the coded values for sodium concentration, extraction temperature and time. The three-dimensional plot is used to understand the interaction between two or three variables as well as to locate their optimum ranges. The response surface plots showing the effect of sodium hydroxide concentration, extraction time and temperature on arabinoxylan yield and purity are shown in Appendix B. The highest extraction yield obtained in this study was higher compared to that 23.8% obtained in literature (Maes & Delcour, 2002) and the highest arabinoxylan content was within the range 45.1 to 86.4% reported in literature Table 2.2. Aguedo *et al.*, (2014) reported a yield of 20.8% and purity of 57.5% whilst Chauvelon *et al.*, (1997) obtained a yield of 19.9% and purity of 76.2%. The high purity of soluble arabinoxylan is attributed to certain pre-treatments. However, these pre-treatments may result in degradation of arabinoxylan and lower yields.

The desirability analysis was performed at mean values with STATISTICA and estimated a yield and purity of 55.99% and 47.45%, respectively (Figure 4.5). Three assays of extraction were performed at mean extraction conditions and yield and purity of 56.39% and 43.75% were obtained. These experimental values agreed with values estimated by the model at 95% confidence level, thus indicating that the model adapted to the experimental results.

Table 4.6: Estimated effects and coefficients for the CCD models for arabinoxylan yield as responses.

Factor	SS <sup>a</sup>	Df <sup>b</sup>	MS <sup>c</sup>	F value	p-value (Prob>F)
<b>(1) NaOH (L)</b>	<b>1100.428</b>	<b>1</b>	<b>1100.428</b>	<b>236.0554</b>	<b>0.000599</b>
NaOH (Q)	356.588	1	356.588	76.4926	0.003148
(2) Time (L)	10.830	1	10.830	2.3232	0.224857
Time (Q)	60.578	1	60.578	12.9948	0.036637
(3) Temp (L)	226.083	1	226.083	48.4977	0.006075
Temp (Q)	11.574	1	11.574	2.4828	0.213178
1L by 2L	33.188	1	33.188	7.1193	0.075806
1L by 3L	5.432	1	5.432	1.1653	0.359427
2L by 3L	2.952	1	2.952	0.6332	0.484339
Lack of Fit	45.175	5	9.035	1.9381	0.310841
Pure Error	13.985	3	4.662		
Total SS	1811.584	17			

<sup>a</sup>Sum of squares

<sup>b</sup>Degrees of freedom

<sup>c</sup>Mean Square

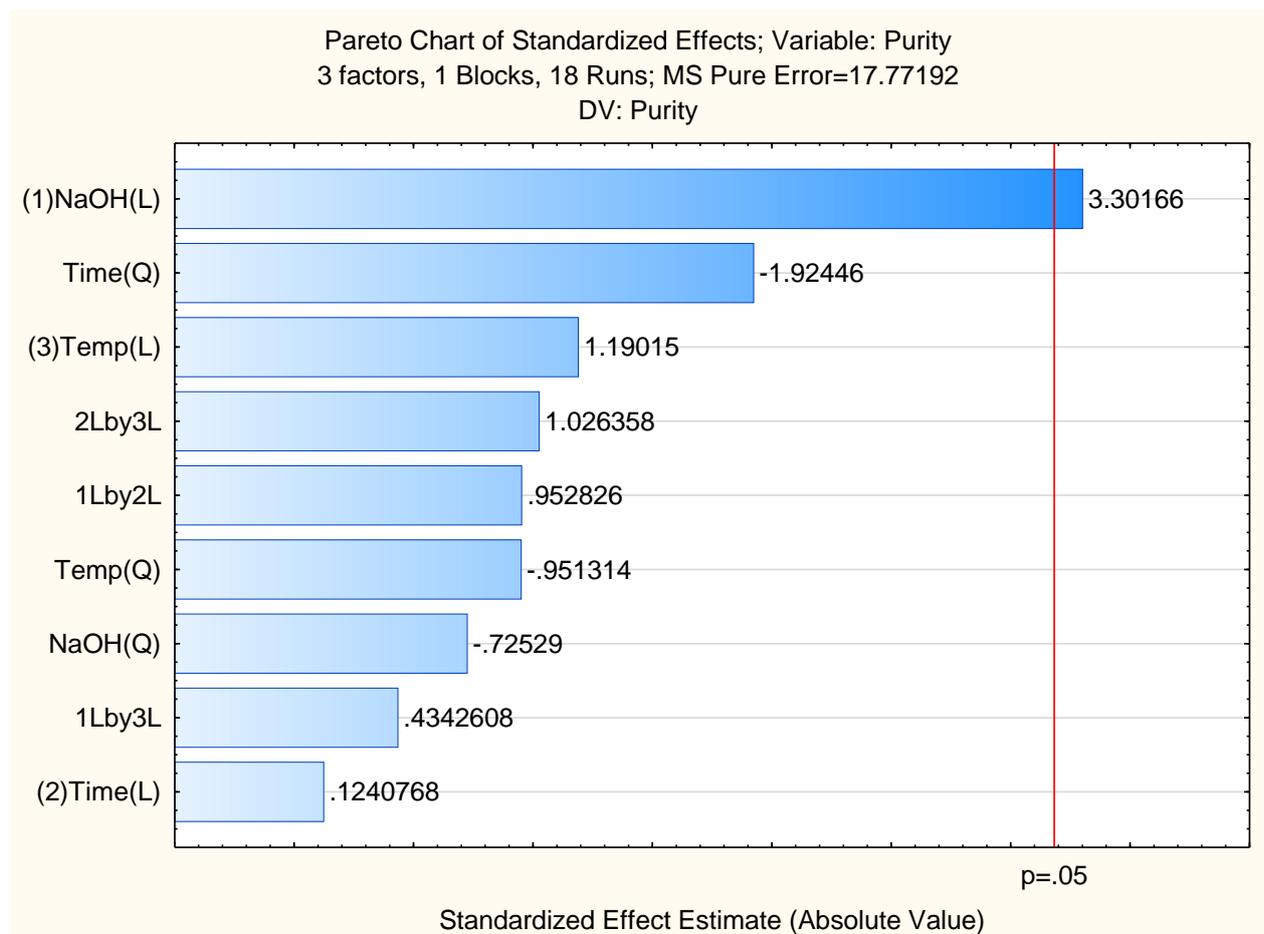


Figure 4.4: Standardized Pareto chart estimating the significant the effects sodium hydroxide, extraction time and temperature on arabinoxylan purity from destarched wheat bran.

Table 4.7: Estimated effects and coefficients for the CCD models for arabinoxylan purity as responses.

Factor	SS <sup>a</sup>	Df <sup>b</sup>	MS <sup>c</sup>	F value	p-value (Prob>F)
<b>(1) NaOH (L)</b>	<b>193.7309</b>	<b>1</b>	<b>193.7309</b>	<b>10.90096</b>	<b>0.045677</b>
NaOH (Q)	9.3488	1	9.3488	0.52605	0.520730
(2) Time (L)	0.2736	1	0.2736	0.01540	0.909101
Time (Q)	65.8190	1	65.8190	3.70354	0.149980
(3) Temp (L)	25.1732	1	25.1732	1.41646	0.319585
Temp (Q)	16.0836	1	16.0836	0.90500	0.411627
1L by 2L	16.1347	1	16.1347	0.90788	0.410972
1L by 3L	3.3515	1	3.3515	0.18858	0.693436
2L by 3L	18.7211	1	18.7211	1.05341	0.380246
Lack of Fit	131.3116	5	26.2623	1.47774	0.397304
Pure Error	53.3158	3	17.7719		
Total SS	514.4089	17			

<sup>a</sup>Sum of squares<sup>b</sup>Degrees of freedom<sup>c</sup>Mean Square

#### 4.4.4 Simultaneous optimisation of process parameters for extraction of arabinoxylan from wheat bran

The highest yield of arabinoxylan achieved experimentally in this study was 63.34% at sodium hydroxide of concentration 1.5 M, extraction time of 5 hrs and temperature of 80°C. Whilst the predicted optimum extraction yield of arabinoxylan was 66.20% at sodium concentration, extraction time and temperature of 1.56M, 4.38 hrs and 95°C, respectively (Table 4.8). An extraction temperature of 95°C would demand high energy levels and specialized equipment. Furthermore, the high temperatures at large scale would not only be energy demanding but economically challenging. The highest arabinoxylan content obtained in this study was 53.14% at sodium hydroxide concentration of 1.5M whereas the predicted optimum arabinoxylan content of 68.13% would require a sodium hydroxide concentration of 5.27 M. High sodium hydroxide concentration would pose environmental challenges as well as equipment damage (Bataillon *et al.*, 1998).

Table 4.8: The comparison between individual and simultaneous optimisation of yield and purity in central composite design

Variable	Yield		Purity	
	Composite optimization	Individual optimisation	Composite optimisation	Individual optimisation
NaOH concentration	1.56	1.50	5.27	1.50
Extraction time	4.38	5.00	8.80	5.00
Extraction temperature	94.86	80.00	133.07	80.00
Output	66.20 <sup>a</sup>	63.34 <sup>a</sup>	68.13 <sup>b</sup>	53.14 <sup>b</sup>

<sup>a</sup>Yield defined as weight of arabinoxylan relative to weight of destarched wheat bran

<sup>b</sup>Purity defined weight of arabinoxylan relative to dry weight of extract

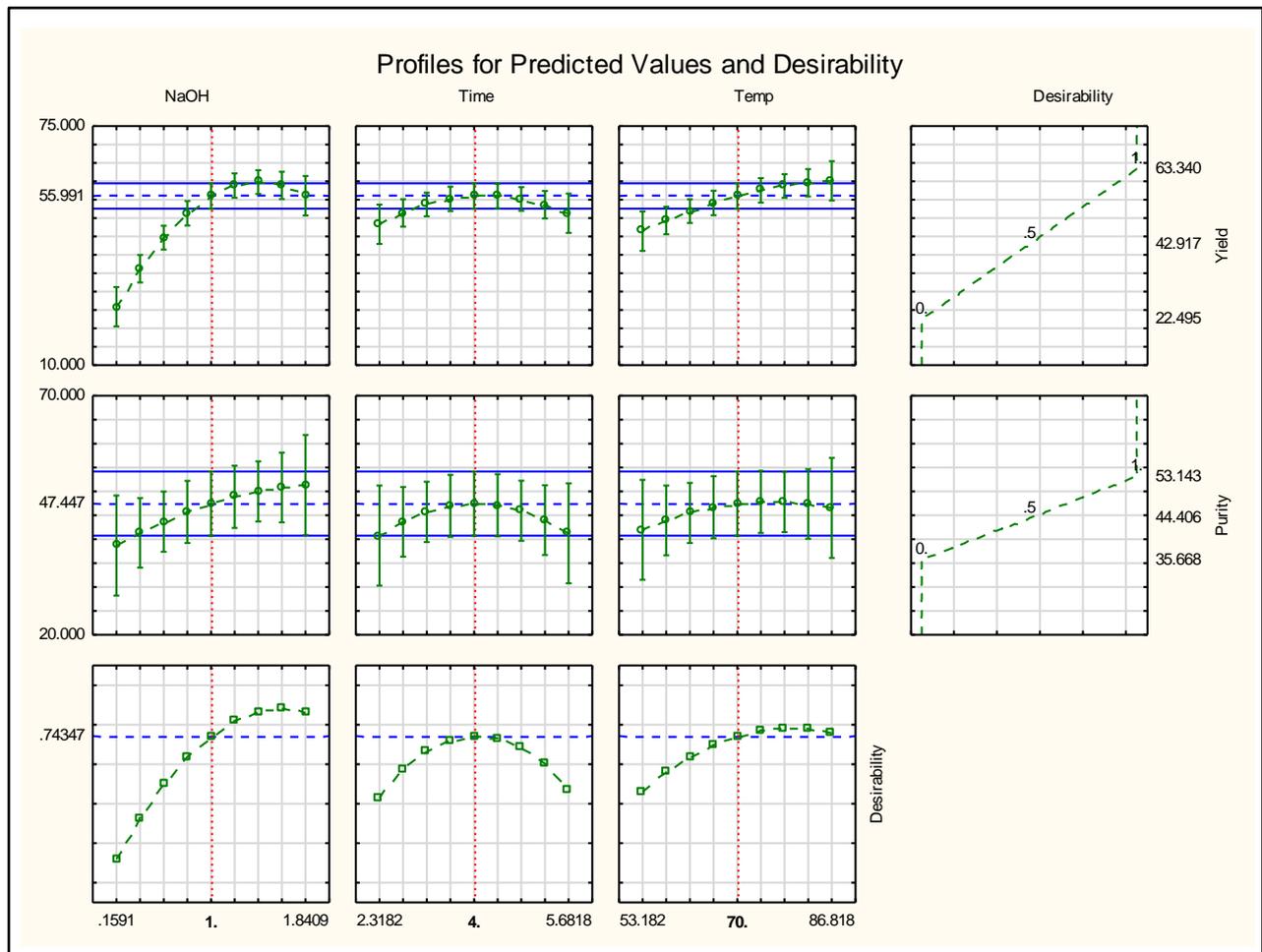


Figure 4.5: The desirability plot of arabinoxylan yield and purity

## 4.5 Conclusion

Soluble polymeric arabinoxylan was extracted from industrial wheat bran using alkaline method. According to the fractional factorial design, sodium hydroxide concentration, extraction temperature and time had a significant effect on the yield of arabinoxylan whilst solid loading was not significant on the yield of arabinoxylan. The yield and purity of arabinoxylan obtained was of the range 22 – 63% and 35 – 53%, respectively. The optimum condition was estimated to be at sodium hydroxide concentration 1.5 M, extraction temperature of 80°C and an extraction time of 5 hrs. However, extreme conditions were avoided as they could result in the degradation of arabinoxylan making it unfit for modification into hydrogels. The optimum yield obtained was higher than 23.8% obtained by Maes and Delcour (2002). The optimum purity was within the range of 45.1 to 86.4% that is reported in literature (Table 2.2). The extracted arabinoxylan had a lignin content of less than 3% and showed no detectable degradation of polymeric chain.

