Ana Catarina Moreira Gameiro

Undergraduate Degree in Chemical and Biological Engineering

Enzymatic hydrolysis of yeast cell walls for the production of value-added products

Dissertation to obtain the Degree of Master in Biotechnology

Supervisor: Dr. Dina Krüger, Lead Scientist for Biocatalysis in R&D department, Ohly GmbH

Jury:

President: Prof. Dr. Pedro Miguel Calado Simões
Examiner: Prof. Dr. Susana Filipe Barreiros

FACULDADE DE CIÊNCIAS E TECNOLOGIA
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At the end of this academic chapter, I want to thank all the involuntary co-authors that have inspired and guided me through all this process.

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Abstract

Currently at Ohly GmbH, the insoluble yeast cell wall (YCW) fraction is generated as a side-stream product from the *Saccharomyces cerevisiae* yeast extract production, which is sold for a low profit margin as an additive and supplement for animal feed. The YCW structure exhibits a great source of health-promoting activity and bioactive properties in humans and animals. Therefore, the main aim of this dissertation was to hydrolyse the autolysed YCW through enzymatic digestion, altering its composition and structure, and thereby obtaining different types of unique hydrolysates, which may be used for possible innovative and valuable applications.

As there wasn't any previous work with YCWs at Ohly GmbH, the first step was to develop an enzymatic conversion method establishing the reaction conditions and a hydrolysate product analysis protocol to standardize the process. Once the protocol was determined, the second step was screening 56 commercial enzyme mixtures, which present different hydrolytic activities (such as phospholipase, β-glucanase, chitinase, mannanase, etc) and also characterize the obtained hydrolysates in visual, analytical and chemical terms, such as viscosity, turbidity, solubilisation and phase separation, reducing and free sugars over reaction time.

Afterwards, a characterization of glucan- and mannan-oligosaccharides was performed, in order to establish a hydrolysate product selection according to the respective number-average degree of polymerization (DP_n), which was also estimated. The DP_n values obtained from the enzymatic hydrolysates were also compared and related by their molecular weight (MW) profile, which was analysed by HPLC-SEC. Thus, with a full and detailed report of each hydrolysate product, ideas of new products development were generated, since different DP_n values can lead to different product applications.

In addition, to obtain a hydrolysate product with an immune-stimulant activity to animal feed, a process optimization (variation of the enzymatic reaction conditions) for a specific commercial enzyme mixture was performed and studied in detail.

Keywords: Yeast cell wall; Enzymatic hydrolysis; Glucan-oligosaccharides; Mannan-oligosaccharides; Number-average degree of polymerisation; Molecular weight.

Resumo

Atualmente, na Ohly GmbH, a fração insolúvel da parede celular da levedura (PCL) é um subproduto da produção de extrato de levedura *Saccharomyces cerevisiae*, sendo vendida a uma margem de lucro reduzida como aditivo e suplemento para alimentação animal. A estrutura da PCL apresenta uma elevada fonte de propriedades bioativas e de atividade de promoção de saúde em seres humanos e animais. Esta dissertação teve como principal objetivo a hidrólise da PCL autolisada por digestão enzimática, alterando a sua composição e estrutura, obtendo assim diferentes tipos de hidrolisados únicos, que poderão ser utilizados para possíveis aplicações inovadoras e de valor acrescentado.

Uma vez que, na Ohly GmbH não existia nenhum trabalho anterior com a PCL, o primeiro passo envolveu o desenvolvimento de um método de conversão enzimática, estabelecendo as condições de reação, e de um protocolo de análise dos produtos hidrolisados para uniformizar o processo. Após a realização do protocolo, procedeu-se ao rastreio de 56 misturas de enzimas comerciais, que apresentam diferentes atividades hidrolíticas (tais como fosfolipase, β-glucanase, quitinase, mananase, etc) e também a caraterização dos hidrolisados obtidos em termos visuais, analíticos e químicos, tais como viscosidade, turbidez, solubilização e separação de fases e açúcares redutores e livres ao longo do tempo de reação.