In conclusion, polymeric arabinoxylan that is suitable for the application of hydrogel formation was extracted using alkaline method from destarched wheat bran.

## Chapter 5: Modification of soluble arabinoxylan into delivery systems

### 5.1 Abstract

Wheat bran arabinoxylan was used to prepare hydrogels for the delivery of gallic acid using coacervation chemical method and side chain removing enzyme  $\alpha$ -arabinofuranosidase produced in house. The soluble arabinoxylan was obtained by alkaline extraction from wheat bran in chapter 4. The aim of this work was to produce wheat bran arabinoxylan hydrogels to be applied as delivery systems for the slow release of gallic acid whilst preserving the functional properties of gallic acid.

Analysis of the size of hydrogels formed indicated that hydrogels formed by both coacervation and enzymatic hydrolysis were in nano-size range (469 – 678nm). However, enzymatically formed hydrogels were more stable as compared to chemically formed hydrogels as they had smaller size distribution and more negative zeta potential. The negative zeta potential meant that the hydrogels were less likely to agglomerate. The functional properties of hydrogels were influenced by the method of encapsulation. The encapsulation efficiency of gallic acid in chemically modified hydrogels and enzymatically modified hydrogels when gallic acid is incorporated during formation of hydrogels was 72.35% and 58.85%, respectively. Encapsulation of gallic acid after formation of hydrogels resulted in slightly lower encapsulation efficiencies as compared to incorporating gallic acid during formation of hydrogels. Although chemically modified hydrogels had a higher encapsulation efficiency for gallic acid than enzymatically formed hydrogels, enzymatically formed hydrogels better preserved the antioxidant capacity of gallic acid.

In conclusion, this study showed that coacervation method and side chain removing enzyme  $\alpha$ -arabinofuranosidase modified the soluble wheat bran arabinoxylan into delivery systems for controlled release of gallic acid. However, encapsulating gallic acid in hydrogels formed by enzymatic hydrolysis better preserved the antioxidant capacity of the incorporated gallic acid.

## 5.2 Introduction

Hydrogels are insoluble three-dimensional network structures formed by the cross linking of hydrophilic polymers with ability to imbibe large amounts of water or biological fluid (Peppas, 2000; Soppimath *et al.*, 2002; Laftah *et al.*, 2011). The cross-linked polymer chains are held together by physical and chemical forces in a network matrix, which attributes to their insolubility property (Gupta *et al.*, 2002). The hydrogels are being applied in a number of industries that include agriculture, textile, cosmetics, water treatment, oil recovery and pharmaceuticals (Gupta *et al.*, 2002). Of particular interest, is the increased application of hydrogels as entrapment matrices for controlled release of chemicals, bioactive substances and drugs. The encapsulation of substances in hydrogels has several advantages, which include protection of encapsulated substance from harsh external conditions thereby allowing delivery in its active form and tailoring the release of encapsulated substance over time or to suit desired purpose (Fang & Bhandari, 2010). Thus, the preparation of hydrogels for delivery systems as well as encapsulating matrix are important factors in the development of hydrogels for substance delivery.

There is increasing interest in the replacement of synthetic polymers with renewable biopolymers for development of delivery system due to limited availability of fossil based raw materials (Karaaslan *et al.*, 2010; Peng, Ren & Sun, 2010). Hemicellulose are a renewable biopolymer making up 20 - 35% of lignocellulosic biomass with xylan being the most abundant (Saha, 2003; Ebringerová *et al.*, 2005). Furthermore, xylans are a good alternative to synthetic polymers as they are biocompatible, non-toxic and biodegradable (Karaaslan, Tshabalala, Yelle & Buschle-diller, 2011; Martínez-López *et al.*, 2013). In pharmaceutical applications, xylans have the advantage of being resistant to degradation in the stomach as well as small intestines and can only be degraded in the colon by micro-organisms (Ebringerová & Heinze, 2000; Kumar & Negi, 2012). Thus, xylan can be used as a delivery system for drugs sensitive to the gastrointestinal environment. Xylan from agro-waste corn cobs was used to incorporate 5-aminosalicylic acid for sustained and site-specific release.

However, the use of xylan as insoluble delivery systems is limited by its water solubility due to its branched structure as well as its low molecular weight (Ebringerová *et al.*, 2005; Zhu Ryberg *et al.*, 2011). Xylan structure is made of  $\beta$ -linked xylose backbone that is branched with either D-glucuronic acid or its 4-O-methyl ester and L-arabinose and the alteration of the side

group substitution can result in the formation of insoluble hydrogels for controlled release of substances (Izydorczyk & Biliaderis, 1995; Ebringerová *et al.*, 2005; Chimphango, van Zyl, *et al.*, 2012b).

Modification of water soluble xylan into insoluble hydrogels using various, chemical, biological and physical methods has been reported. However most chemical methods for hydrogel formation use toxic cross linkers or result in residual monomers that may not be favorable for encapsulation and release of substances (Marcelino *et al.*, 2015; Ullah *et al.*, 2015). Nano-sized hydrogels that showed sustained release of horseradish peroxidase were formed from selective hydrolysis of oat spelt xylan with  $\alpha$ -arabinofuranosidase (Chimphango, van Zyl, *et al.*, 2012b). Such green or mild methods are advantageous in preparation of delivery systems for sensitive chemicals or bioactive substances such as gallic acid.

Gallic acid (3,4,5-trihydroxybenzoic acid) is a polyphenol found in tea, fruits, cereals and herbs with therapeutic properties such as anti-oxidative, anti-inflammatory, anti-carcinogenic and anti-microbial properties (Yen, Duh & Tsai, 2002; Kim, Jun, Suk, Choi, Lim, Park, Lee, Shin, Kim & Shin, 2006; Chanwitheesuk, Teerawutgulrag, Kilburn & Rakariyatham, 2007). However, its application is limited by its instability, low aqueous solubility, low-bioavailability and unpleasant taste (Fang & Bhandari, 2010; Tavano, Muzzalupo, Picci & Cindio, 2014). Due to these characteristics, gallic acid was selected as a model substance for encapsulation in this study.

In this chapter, hydrogels formed were produced from wheat bran xylan by selective hydrolysis with  $\alpha$ -arabinofuranosidase enzyme and coacervation method that is based on neutralization of alkaline solution with acid. The objectives were to investigate the encapsulation efficiencies and antioxidant capacities of the encapsulated gallic acid in the formed hydrogels and to subsequently assess the release characteristics of GA.

## 5.3 Material and methods

### 5.3.1 Materials

The wheat bran arabinoxylan was obtained as described in chapter 4. The oat spelt xylan was obtained from colleagues. The gallic acid, 1,1'-diphenyl-2-picrylhydrazyl (DPPH), *p*-nitrophenol (*p*NP) and *p*-nitrophenyl- $\alpha$ -arabinofuranoside (*p*NPA) were purchased from Sigma-Aldrich. Commercial AbfB was obtained from Megazyme. Polyoxoethylen (20) sorbitan monolaurate (Tween<sup>®</sup> 20) was purchased from Merck Millipore.

### 5.3.2 Production of recombinant $\alpha$ -L-arabinofuranosidase

The  $\alpha$ -L-arabinofuranosidase (AbfB) was produced from recombinant *Aspergillus niger* D15 strain carrying the AbfB gene according to (Chimphango, Rose, *et al.*, 2012). The *A. niger* spores carrying the gene (*A. niger* D15 [abfB]) were obtained from colleagues in the Microbiology Department of Stellenbosch University. The *A. niger* D15 [abfB] was grown by streaking 2  $\mu$ L of spore solution on plates containing 25 ml of minimal medium with nitrate (1% glucose, 0.2% trypticase (BBL), 0.1% yeast extract, 0.1% (*w/v*) casamino acids) and incubated at 30°C for 4 days. The spores were then harvested by pouring 20 ml of sterile 0.9% of physiological salt (NaCl) on three plates, after which a wooden stick with end covered in cotton wool was used to loosen the spores. The spore solution was poured back into bottle with rubber stopper lid and stored at 4°C. Cultivation was performed in 125 mL Erlenmeyer shake flasks by inoculating  $1 \times 10^6$  spores/ml in 25 mL of double strength minimal medium (2xMM) supplemented with 10% glucose. The cultivation occurred at 30°C with shaking on a rotary shaker (200 rpm) for 4 days. After cultivation, the enzyme was harvested by filtering through a Mira cloth (Merck) placed on a beaker and squeezing out the enzyme. The recovered enzyme was stored in falcon tubes at 4°C.

#### 5.3.2.1 Recombinant $\alpha$ -L-arabinofuranosidase characterisation

The AbfB enzyme activity was obtained by determining the rate of release of *p*-nitrophenol (*p*NP) (Sigma-Aldrich) from *p*-nitrophenyl- $\alpha$ -arabinofuranoside (*p*NPA) (Sigma-Aldrich). A volume of 25  $\mu$ L 5 mM *p*NPA (made in 0.05 M citrate buffer, pH 5.0), 25  $\mu$ L dH<sub>2</sub>O, 25  $\mu$ L 0.05 M citrate buffer, pH 5.0 and 25  $\mu$ L of recombinant AbfB enzyme was mixed in a microtiter plate. The reaction was carried out at 40°C for 10 mins and the absorbance was measured at wavelength of 405 nm using spectrophotometer (xMark<sup>™</sup> microplate spectrophotometer,

BIO-RAD, South Africa). Commercial AbfB (Megazyme) was used as a positive control. Different concentrations of  $\rho$ NP were prepared by serial dilution and assayed under similar conditions to obtain a standard curve that was used to determine AbfB enzyme activity. The AbfB enzyme activity was calculated as the amount micromoles of  $\rho$ NP released by the enzyme from  $\rho$ NPA per minute per milliliter expressed as units per milliliter (Chimphango, Rose, *et al.*, 2012).

### **5.3.2.2 The total protein content was determined according to Bradford**

The total protein of recombinant AbfB was determined using BCA protein Assay kit (Novagen®, UK). BCA working reagent was prepared by mixing BCA solution (bicinchoninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 M NaOH, pH 11.25) with 4% cupric sulfate in a ratio of 1:50. A volume of 50  $\mu$ L was mixed with 1 mL BCA working reagent and the reaction was incubated at 37°C for 30 minutes. After incubation, the absorbance was measured and recorded at 562 nm within 10 mins. BSA standards were used to generate a standard curve for determination of protein content.

### **5.3.3 Enzymatic formation of hydrogels and encapsulation of gallic acid**

The activity of recombinant AbfB was assessed according to Chimphango *et al.* (2012) on xylan extracted from wheat bran (Essential Grains, Pioneer Foods, Paarl, South Africa) using alkaline method adapted from Aguedo *et al.* (2014) and oat spelt xylan. A solution of 3% (*w/v*) xylan extracted from wheat bran and oat spelt xylan was prepared according to De Wet *et al.* (2008). The reaction mixture contained 2.5 mL of substrate solution and 3 mL recombinant AbfB. The reaction was carried out in an incubator at 40°C and was terminated after 24 hrs by placing test tubes on ice for 10 mins. The formation of the hydrogels was assessed by visual inspection, change in viscosity using a rheometer (Physica MCR501, Anton Paar) equipped with double gap measuring system and HPLC analysis to measure the release of arabinose. For *in situ* encapsulation, gallic acid (20% *w/w* of total solids) was added before 24 hr incubation at 40°C, whilst for *ex situ*, the encapsulation was performed after terminating the enzymatic reaction. Both encapsulation reactions were stored at 4°C for 24 hrs before analysis.

### **5.3.4 Chemical formation of hydrogels and encapsulation of gallic acid**

A coacervation method based Garcia *et al.*, (2001) was used to modify xylan extracted from wheat bran. Firstly, xylan solution was made by stirring 100 mg of wheat bran xylan with 5 mL of 1 N sodium hydroxide. A 1 N solution of acetic acid was added to the xylan solution to achieve a neutral solution, resulting in the spontaneous formation of hydrogels. Polyoxoethylen (20) sorbitan monolaurate (Tween<sup>®</sup> 20) (1.5% v/v) was added to the reaction to increase the stability of hydrogels. Gallic acid (20% w/w of total solids) was added for the encapsulation reaction before as well as after neutralisation.

### **5.3.5 Hydrogel characterization**

#### **5.3.5.1 Size, polydispersity index and zeta potential**

The mean particle size of hydrogels, polydispersity index (PDI) and zeta potential was measured using Zetasizer (Nano-ZS90, Malvern instruments, UK) using dynamic light scattering (DLS) principle at a temperature of 25°C. The laser beam was operating at wavelength of 633 nm and scattering angle of 90°. The samples were diluted with water purified by reverse osmosis before measurements and each sample was measured three times.

#### **5.3.5.2 Infrared spectra**

FTIR spectra were obtained by Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy method using Thermo NICOLET iS10 spectrophotometer with a Smart Orbit ATR sampling accessory with diamond crystal (Thermo SCIENTIFIC, Madison, USA) in absorbance mode with wave number region 4 000 – 500 cm<sup>-1</sup>. The spectra were averaged for 64 scans with a resolution of 4 cm<sup>-1</sup>. The signals were processed using Origin software.

#### **5.3.5.3 Scanning electron microscopy**

The structure and surface morphology of the hydrogels was studied by scanning electron microscopy (Zeiss MERLIN, Germany) at beam strength of 3 kV. The hydrogel samples were carbon coated and the images were obtained in secondary electron imaging mode.

#### **5.3.5.4 Encapsulation efficiency**

The amount of gallic acid (GA) loaded into xylan hydrogels was measured following method of Tavano *et al.* (2014). The encapsulation efficiency was determined by dissolving samples in 5 mL of methanol. The amount of gallic acid was quantified using spectrophotometer (A & E

Lab Instruments, China) at wave length 280 nm against a predetermined gallic acid standard calibration curve shown in Appendix C. The encapsulation efficiency was calculated as a percentage of gallic acid entrapped in hydrogels with respect to the total amount of gallic acid added.

### 5.3.5.5 Evaluation of antioxidant capacity

The antioxidant activity (AA %) was measured using 1,1'-diphenyl-2-picrylhydrazyl (DPPH). Hydrogels encapsulating GA and empty hydrogels were dissolved in 10 mL of 80% ethanol. An aliquot of 1 mL of ethanol solution was allowed to react with 3 mL of 0.1 mM DPPH prepared in methanol for 30 mins in the dark at room temperature. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer. The antioxidant activity (AA%) was expressed as follows:

$$AA \% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (5.1)$$

Where  $A_{control}$  and  $A_{samples}$  are the absorbances of DPPH without and with the presence of sample, respectively.

### 5.3.6 Gallic acid release profile

The release of gallic acid entrapped in the hydrogels was evaluated by placing approximately 15 mg of the hydrogels entrapping gallic acid in 20 mL of PBS buffer (pH = 7,4). The release experiments were carried out in a water bath at 25°C with shaking for 48 hrs. Samples of 4ml were drawn at time intervals after centrifugation at 8000 rpm for 10 mins and the concentration of gallic acid was determined using a UV-Vis spectrophotometer at 259 nm. The removal of 4mL of sample was replaced by equal volume of fresh PBS buffer.

### 5.3.7 Statistical Analysis

All values were expressed as a mean  $\pm$  standard deviations (SD) of three determinations. Statistical significance ( $p < 0.05$ ) was determined using one-way analysis of variance (ANOVA) using STATISTICA version 13. 2 (Statsoft, USA).

## 5.4 Results and discussion

### 5.4.1 Enzymatic hydrolysis of arabinoxylan with $\alpha$ -arabinoxylan.

The recombinant  $\alpha$ -arabinofuranosidase that was used in this study was produced in house and the freeze cultures were donated by colleagues from Microbiology department of Stellenbosch University. The recombinant  $\alpha$ -arabinofuranosidase was used to precipitate soluble wheat bran and oat spelt arabinoxylan into hydrogels that were visually observed settling at the bottom of the reaction tubes (Figure 5.1). The oat spelt was used as a control in the enzymatic hydrolysis experiments. The enzyme cleaved arabinose side groups along the wheat bran and oat spelt arabinoxylan backbone resulting in the liberation of 6.81% and 5.06% arabinose, respectively and forming hydrogels (Figure 5.2). The enzymatic formation of arabinoxylans of hydrogels or precipitation of soluble arabinoxylans was due to association of unsubstituted linear arabinoxylan chains forming xylan-xylan interactions (Andrewartha *et al.*, 1979; Linder *et al.*, 2003; Chimphango, Rose, *et al.*, 2012).

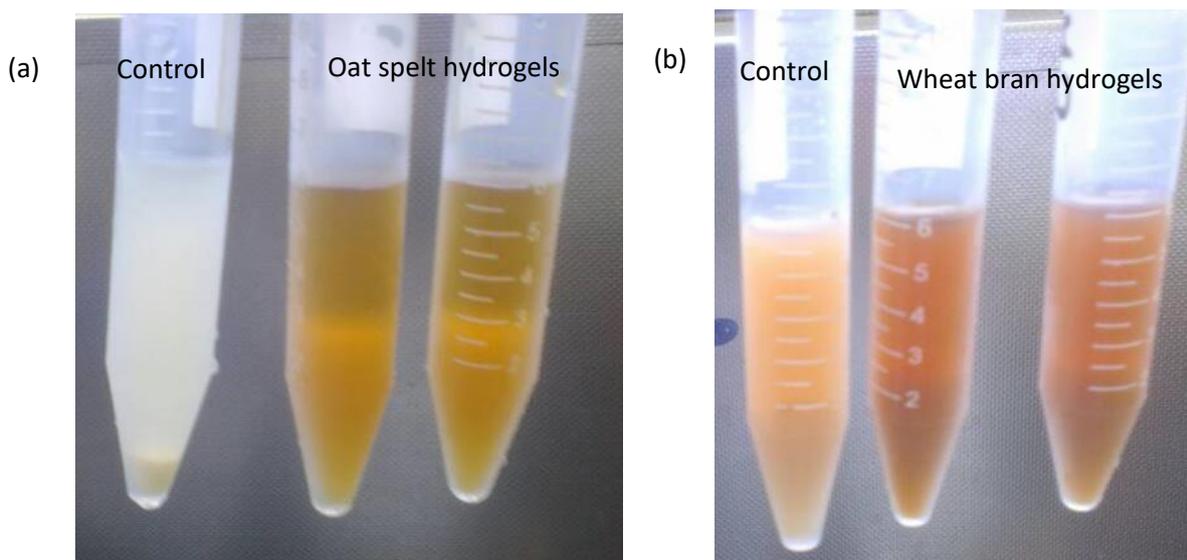


Figure 5.1 Enzymatic precipitation of (a) oat spelt (b) wheat bran with  $\alpha$ -arabinofuranosidase enzyme to form insoluble hydrogels

The removal of arabinose from soluble wheat bran arabinoxylan using recombinant  $\alpha$ -arabinofuranosidase resulted in reduced intrinsic viscosity whilst an opposite effect occurred with the hydrolysis of soluble oat spelt arabinoxylan (Figure 5.3). Bergmans *et al.* (1996a) also observed that the reduction in intrinsic viscosity and partial aggregation of wheat bran xylan

when (1→4)- $\beta$ -D-arabinoxylan arabinofuranohydrolase purified from *Aspergillus awamori* hydrolyzed 4% arabinose from the xylan backbone. According to Andrewartha *et al.* (1979) who also observed similar results, the increase in intrinsic viscosity was attributed to the increase in flexibility of arabinoxylan structure after the removal of arabinose along the backbone. The presence of arabinose along the arabinoxylan backbone results in a stiff rod-like conformation that causes steric hindrances and prevents xylan-xylan associations leading to arabinoxylan having a high intrinsic viscosity in aqueous solution (Andrewartha *et al.*, 1979; Ebringerová & Heinze, 2000). However, the enzymatic hydrolysis of soluble oat spelt arabinoxylan with recombinant  $\alpha$ -arabinofuranosidase resulted in increased intrinsic viscosity (Figure 5.4). These results are consistent with those obtained in literature (Chimphango, van Zyl, *et al.*, 2012b; Gomes *et al.*, 2015). These inconsistencies in the rheology behaviours after enzymatic hydrolysis of side groups are the result of variations in the degree of substitution as well as pattern of distribution of arabinose along the different xylan backbone, which in turn affects the susceptibility of the xylans to enzymatic hydrolysis (Andrewartha *et al.*, 1979; Izydorczyk & Biliaderis, 1995; Ebringerová & Heinze, 2000). Furthermore, wheat bran and oat spelt possibly have structural variances because of their different botanical origin (Izydorczyk & Biliaderis, 1995) and hence they would behave different in aqueous solution and upon enzymatic hydrolysis of arabinose side group.

#### **5.4.2 Chemical modification of wheat bran arabinoxylan**

The chemically induced hydrogels formed spontaneously upon addition of acetic acid on alkaline xylan solution in a coacervation process. The pH of hydrogel solution was neutral after addition of acid. The final pH after coacervation process was shown to have influence on the stability of particles formed and a pH of 7 is suitable for formation of stable hydrogels (Garcia *et al.*, 2001). Encapsulation of gallic acid in chemically formed hydrogels by coacervation method occurred during and after addition of acid solution. The properties of these chemically formed hydrogels are compared to the enzymatically formed hydrogels in the rest of this chapter.

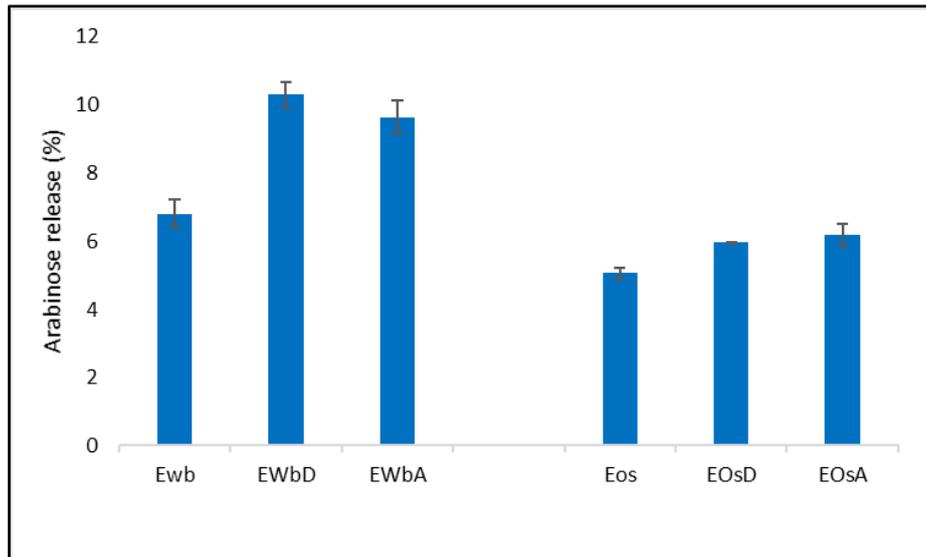


Figure 5.2: Release of arabinose from Wheat bran and Oat spelt after enzymatic hydrolysis with  $\alpha$ -arabinofuranosidase. Ewb, EWbD and EWbA represent wheat bran enzyme hydrolysate without any encapsulation, with encapsulation occurring during and after the formation of hydrogels, respectively. Eos, EOsd and EOsa represent oat spelt enzyme hydrolysate without any encapsulation, with encapsulation occurring during and after the formation of hydrogels, respectively

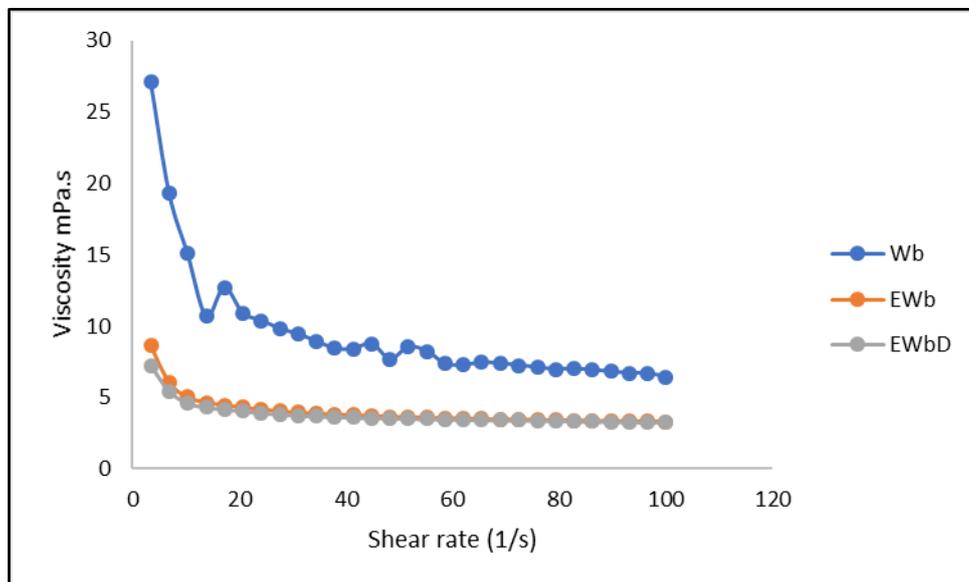


Figure 5.3: Change of viscosity with increasing shear rate for unmodified (Wb), enzymatically modified wheat bran without gallic acid (EWb) and enzymatically modified wheat bran with gallic acid added during modification

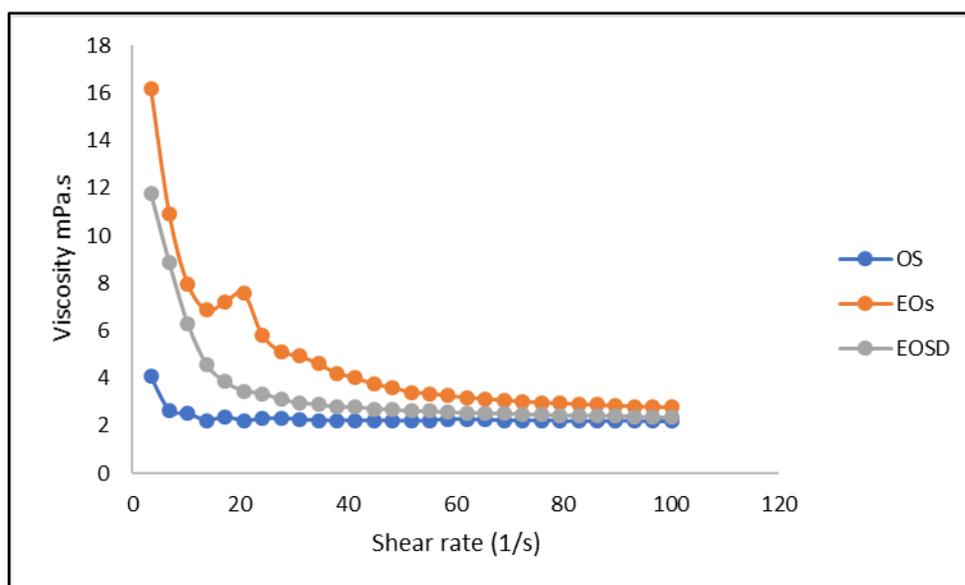


Figure 5.4: Change of viscosity with increasing shear rate for unmodified (OS) enzymatically modified Oat spelt without gallic acid (Eos) and enzymatically formed with gallic acid gallic acid added during modification

### 5.4.3 Size, polydispersity index and zeta potential of hydrogels

The wheat bran hydrogels formed by enzymatic hydrolysis with  $\alpha$ -arabinofuranosidase and coacervation method were analysed for size, polydispersity index and zeta potential using dynamic light scattering principle and the results are shown on Table 5.1. Nano-sized wheat bran arabinoxylan hydrogels were obtained after enzymatic and chemical modification. Encapsulation of gallic acid during and after the formation of hydrogels resulted in slight increase of average size of the hydrogels for both the enzymatic and chemical encapsulation. Enzymatically modified hydrogels had a significantly ( $p < 0.05$ ) higher average size of 597.47 nm compared to chemically modified hydrogels with an average size of 468.96 nm. Although chemically formed hydrogels had smaller size, the enzymatically formed hydrogels with larger size would be preferred for slow release of substances because larger hydrogels have smaller initial burst release of encapsulated substance and longer sustained release (Hans & Lowman, 2002). Furthermore, the chemically modified hydrogels showed a very broad size distribution with a polydispersity index of 1 whilst enzymatically formed hydrogels were more homogenous with monodisperse particles. According to Lamara *et al* (2016) hydrogels with PDI values of 0.01 to 0.5 - 0.7 are monodispersed and hydrogels with PDI greater than 0.7 have a broad size distribution that is heterogenous particle size. Furthermore, the chemically

modified hydrogels had a less negative zeta potential as compared to enzymatically formed hydrogels. Zeta potential is an important indicator of the stability of the hydrogels in solutions and hydrogels with same surface charge repel each other thereby reducing agglomeration and giving stability (Nourbakhsh, Emam-Djomeh, Madadlou, Mousavi, Moosavi-movahedi & Guanasekaran, 2016).