Posteriormente, realizou-se uma caracterização de gluco- e mano-oligossacarídeos, de maneira a estabelecer uma seleção de produtos hidrolisados de acordo com o respetivo grau médio de polimerização (GP_n), que também foi estimado. Os valores de GP_n obtidos dos hidrolisados enzimáticos foram também comparados e relacionados com o seu respetivo perfil de peso molecular (PM), o qual foi analisado por HPLC-SEC. Assim, com um relatório completo e detalhado de cada produto hidrolisado, foram geradas ideias de desenvolvimento de novos produtos, uma vez que diferentes valores de GP_n podem conduzir a diferentes aplicações de produto.

Adicionalmente, de modo a obter um produto hidrolisado com uma atividade imune-estimulante para alimentação animal, realizou-se e estudou-se em detalhe um processo de otimização (variação das condições de reação enzimática) para uma mistura de enzima comercial específica.

Palavras-chave: Parece celular da levedura; Hidrólise enzimática; Gluco-oligossacarídeos; Mano-oligossacarídeos; Grau médio de polimerização; Peso molecular.

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List of Abbreviations and Symbols

ADP	Adenosine-5'-diphosphate
AGP	Antibiotic growth promotors
AMP	Adenosine monophosphate
Asn	Asparagine
ATP	Adenosine-5'-triphosphate
CAZyme	Carbohydrate-active enzyme
CMP	Cytidine monophosphate
CO_2	Carbon dioxide
CW	Cell wall
CWP	Cell wall proteins
Da	Dalton
ddH_2O	Double-distilled water
DM	Dry matter
DNS	Dinitrosalicylic acid
DP	Degree of polymerisation
DP_n	Number-average degree of polymerisation
DW	Dry weight
F-6-P	Fructose-6-phosphate
FDA	Food and drug administration
Fdase	Phosphodiesterase
g	Gram(s)
G-6-P	D-glucose-6-phosphate
G6P-DH	Glucose-6-phosphate dehydrogenase
GH	Glycoside hydrolase
GlcNAc	*N*-acetylglucosamine
GMO	Genetically modified organism
GMP	Guanosine monophosphate
GOD	Glucose oxidase
GPI	Glycosylphosphatidylinositol
GRAS	Generally recognized as safe
GT	Glycosyltransferase
h	Hour(s)
HK	Hexokinase
HPAE	High performance anion-exchange chromatography
HPLC	High performance liquid chromatography

IMP	Inosine monophosphate
L	Litter(s)
LPHase	Laminaripentaose-producing β-(1,3)-glucanase
m	Meter(s)
M-6-P	Mannose-6-phosphate
min	Minute(s)
MOS	Mannan-oligosaccharides
MPR	Microplate reader
MSG	Mono sodium glutamate
MW	Molecular weight
MW_n	Number-average molecular weight
NADP	Nicotinamide-adenine dinucleotide phosphate
NADPH	NADP reduced form
O_2	Oxygen
Opt.	Optimum
Pa	Pascal
PAD	Pulsed amperometric detection
PGI	Phosphoglucose isomerase
PH	Polysaccharide lyase
PMI	Phosphomannose isomerase
RNA	Ribonucleic acid
rpm	Revolutions per minute
RS	Reducing sugars
s	Second(s)
SEC	Size exclusion chromatography
Ser	Serine
SM	Starting material
SN	Supernatant
T	Temperature
Thr	Threonine
UMP	Uridine monophophate
UV	Ultraviolet
VIS	Visible
w/v	Solute’s weight per total solution’s volume
w/w	Solute’s weight per total solution’s weight
YCW	Yeast cell wall
YE	Yeast extract
λ	Wavelength

CHAPTER 1

1. Introduction

1.1. Ohly GmbH

Ohly GmbH is one of the world's leading producers and suppliers of yeast extracts, yeast based flavours and specialty powders for the health markets (nutraceutical), biotechnology (fermentation), food and animal feed industries. The company is part of the ABF Ingredients group, a division of Associated British Foods which comprises of companies such as AB Enzymes, ABITEC, PGP International and, evidently, Ohly GmbH. The company focuses in high value ingredients in food and non-food areas. Thus, Ohly's wide range of products are based on baker's, Torula and brewer's yeast such as yeast extracts, autolysed yeast, inactive dry yeast, yeast based flavours, smoked yeast, yeast cell walls, yeast beta-glucans, active yeast as well as bacteria starter cultures and speciality powders [1].