Table 5.1: Size, Polydispersity index and zeta potential of hydrogels

Sample	Average size (nm)	Polydispersity index (PI)	Zeta potential (mV)
CWb <sup>a</sup>	468.96 ± 19.42	1.00	-3.95
CWbD <sup>b</sup>	599.47 ± 23.48	0.96	-2.90
CWbA <sup>c</sup>	601.93 ± 74.55	1.00	-2.89
EWb <sup>d</sup>	597.47 ± 24.47	0.33	-8.84
EWbD <sup>e</sup>	657.57 ± 77.06	0.30	-7.12
EWbA <sup>f</sup>	677.67 ± 51.59	0.28	-6.12
Oat Spelt	3 177.66 ± 84.00	0.33	-10.70
EOsD <sup>g</sup>	3 473.86 ± 57.00	0.39	-10.69

<sup>a</sup>chemical modified wheat bran hydrogels,

<sup>b</sup>chemically modified wheat bran hydrogels with gallic acid added during formation of hydrogels,

<sup>c</sup>chemically modified wheat bran hydrogels with gallic acid added after the formation of hydrogels,

<sup>d</sup>enzymatically formed wheat bran hydrogels,

<sup>e</sup>enzymatically formed wheat bran hydrogels with gallic acid added during the formation of hydrogels,

<sup>f</sup>enzymatically formed wheat bran hydrogels with gallic acid added after the formation of hydrogels,

<sup>g</sup>enzymatically formed oat spelt hydrogels with gallic acid added during the formation of hydrogels

The negative charge of the hydrogels formed was probably as a result of adsorption of hydroxyl ions in solution. However, the less negative surface charge of chemically modified hydrogels as compared to enzymatically modified hydrogels could have probably lead to greater agglomeration, which in turn resulted in broad size distribution shown by the PDI value of 1. In addition, since chemically modified hydrogels had a smaller average size, this means that there was large surface area and less surface charge thereby favouring agglomeration of the hydrogels. In a study, cellulose based nanoparticles were found to remain stable until a zeta potential of -8.3 mV and a pH of 3.4. Furthermore, pH was shown to affect the stability of the formed nanoparticle and the latter was found to be stable between the pH of 3.4 and 10 (Hornig & Heinze, 2008). The degree of substitution, nature of

substituents along the polymer backbone as well as method of preparation of hydrogels affect the size, PDI and zeta potential of hydrogels (Hornig, Bunjes & Heinze, 2009). Since the DS and character of substituent was the same for chemically and enzymatically modified hydrogels, the differences in the size, PDI and zeta potential could probably be due to differences in the preparation techniques (Hornig, Bunjes & Heinze, 2009). This shows that the preparation technique of hydrogels is an important factor in the formation of stable hydrogels.

#### **5.4.4 Encapsulation efficiency of gallic acid in xylan hydrogels**

The wheat bran arabinoxylan based hydrogels were used to encapsulate gallic acid both during and after the formation of hydrogels with enzymatic hydrolysis and coacervation method. It was observed that the encapsulation efficiencies were higher for *in situ* encapsulation than encapsulation after the formation of the hydrogels for both enzymatic and chemical encapsulation. Despite forming less stable hydrogels, the encapsulation efficiency of gallic acid in chemically modified hydrogels was 72.35 and 67.65% for encapsulation during and after the formation of hydrogels, respectively (Table 5.2). *In situ* encapsulation of gallic acid in enzymatic hydrogels resulted in encapsulation efficiency 58.85% and 51.85% (Table 5.2) for encapsulation after the formation of hydrogels. The high encapsulation efficiency in chemically modified hydrogels is probably due to their smaller average size compared to enzymatically formed hydrogels. The lower encapsulation efficiency obtained with enzymatic hydrogels could be probably attributed to the partial hydrolysis of arabinose along the xylan backbone during formation of wheat bran arabinoxylan hydrogels. The difference in encapsulation efficiency of gallic acid in chemically modified and enzymatically hydrogels can also be attributed to differences in the crosslinking densities and network structure as will be seen in Section 5.4.7 below. A high crosslinking density restricts gallic acid incorporation and results in lower gallic acid encapsulation (Sun *et al.*, 2013). Although chemically modified hydrogels had high encapsulation efficiency, the lower encapsulation capacity of enzymatically formed hydrogels is advantageous for sustained release of gallic acid as high encapsulation capacity results in burst release of gallic acid (Hans & Lowman, 2002; Holland, Tabata & Mikos, 2003). The process parameters of hydrogel formation can be varied to obtain higher or lower encapsulation efficiency to suit the desired need. These encapsulation efficiencies obtained in this study were comparable to that of

Robert *et al.*, (2012) who observed encapsulation efficiency of between 47% and 83% of gallic acid incorporated into both native and acetylated inulin and starch by spray drying at varied operating conditions.

Table 5.2: Efficiency of encapsulation and antioxidant activity of gallic acid

Sample	Efficiency of encapsulation (%)	Antioxidant activity (%)
CWbD <sup>a</sup>	72.35 ± 0.98	80.26 ± 0.15
CWbA <sup>b</sup>	67.65 ± 1.39	78.67 ± 1.00
EWbD <sup>c</sup>	58.12 ± 2.72	91.46 ± 0.05
EWbA <sup>d</sup>	51.85 ± 2.06	91.85 ± 0.00
Oat spelt <i>in situ</i>	67.75 ±	89.33 ± 0.15

<sup>a</sup>Chemically modified wheat bran hydrogels with gallic acid added during formation of hydrogels,

<sup>b</sup>Chemically modified wheat bran hydrogels with gallic acid added after the formation of hydrogels,

<sup>c</sup>Enzymatically formed wheat bran hydrogels with gallic acid added during the formation of hydrogels,

<sup>d</sup>Enzymatically formed wheat bran hydrogels with gallic acid added after the formation of hydrogels

#### 5.4.5 Antioxidant Capacity of encapsulated gallic acid

The antioxidant capacity of encapsulated gallic acid in chemically and enzymatically formed hydrogels was evaluated by the measuring the ability to scavenge the DPPH radical. Gallic acid donates hydrogen atom to DPPH thereby acting as a radical scavenger (Tavano *et al.*, 2014). The antioxidant activity of gallic acid encapsulated during and after formation of hydrogels formed by coacervation was 80.26 and 78.67%, respectively (Table 5.2). However, the antioxidant activity of gallic acid encapsulated in enzymatically formed hydrogels was both approximately 91% both *in situ* and *ex situ* encapsulation (Table 5.2). The antioxidant activity of gallic acid encapsulated in enzymatically formed hydrogels did not differ from that of pure GA (91.46%) but antioxidant capacity of the gallic acid encapsulated in chemically modified hydrogels was lower compared to that of pure GA. The reduced antiradical activity of gallic acid encapsulated in chemically modified hydrogels could have been caused by the alkaline and acid solution used in the coacervation method. Neo *et al.*, (2013) reported antioxidant activities of gallic acid encapsulated in electro spun zein fibers between 58.08 to 88.60%. Empty wheat bran hydrogels showed 33% scavenging activities against DPPH radical and this could be attributed to the presence of phenolic compounds present together with arabinoxylan after alkaline extraction (Zhou, Laux & Yu, 2004; Koegelenberg & Chimphango,

2017). Nevertheless, gallic acid encapsulated in chemically and enzymatically formed hydrogels showed significantly ( $p>0.05$ ) higher antioxidant activity than empty hydrogels thus indicating that gallic acid was incorporated into both the chemically and enzymatically formed hydrogels.

#### 5.4.6 Structural properties of unmodified and modified hemicelluloses

The IR spectroscopy was used to study the chemical structure of unmodified hemicellulose, enzymatically and chemically modified hemicellulose hydrogels, encapsulation of gallic acid and pure gallic acid. In Figure 5.5, the spectra of arabinoxylans is typical of that recorded in literature (Kacurakova, Ebringerova, Hirsch & Hromadkova, 1994; Kacurakova, Belton, Wilson, Hirsch & Ebringerova, 1998; Peng, Wang, Hu, Yu, Liu, Zhang & Ruan, 2012). The band observed in the region 3400 to 3300  $\text{cm}^{-1}$  may be attributed to hydroxyl stretching whilst that at 2903  $\text{cm}^{-1}$  corresponds to the C-H stretching of  $\text{CH}_2$  (Peng, Wang, *et al.*, 2012). The multiple peaks observed in the region 1500 to 800  $\text{cm}^{-1}$  are attributed to C-C, C-O, C-OH and C-O-C stretching. Specifically in this region, the low intensity peaks observed at 1164 and 895  $\text{cm}^{-1}$  indicate the presence of arabinose substituent on the xylan backbone chain (Kacurakova *et al.*, 1994). When wheat bran arabinoxylan was hydrolysed with  $\alpha$ -arabinofuranosidase to form hydrogels, the bands 1164 and 895  $\text{cm}^{-1}$  increased in intensity (Figure 5.5b). This increase in intensity shown is probably due to decrease in the amount of arabinose substituted on the xylan backbone by enzymatic hydrolysis. Kacurakova *et al.*, (1994) also observed that with increasing arabinose substitution on the xylan backbone, the absorption intensity of bands 1164 and 895  $\text{cm}^{-1}$  reduced. Furthermore, the slight difference in intensities of bands the region 1500 – 1100  $\text{cm}^{-1}$  indicated that there was altered substitution pattern of arabinose along the arabinoxylan backbone. Enzymatic hydrolysis of wheat bran arabinoxylan with  $\alpha$ -arabinofuranosidase also resulted in shifting of band from 1584  $\text{cm}^{-1}$  to 1643  $\text{cm}^{-1}$  in the modified arabinoxylan.

The gallic acid spectra also showed bands at 3488 and 3250  $\text{cm}^{-1}$ , which could be attributed to O-H stretching (Figure 5.6). Multiple peaks were also observed for gallic acid in the region 1650 to 1349  $\text{cm}^{-1}$ , which corresponds to C-C and C-H bendings and in the region 1313 to 1170  $\text{cm}^{-1}$ , which corresponds to C-H and O-H stretching of aromatic ring. The absorbance peaks observed in the 1004 to 623  $\text{cm}^{-1}$  correspond to C-O bending (Neo *et al.*, 2013). The enzymatic arabinoxylan hydrogels containing encapsulated gallic acid did not show a large remarkable

difference from empty enzymatic hydrogels because the empty hydrogels also showed absorbance peaks for C-C, C-O, C-OH, and C-O-C stretching in the region 1500 – 800  $\text{cm}^{-1}$ , which overlaid with gallic acid absorbance peaks in the same region (Figure 5.6). However, absorbance peak around 1337  $\text{cm}^{-1}$  increased in intensity for gallic acid loaded enzymatic hydrogels. Furthermore, new low intensity bands appeared at 778 and 826  $\text{cm}^{-1}$  in the spectra of gallic acid loaded enzymatic hydrogels that were not apparent in the empty enzymatic hydrogels. The spectra of *in situ* encapsulated enzymatic hydrogels and hydrogels with gallic acid incorporated after formation was the same. The empty chemical modified hydrogels differed from unmodified wheat bran arabinoxylans as high intensity bands were observed at 1548 and 1404  $\text{cm}^{-1}$ . The encapsulation of gallic acid in chemically modified hydrogels did not result in any difference in the spectra of gallic acid loaded hydrogels and this could be due to the overlaying of absorbance peaks in the same region (Figure 5.7).

#### **5.4.7 Morphology properties of hydrogels using Scanning electron microscopy**

The scanning electron microscopy images showing the morphology of the hydrogels are shown in Figure 5.8 and Figure 5.9. The empty enzymatically modified hydrogels showed a porous honeycomb structure whilst the empty chemically modified hydrogels showed a more rough and compact structure. This difference in structure could be attributed to the difference in the method of hydrogel formation. The structure of enzymatically modified hydrogels encapsulating gallic acid before and after formation of hydrogels differed from the structure of empty enzymatically modified hydrogels. The enzymatically modified hydrogels encapsulating gallic acid showed a less porous structure compared to the empty enzymatically modified hydrogels and thus showing that the encapsulation of gallic acid occurred. An imperfect honeycomb structure has been recorded in literature for arabinoxylan hydrogels formed by oxidative cross-linking (Iravani, Fitchett & Georget, 2011). The chemically modified hydrogels encapsulating gallic acid showed a smoother surface structure compared to the empty chemically modified hydrogels. The change in the morphology of the hydrogels after encapsulation is attributed to gallic acid encapsulation.

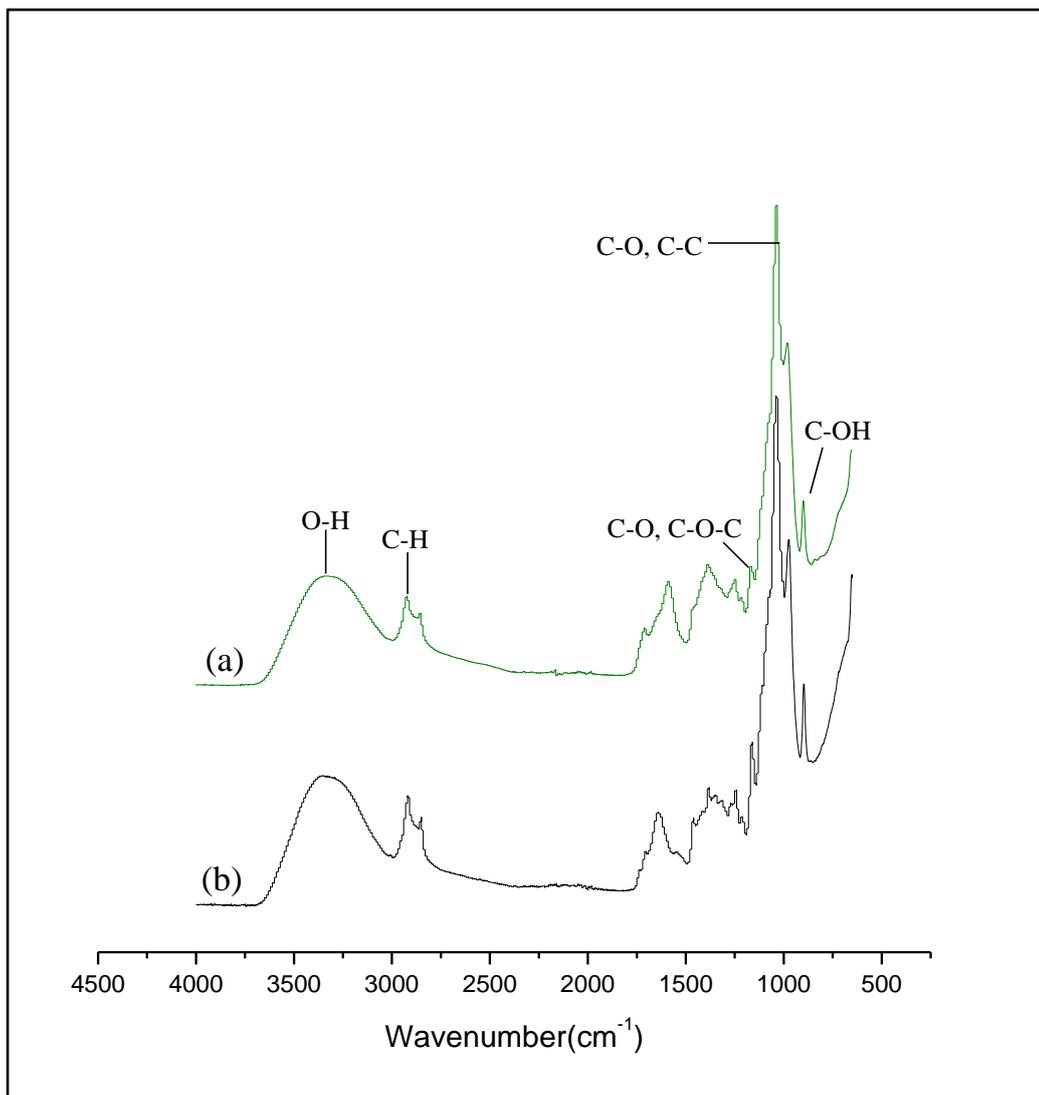


Figure 5.5: IR spectra comparing (a) wheat arabinoxylan (b) enzymatically modified arabinoxylan chemical structure

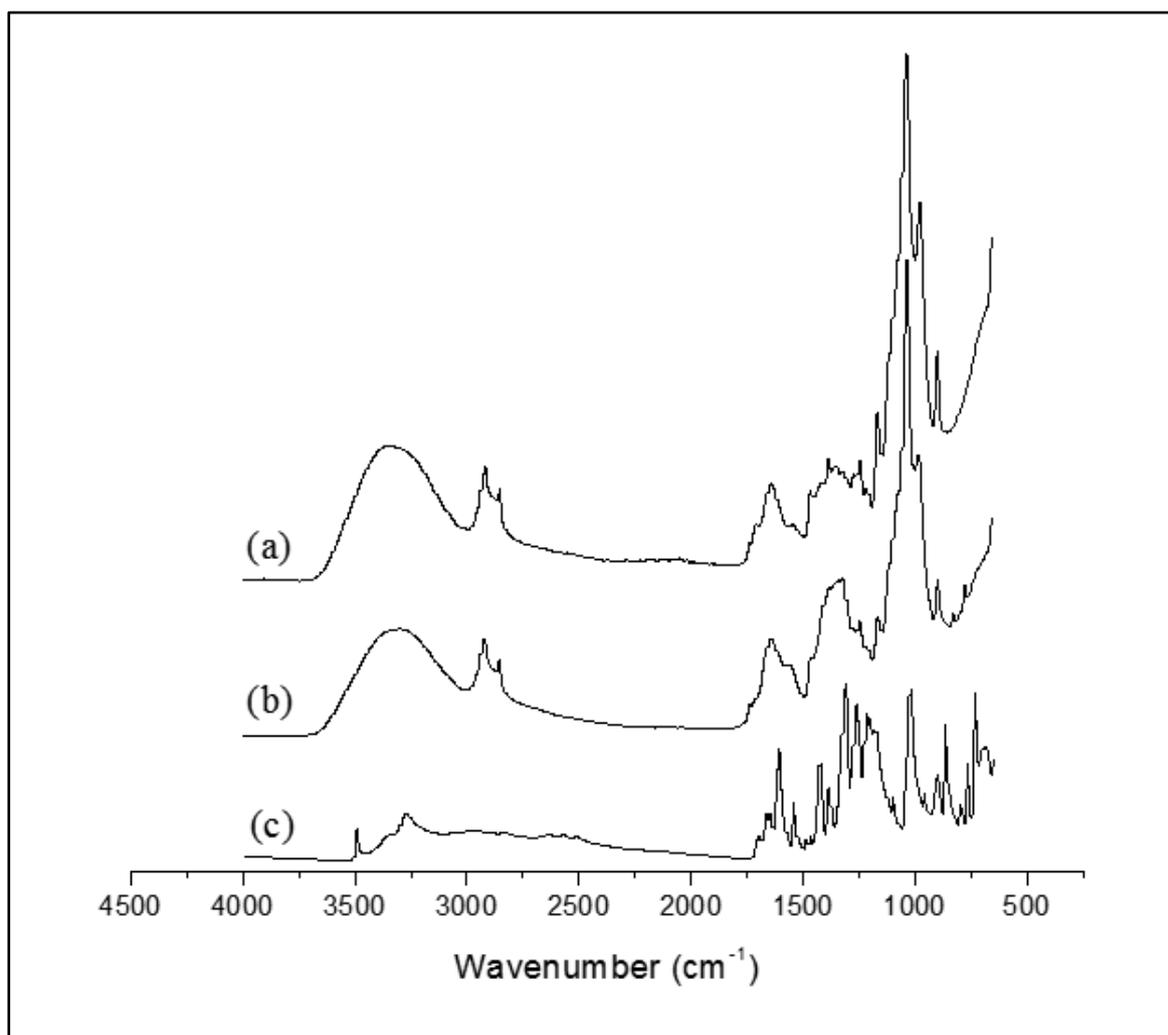


Figure 5.6: IR spectra comparing (a) enzymatic empty arabinoxylan hydrogels (b) gallic acid loaded hydrogels (c) gallic acid chemical structure

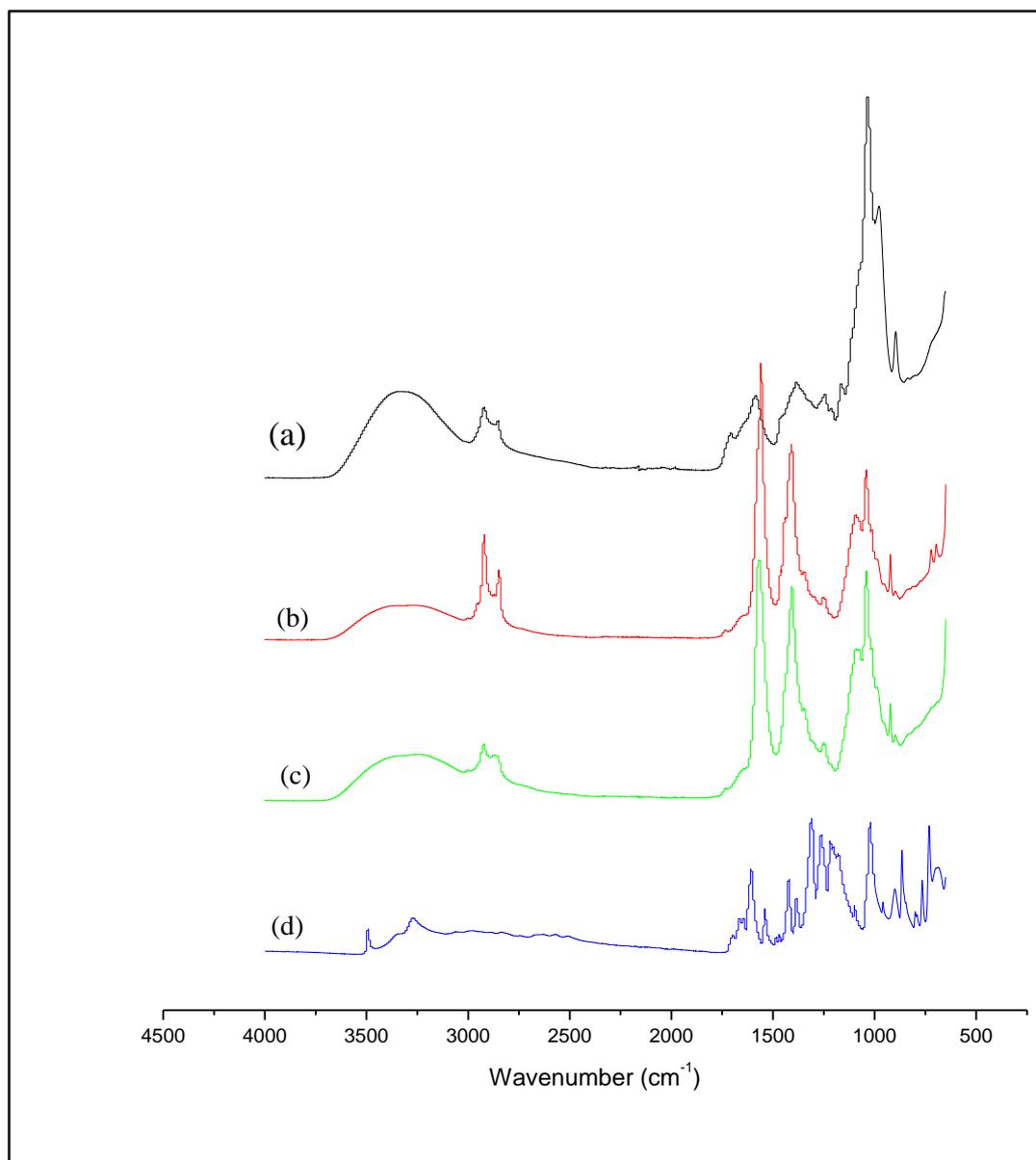


Figure 5.7: IR spectra comparing (a) wheat bran arabinoxylan (b) chemically modified arabinoxylan hydrogels (c) chemical hydrogels with gallic acid loaded during encapsulation (d) gallic acid chemical structure