Ohly's history goes back to 1836, when it was founded as "Heinrich Helbing Korn Distillery" by Heinrich Helbing in Hamburg, Germany producing yeast and spirits only for the local market. In the mid 1930's, Götz Ohly was the first to develop yeast extracts, whereby in 1961 the company was converted to "Deutsche Hefewerke GmbH" [1].

Figure 1.1 Ohly GmbH in 1923 with the name "Heinrich Helbing Korn Distillery".

In 1994, Burns Philp, a group in the food manufacturing business which purchased previously Provesta Corporation in Hutchinson (US), acquired Deutsche Hefewerke GmbH and since then, a close collaboration with Provesta started. In 2004, ABF Ingredients acquired parts of the Burns Philp group including Provesta and Deutsche Hefewerke and, finally, in 2007 these two companies merged and started to operate under the well-established brand Ohly, derived from the surname of the first yeast extract producer [1].

The company currently has three manufacturing plants. In Hamburg, yeast derivatives based on baker's yeast and starter cultures are produced. The Hutchinson, Minnesota (US) site specialises in the continuous fermentation of Torula yeast for the manufacture of Torula yeast based flavour ingredients and in Boyceville; Wisconsin (US), a variety of Torula, brewer's and baker's yeast extracts as well as a specialty powders range of products are manufactured [1].

Figure 1.2 Current Ohly GmbH plant in Hamburg, Germany.

1.2. Contextualization

The side-stream products valorisation enables to reduce waste removal costs and entry of new and value-added products to the market and/or improves sustainability in the process chain. Since the food manufacturers immediately benefit, there is a large demand and need of innovative methods to deal with side-stream products and concern about the environment issues regarding the waste disposal of them [2].

Currently at Ohly GmbH, the insoluble cell wall fraction is generated as a side-stream product from the yeast extract production, which is sold for a low profit margin as an additive incorporated into feeds for cattle, swine and poultry in the feed industries. Therefore, the aim of this project was to develop a method for the enzymatic hydrolysis of yeast cell walls and also to standardize a hydrolysate product characterization protocol in visual, analytical and chemical terms. Afterwards, based on the result of the screening of 56 commercial enzyme mixtures, the resulting hydrolysate products will lead to ideas for new product development with valuable and useful applications in food, fermentation and animal feed areas.

CHAPTER 2

2. Theoretical Background

2.1. *Saccharomyces cerevisiae*

The metabolic process called fermentation was considered for several thousands of years an effective and low-cost resource to preserve the quality and safety (shelf life) of food. Moreover, fermentation also has the property of enhancing nutritional quality and digestibility, provides dietary enrichment through aroma and flavour production and modifies textures of food substrates. All these changes are produced by micro-organisms, which are naturally present (spontaneous fermentation) or added (inoculated fermentation) in raw materials composed by complex carbohydrates and proteins. These complex compounds are broken down into more easily digestible elements by micro-organisms, where yeasts stand out due to its wide use for commercial purposes in food and beverage industries [3,4].

Yeasts have been well known to humans due to their use in fermentation processes, namely for the production of alcoholic beverages and bread leavening. Since their identification and isolation by Louis Pasteur[1], yeasts started being produced and commercialized in the end of the 19th century. The current technology allows the isolation, construction and industrial production of yeast strains with specific properties to satisfy the demands of the food and beverage industries [3].