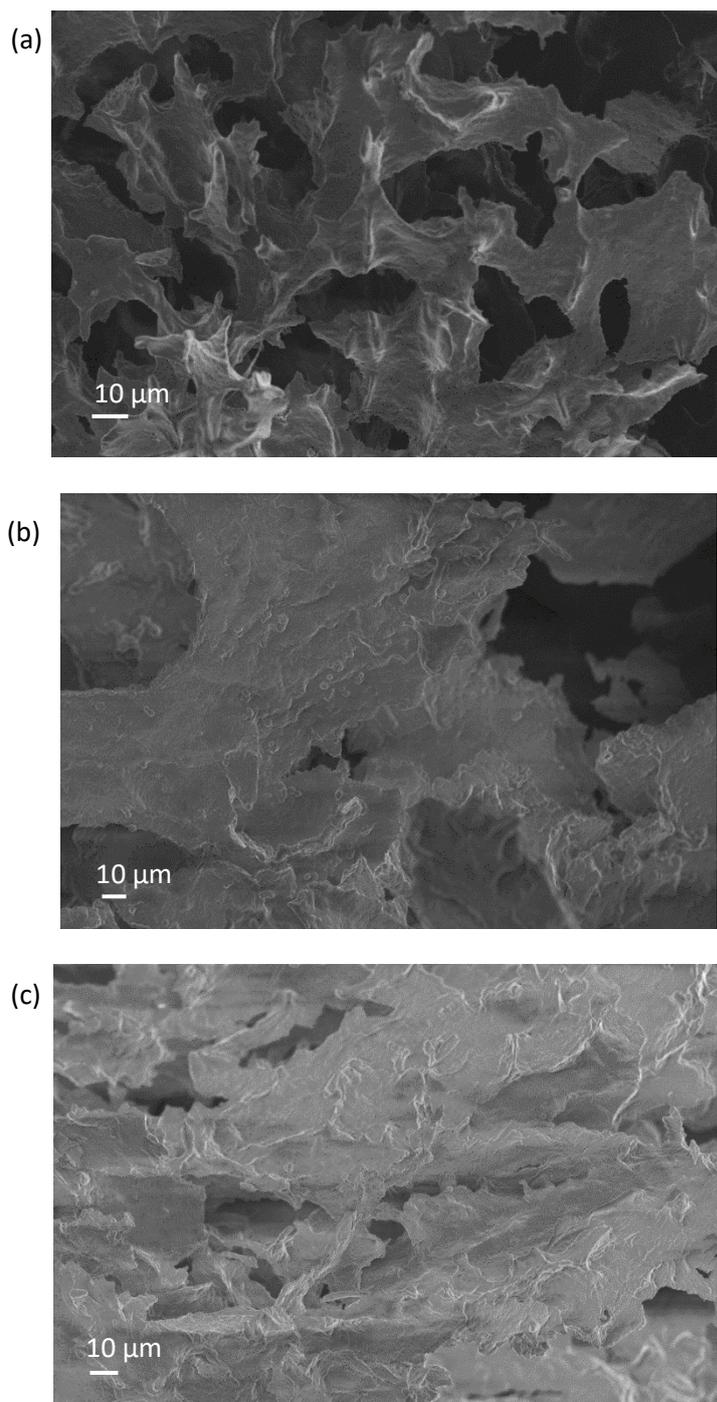


Figure 5.8: Scanning electron microscopy images  $\times 500$  magnification for (a) empty enzymatically formed hydrogels (b) enzymatically formed hydrogels that encapsulated gallic acid during hydrogel formation (c) enzymatically formed hydrogels that encapsulated gallic acid during hydrogel formation

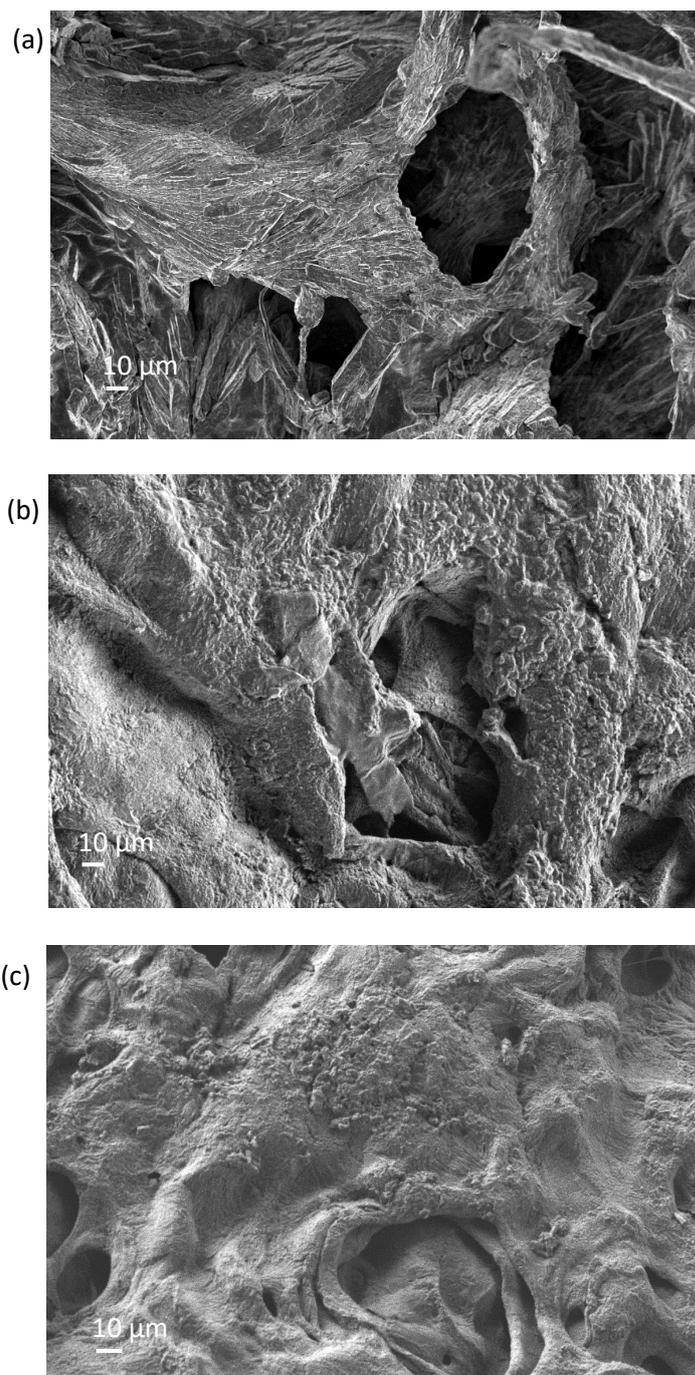


Figure 5.9: Scanning electron microscopy images at  $\times 500$  magnification for (a) empty chemically formed hydrogels (b) chemically formed hydrogels that encapsulated gallic acid during hydrogel formation (c) chemically formed hydrogels that encapsulated gallic acid during hydrogel formation

### 5.4.8 In vitro release of gallic acid from hydrogels

The release of gallic acid from chemically and enzymatically formed hydrogels was performed for 8 hrs in saline PBS buffer to simulate biological fluid. The cumulative release profiles are shown in Figure 5.1. The release of gallic acid from chemical and enzymatic hydrogels where gallic acid was encapsulated after the formation of hydrogels increased for 4 and 2 hrs, releasing 57 and 58% of gallic acid, respectively. This shows that encapsulation after hydrogel formation lead to burst release of gallic acid probably due to poorly entrapped gallic acid. Burst release is a common challenge in formulating drug delivery systems and some of the factors that have been recorded to result in burst release include particle size of hydrogels and substance loading (Hans & Lowman, 2002). However, the release of gallic acid from enzymatic and chemical hydrogels where gallic acid was encapsulated during the formation of hydrogels showed sustained release. The sustained release of gallic acid from enzymatically formed hydrogels could be attributed to their stability, small size and low encapsulation efficiency. The burst release of gallic acid from hydrogels that encapsulated gallic acid after formulation could also be a result of instability of hydrogels as shown by the less negative zeta potential and large polydispersity index. However, encapsulating the gallic acid during formation of chemical hydrogels reduced the burst release effect.

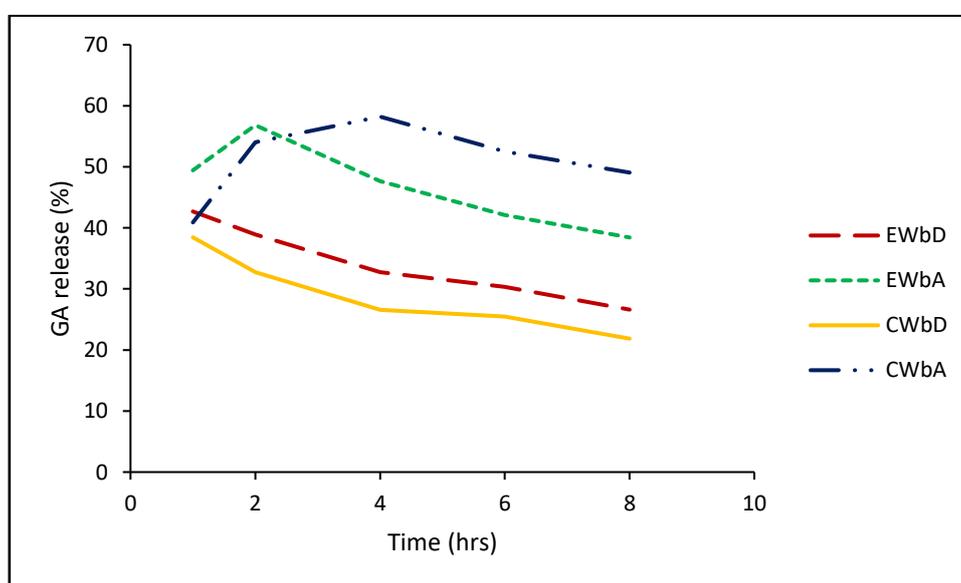


Figure 5.10: Gallic acid release from enzymatic hydrogels where gallic acid was added before (EWbD) and after (EWbA) formation of hydrogels, and from chemical hydrogels where gallic acid was added before (CWbD) and after (CWbA) formation of hydrogels.

## 5.5 Conclusion

Wheat bran arabinoxylan was modified into hydrogels using coacervation method and enzymatic hydrolysis with  $\alpha$ -arabinofuranosidase. The arabinoxylan hydrogels were functionalized with gallic acid as delivery system. Chemically formed hydrogels had a smaller average particle size with very broad size distribution and less negative zeta potential than enzymatically formed hydrogels. Although the enzymatically formed hydrogels had a larger average size particle, they were more stable due to their more negative surface charge that reduced agglomeration resulting in monodisperse particles. Despite encapsulation efficiency of gallic acid being higher in chemically modified hydrogels than in enzymatic hydrogels formed hydrogels, the antioxidant capacity was retained better in enzymatically formed hydrogels. This showed that use of enzymes is more favorable for producing more stable hydrogels and in encapsulating sensitive substances and preserving their integrity. The release of gallic acid from hydrogels was more sustained when encapsulation was performed during hydrogel formation than when gallic acid was encapsulation after hydrogel formation for both chemical and enzymatic encapsulation. This shows that the method of hydrogel preparation as well as encapsulation influence the functional properties of the delivery systems and they should be carefully considered in the formulation of the delivery systems. Based on these studies, it can be concluded that encapsulating gallic bioactive or chemical substances in enzymatically formed hydrogels is favorable for sensitive substances. Furthermore, enzymatically formed hydrogels are more stable as compared to chemically formed hydrogels. However, when incorporating a substance that is not too sensitive, chemically formed hydrogels may be considered because of their encapsulation efficiency.

## Chapter 6: Conclusions

### 6.1 Major findings from present study

Wheat bran is an agro-residue that is rich source of renewable xylan biopolymer, which has a potential to replace synthetic polymers. Soluble arabinoxylan was extracted from industrial wheat bran using mild alkaline method up to a yield of 63%. NaOH concentration, extraction temperature and time had a significant effect on the yield of hemicellulose extracted from wheat bran. The optimum conditions were obtained with NaOH concentration 1.5 M at 80°C for 5 hrs. The extracted arabinoxylans were in oligomeric and polymeric form, which indicates that the extraction conditions didn't not degrade the hemicellulose to monomers, hence making them suitable for hydrogel formation. Despite a purity of 53%, the extracted arabinoxylans successfully formed insoluble entrapment material for the delivery of gallic acid. The insoluble entrapment materials were formed by coacervation chemical method and enzymatic hydrolysis using side chain removing  $\alpha$ -arabinofuranosidase. The entrapment materials formed by enzymatic hydrolysis were more stable and better preserved the integrity of incorporated gallic acid. The stability of enzymatically formed hydrogels was due to reduced steric hinderances and the inter-chain bonding of linear hemicellulose chains after enzymatic hydrolysis. However, chemically formed hydrogels were smaller compared to enzymatically formed hydrogels and had a higher encapsulation efficiency. The encapsulation of gallic resulted in altered chemical structure and morphology of the hydrogels. The formed hydrogels released the gallic acid in its active form.

Wheat bran arabinoxylans can be modified by coacervation or side chain removing enzyme  $\alpha$ -arabinofuranosidase into hydrogels that can function as delivery systems for chemical or bioactive substances. The method of preparation of hydrogels as well as encapsulation affect the functionality of entrapment materials. However biological formation of hemicellulose entrapment matrices using side chain removing enzymes offers a green alternative that allows for the protection of sensitive encapsulated substances such as bioactive agents.

## 6.2 Recommendations for future work

Further research is required in the characterization of extracted hemicellulose such as the degree of polymerization (DP) that can be calculated using the molecular weight of hemicellulose. The molecular weight can be determined using size exclusion chromatography. The degree of substitution (DS) of polymer is an important factor of consideration when forming hydrogels. Therefore, studies on the determination of DS of wheat bran arabinoxylan using NMR would be relevant. Subsequently, the effect of DP and DS on the extracted arabinoxylan on the properties of hydrogels would help form better delivery systems.

A bio-refinery approach that involves the co-production of hemicellulose and other components of lignocellulosic matrix of wheat bran such as cellulose and lignin would increase value of wheat bran. The solid residue of wheat bran after hemicellulose extraction could be further fractionated to obtain other lignocellulose components such as cellulose, which can be used for various value-added applications. Wheat bran hemicellulose is also known to contain glucuronic or methyl glucuronic acid, acetyl groups, galactose and ferulic acid in small amounts. Therefore, complete compositional analysis of the hemicellulose would be recommended and subsequently investigate the synergic effect  $\alpha$ -L-arabinofuranosidase with other enzymes such as  $\alpha$ -D-glucuronidase on the formation of hydrogels

The enzymatic hydrolysis of soluble arabinoxylan into insoluble hydrogels can be optimized for enzyme dosage and xylan concentration to obtain best suiting condition for the formation of stable hydrogels. Furthermore, the release mechanisms of gallic acid from the enzymatically and chemically modified hydrogels can be further investigated. The scale up of hemicellulose extraction from wheat bran and modification can be also investigated. Further research is required in the techno-economic analysis of the whole process of extraction and modification of hemicellulose.

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## Appendix A: Mind map for project

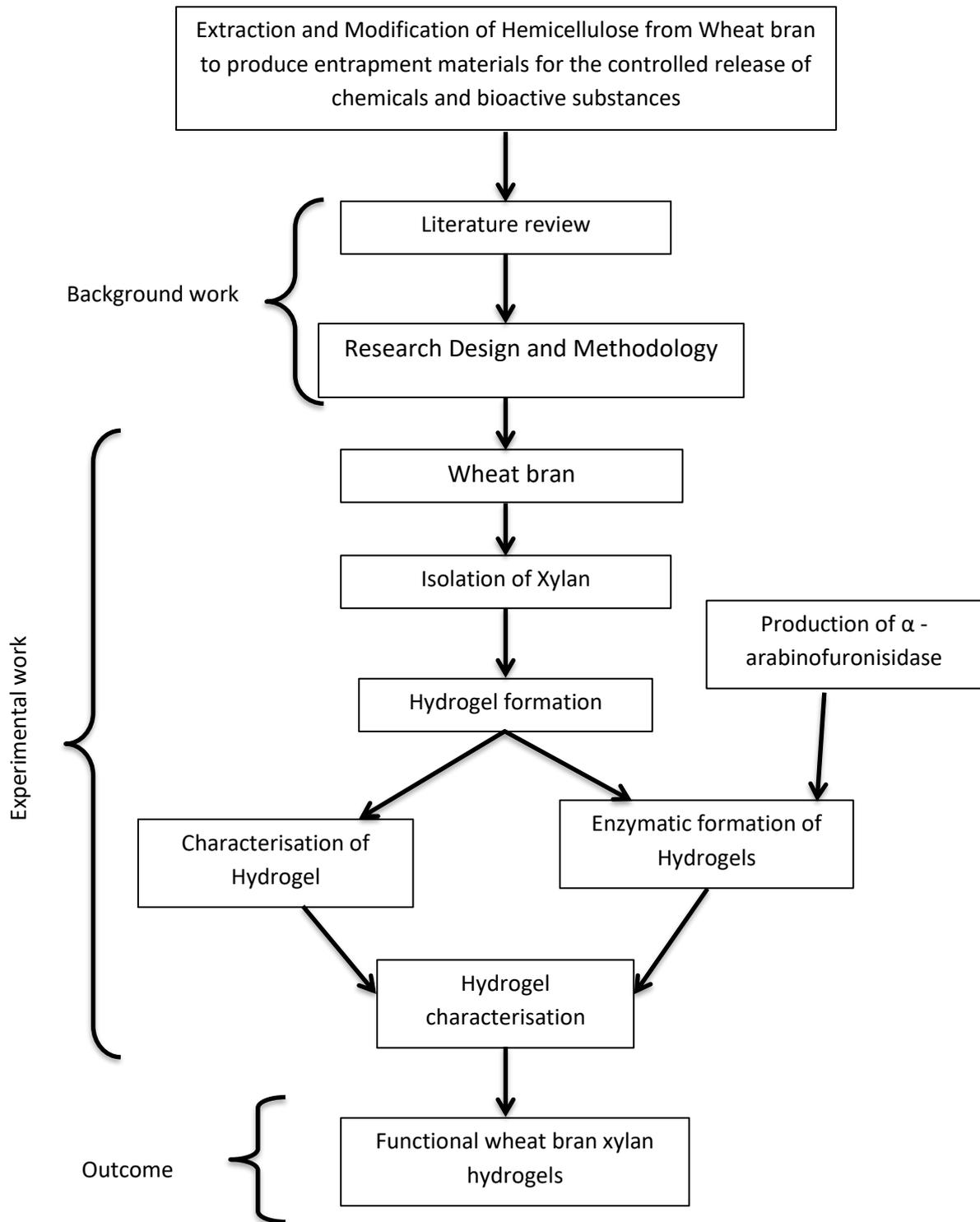
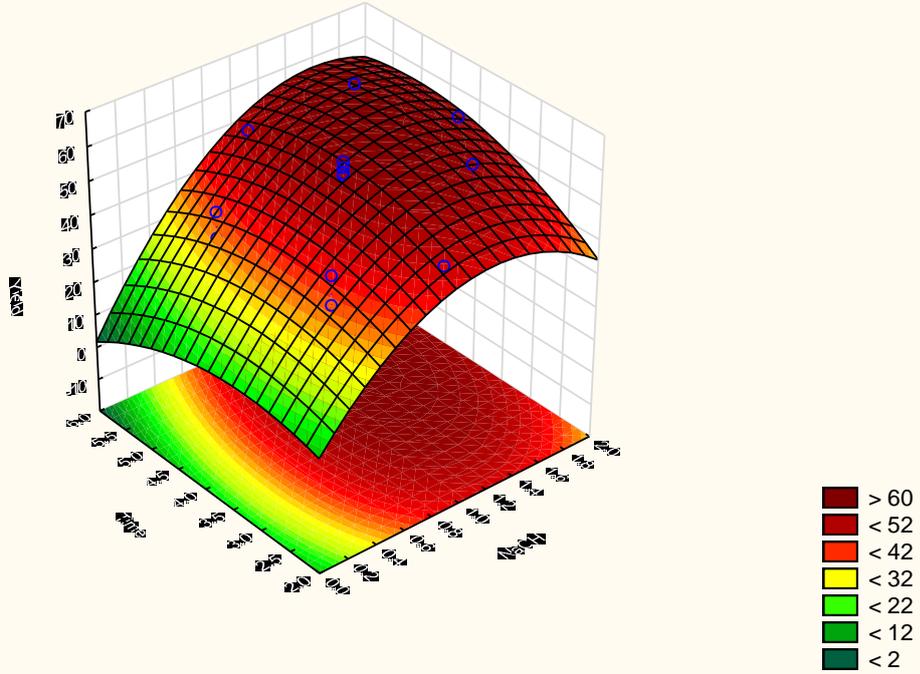


Figure A.0.1: Mind map for this study

## Appendix B: Response surface plots

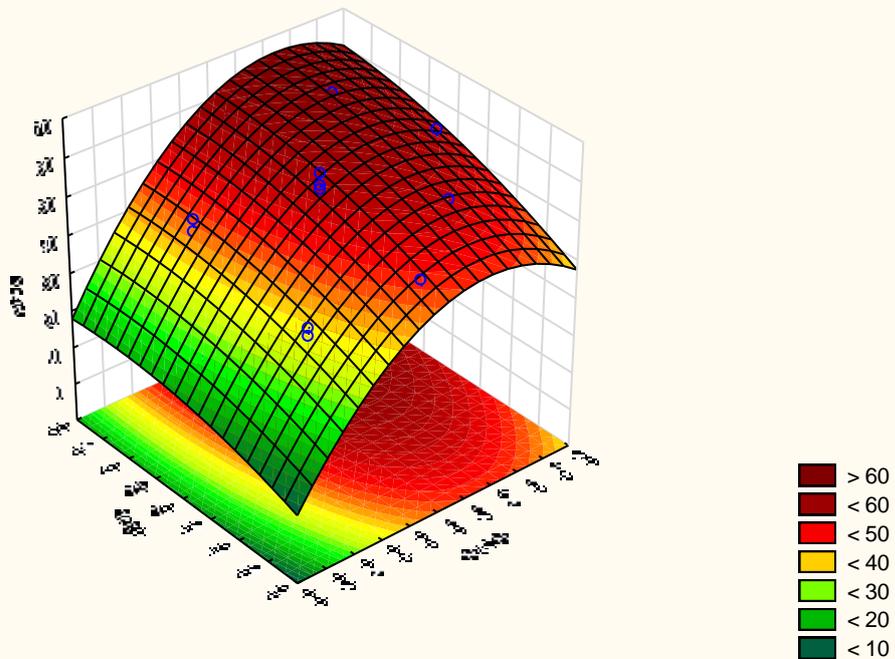
(a)

Fitted Surface; Variable: Yield  
 3 factors, 1 Blocks, 18 Runs; MS Pure Error=4.661734  
 DV: Yield



(b)

Fitted Surface; Variable: Yield  
 3 factors, 1 Blocks, 18 Runs; MS Pure Error=4.661734  
 DV: Yield



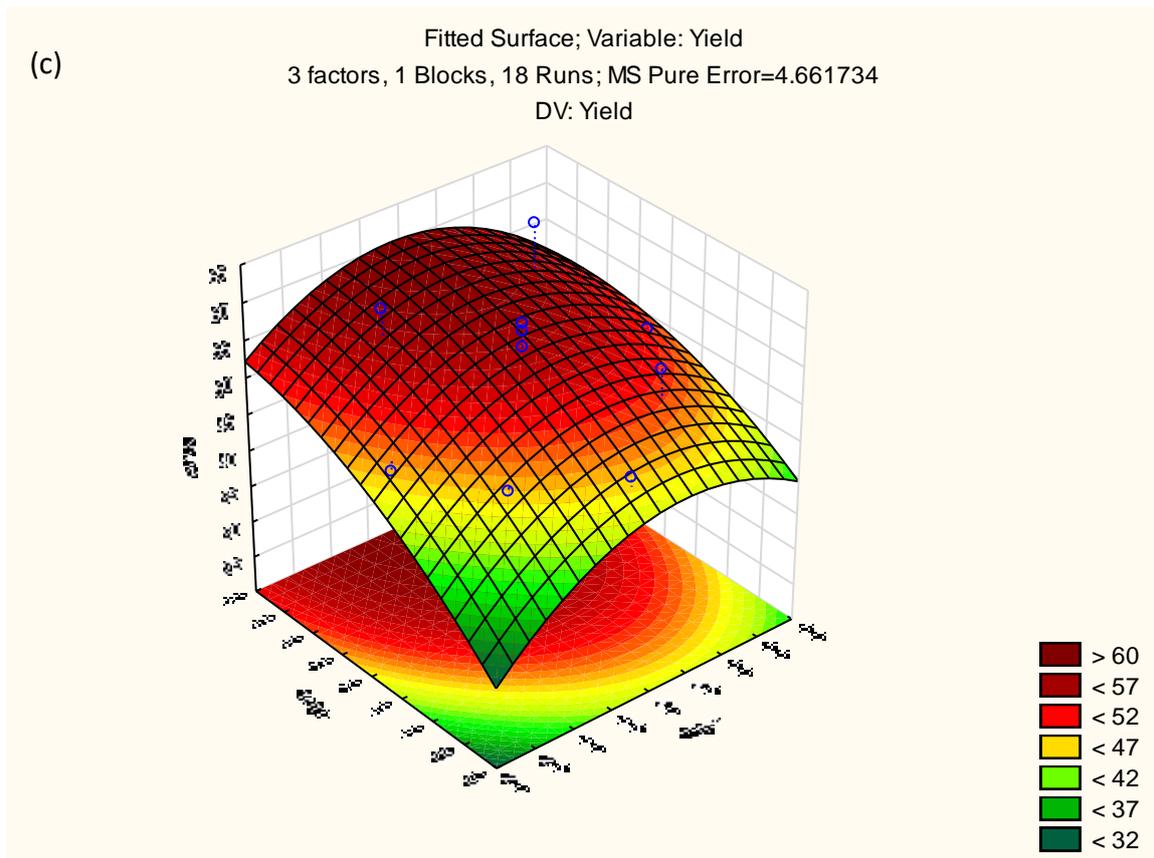
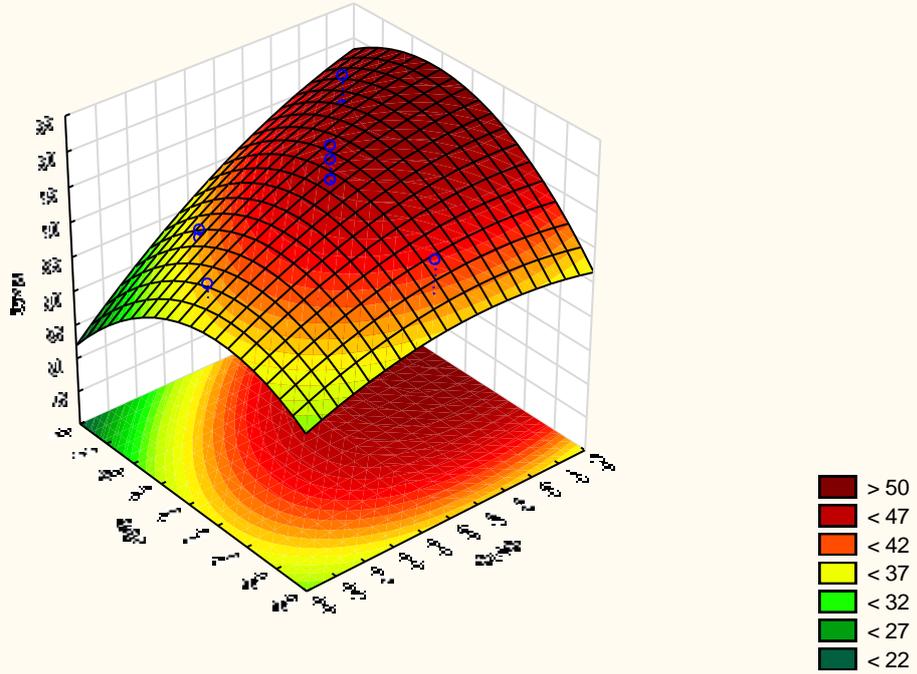


Figure B.0.1: Response surface plots for arabinoxylan yield as a function of (a) Sodium hydroxide concentration and time (b) sodium hydroxide concentration and extraction temperature (c) extraction time and temperature.

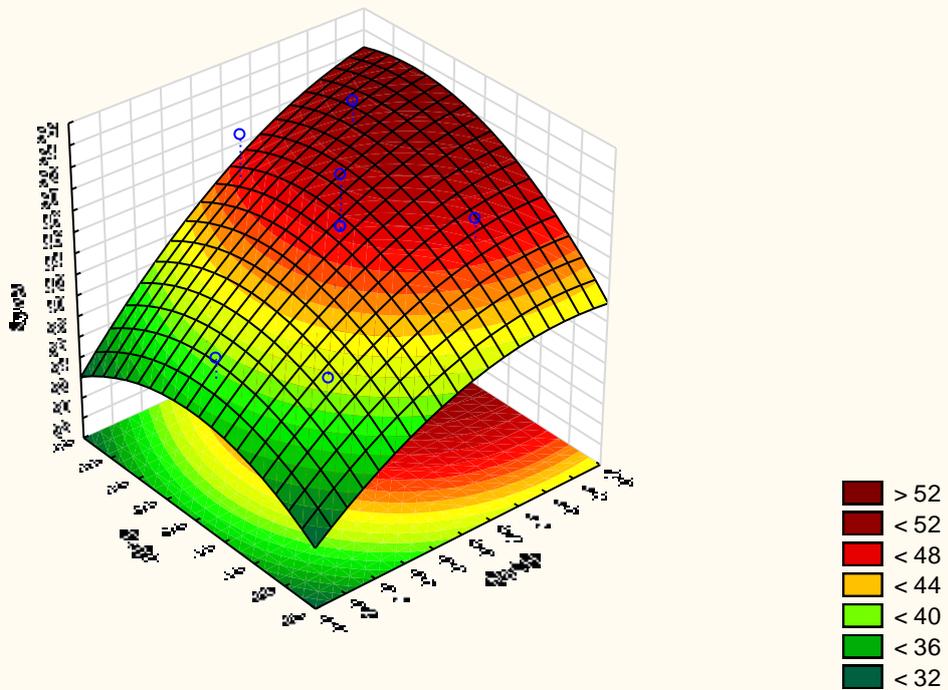
(a)

Fitted Surface; Variable: Purity  
 3 factors, 1 Blocks, 18 Runs; MS Pure Error=17.77192  
 DV: Purity



(b)

Fitted Surface; Variable: Purity  
 3 factors, 1 Blocks, 18 Runs; MS Pure Error=17.77192  
 DV: Purity



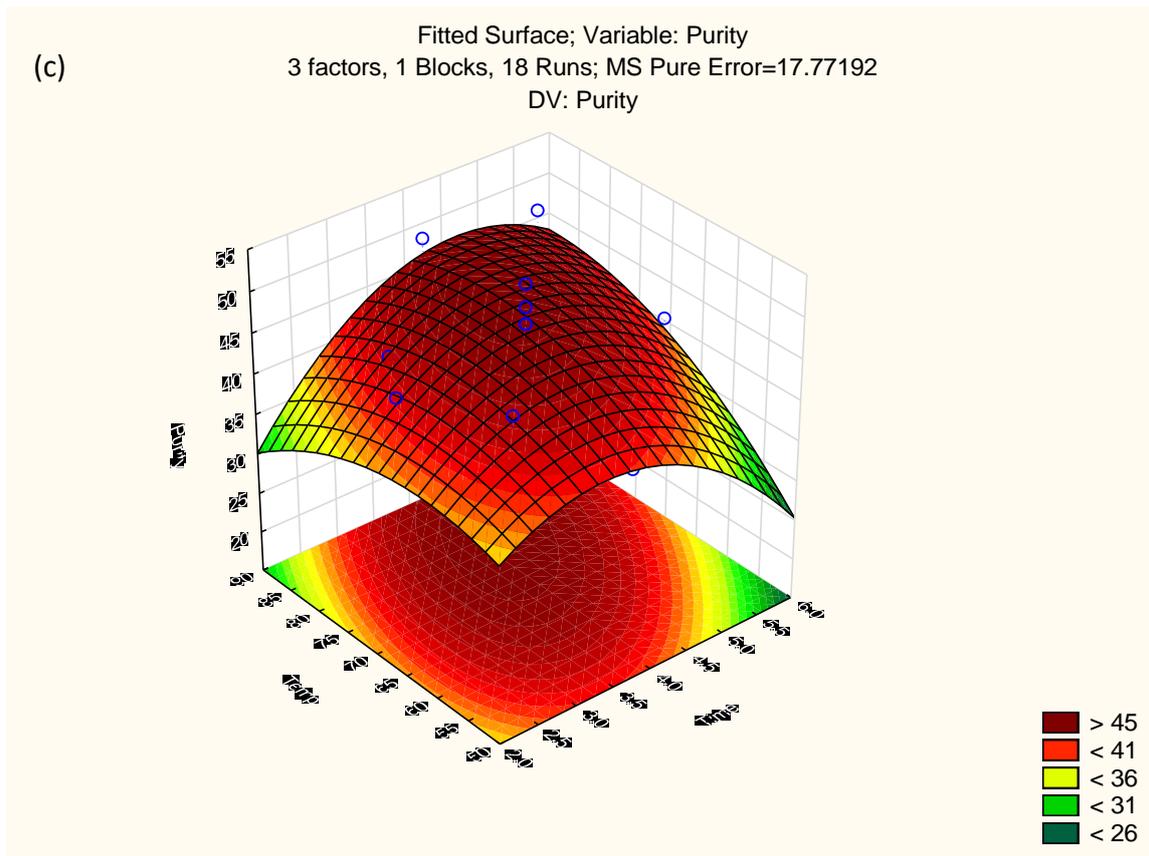


Figure B.0.2: Response surface plots for arabinosyln purity as a function of (a) Sodium hydroxide concentration and time (b) sodium hydroxide concentration and extraction temperature (c) extraction time and temperature.

## Appendix C: Raw data obtained from CCD study

Table C.0.1: The experimental results and composition of xylan under process variables by CCD

Run	NaOH concentration (M)	Time (hrs)	Temperature (°C)	Glucose (g/L)	xlyose (g/L)	Arabinose (g/L)	Glucose %	Xylose %	Arabinose %	AX %	A/X	Lignin %
1	0.50	3.00	60.00	0.13	0.63	0.43	5.46	27.29	18.53	40.32	0.68	1.39
2	0.50	3.00	80.00	0.09	0.59	0.43	3.67	23.50	17.03	35.67	0.72	2.54
3	0.50	5.00	60.00	0.12	0.59	0.41	5.39	26.06	17.96	38.74	0.69	1.78
4	0.50	5.00	80.00	0.09	0.60	0.44	3.82	26.00	19.24	39.81	0.74	2.74
5	1.50	3.00	60.00	0.13	0.77	0.41	5.68	33.57	18.00	45.38	0.54	1.99
6	1.50	3.00	80.00	0.11	0.73	0.39	4.77	31.67	17.12	42.93	0.54	2.16
7	1.50	5.00	60.00	0.14	0.83	0.44	6.15	36.32	19.46	49.09	0.54	2.21
8	1.50	5.00	80.00	0.13	0.87	0.50	5.75	38.31	22.08	53.14	0.58	1.67
9	0.16	4.00	70.00	0.12	0.58	0.46	5.28	25.49	20.52	40.49	0.80	2.81
10	1.84	4.00	70.00	0.13	0.79	0.48	5.62	35.09	21.35	49.66	0.61	2.94
11	1.00	2.32	70.00	0.12	0.75	0.41	5.14	33.28	18.29	45.38	0.55	2.06
12	1.00	5.68	70.00	0.09	0.60	0.34	3.94	26.81	14.93	36.73	0.56	2.14
13	1.00	4.00	53.18	0.12	0.67	0.33	5.17	29.14	14.29	38.22	0.49	1.77
14	1.00	4.00	86.81	0.11	0.81	0.49	5.05	35.82	21.48	50.42	0.60	2.19
15	1.00	4.00	70.00	0.11	0.69	0.40	4.91	30.25	17.57	42.09	0.58	1.68
16	1.00	4.00	70.00	0.14	0.86	0.50	6.26	37.73	21.82	52.40	0.58	1.84
17	1.00	4.00	70.00	0.13	0.79	0.44	5.76	34.57	19.57	47.64	0.57	1.96
18	1.00	4.00	70.00	0.13	0.79	0.44	5.93	34.90	19.23	47.64	0.55	1.82

<sup>a</sup>AX (Arabinoxylan) content = 0.88(xylose + Arabinose)

## Appendix D: Medium preparation for production of recombinant AbfB

### Spore plates medium

The composition of spore plates was minimal media with nitrate, 1% glucose, 0.2% trypticase BBL, 0.1% yeast extract, and 0.1% (w/v) casamino acid. The following components were mixed with 500 mL of distilled water and autoclaved at 120°C for 20 mins.

Any cheap agar	9.0 g
Neopepton (trypticase BBL)	1.0 g
Yeast extract (Difco)	0.5 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.2 g
Glucose	5.0 g
Casamino acids	1.0 g

After sterilisation the following sterile solutions were added

50x AspA with nitrate	10 mL
1000x Trace elements	0.5mL

### 2x MM (double strength minimal medium with 10% glucose)

The following components were mixed with 750 mL of distilled water and the sterilised by autoclaving at 120°C for 20 mins:

Yeast extract	7.5 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.6 g
Glucose	75 g
Casamino acids	3 g

After sterilisation the following sterile solutions were added:

50x AspA with nitrate	10 mL
1000x Trace elements	0.5mL

**50x AspA with Nitrate**

The following components were dissolved in 500 mL distilled water:

NaNO <sub>3</sub>	150 g
KCl	13 g
KH <sub>2</sub> PO <sub>4</sub>	38 g

The pH of solution was adjusted to pH 5.5 using 10 N KOH. The solution was sterilised by autoclaving at 120°C for 20 mins.

**Trace elements 1000x**

The following components were added one by one in the order listed.

dH <sub>2</sub> O	80 ml
ZnSO <sub>4</sub> .7H <sub>2</sub> O	2.2 g
H <sub>3</sub> BO <sub>3</sub>	1.1 g
MnCl <sub>2</sub> .4H <sub>2</sub> O	0.5 g
FeSO <sub>4</sub> .7H <sub>2</sub> O	0.5 g
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.17 g
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.16 g
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.15 g
EDTA	5.0 g

The solution was boiled and allowed to cool to 60°C. The solution pH was adjusted to pH 6.5 using KOH. The volume of solution was adjusted to 100 mL and then the solution was autoclaved.

## Appendix E: Gallic acid standard calibration curve

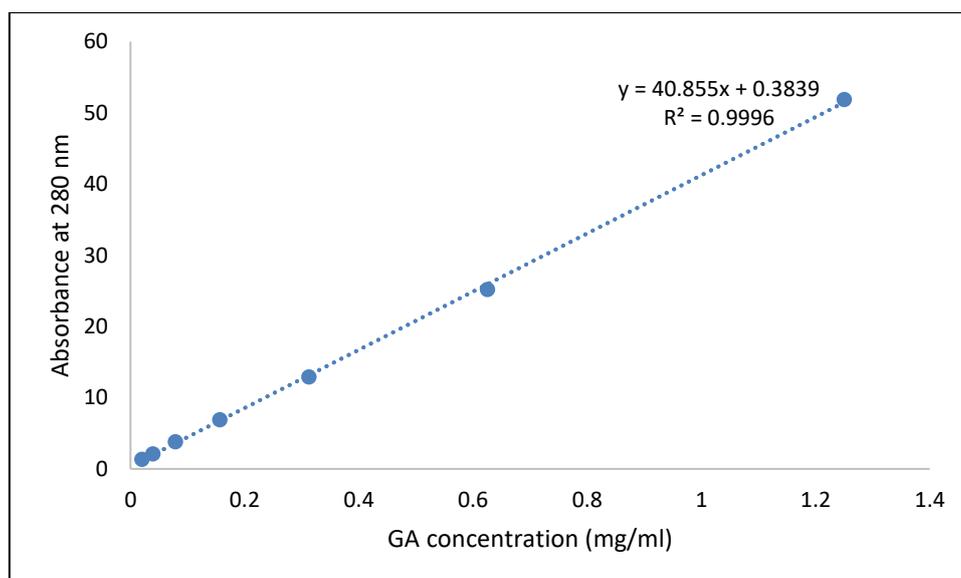


Figure E.0.1: Standard calibration curve of gallic acid in methanol used to determine encapsulation efficiency

## Appendix F: Raw data for size, polydispersity index and zeta potential of hydrogels

The size, polydispersity and zeta potential of hydrogels was measured with different samples and the average and standard deviation were calculated and shown on the table below

Table F.0.1: Size, polydispersity index and zeta potential of hydrogels

Average Size					
Sample	1	2	3	Average Size	Std
CWb	478.95	446.57	481.35	468.96	19.42
CWbD	608.2	573.4	618.1	599.90	23.48
CWbA	516	640.5	649.3	601.93	74.55
Ewb	624	575.8	592.6	597.47	24.47
EWbD	694.8	709.7	569.4	657.97	77.06
EWbA	735	635	663	677.67	51.59
Polydispersity Index (PI)					
	1	2	3	Average PI	Std
CWb	1.00	1.00	1.00	1.00	0.00
CWbD	1.00	0.88	1.00	0.96	0.07
CWbA	1.00	1.00	1.00	1.00	0.00
Ewb	0.26	0.34	0.38	0.33	0.06
EWbD	0.10	0.37	0.43	0.30	0.18
EWbA	0.21	0.32	0.32	0.28	0.07
Zeta Potential (mV)					
	1	2	3	Average ZP	Std
CWb	3.19	3.68	4.99	3.95	0.93
CWbD	2.33	3.01	3.37	2.90	0.53
CWbA	2.63	2.54	3.49	2.89	0.52
Ewb	7.92	8.78	9.83	8.84	0.96
EWbD	6.55	6.41	8.39	7.12	1.10
EWbA	5.9	7.48	4.97	6.12	1.27

CWb - chemical modified wheat bran hydrogels,

CWbD - chemically modified wheat bran hydrogels with gallic acid added during formation of hydrogels,

CWbA - chemically modified wheat bran hydrogels with gallic acid added after the formation of hydrogels,

Ewb - enzymatically formed wheat bran hydrogels,

EWbD- enzymatically formed wheat bran hydrogels with gallic acid added during the formation of hydrogels,

EWbA- enzymatically formed wheat bran hydrogels with gallic acid added after the formation of hydrogels