Consumers have a positive image of yeasts, since they are considered as a safe source of ingredients and additives in food processing. Among several yeast strains, *Saccharomyces cerevisiae*, also known as baker's and brewer's yeast, is a non-pathogenic organism and the most common food grade yeast, since in its inactive form it is used in the health food industry for human consumption as nutritional supplements, food additives, conditioners and flavouring agents, in the production of microbiology media, as well as livestock animal feeds. In its active form, baker's yeast is used in bread making and bakery, while brewer's yeast is used for alcohol fermentation and other fermentative

[1] Louis Pasteur (1822–1895) was a French chemist and microbiologist renowned for his discoveries of the principles of vaccination, microbial fermentation and pasteurization.

processes, such as beer and wine production. It's also considered as an alternative source of protein to human and animal consumption, as it provides high contents of proteins, vitamins (mainly the B group), trace minerals, such as chromium and selenium, amino acids (mainly lysine), lipids and complex carbohydrates. Thus, the USA's Food and Drug Administration (FDA) award of "generally recognized as safe" (GRAS) status to *S. cerevisiae* [3–7].

Baker's yeast biomass is not only a valued raw material with different uses, both in the past and present, it is also important to highlight its promising potential applications in the area of production of ingredients for functional foods (i.e. β-glucan and flavouring compounds) and in the bioremediation area as an agent of detoxifying effluents containing heavy metals (Figure 2.1) [8].

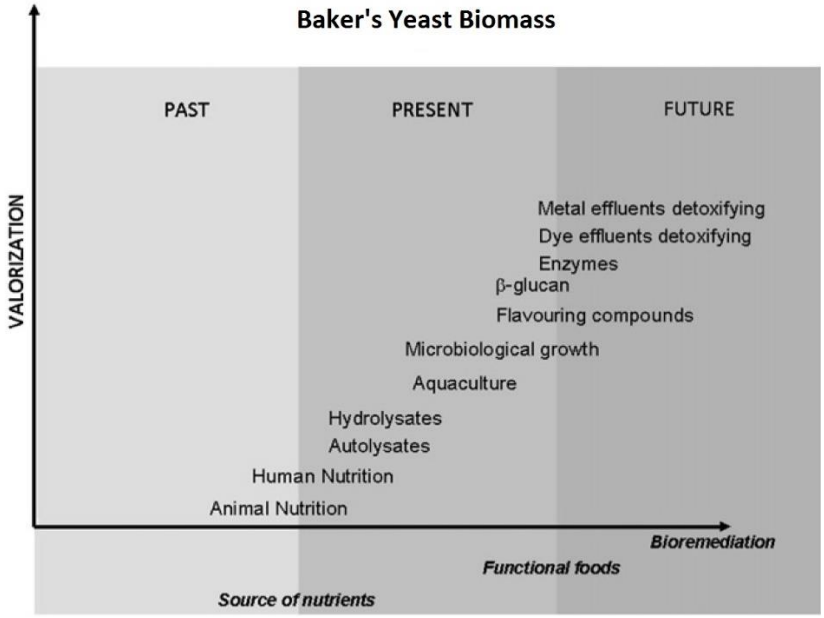

Figure 2.1 Schematic representation of the baker's yeast biomass valorisation though the time (edited) [8].

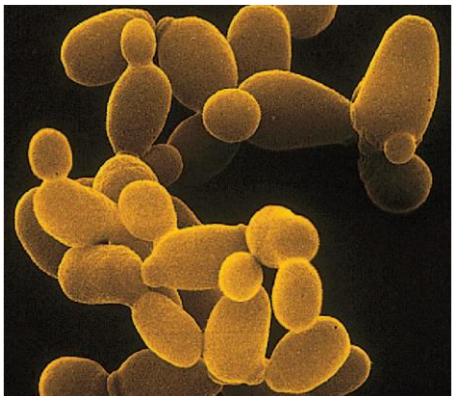

Figure 2.2 Scanning electron micrograph of the microscopic, unicellular yeast, *Saccharomyces cerevisiae* (x21,000) [9].

In biological terms, *S. cerevisiae* belongs to the fungus kingdom, a group of unicellular eukaryotic micro-organisms, which has a single nucleus and reproduces either asexually by budding and transverse division or sexually through spore formation. From each bud, a new yeast cell can grow and some cells group together to form colonies (Figure 2.2). Generally, the size of the yeast cells is considerably different than that of bacteria. Yeast cells are larger, approximately 5–10 µm, and commonly spherical to egg shaped. Regarding the cell biology, yeast cells don't have flagella, but possess most of the other eukaryotic organelles (Figure 2.3) [7,9].

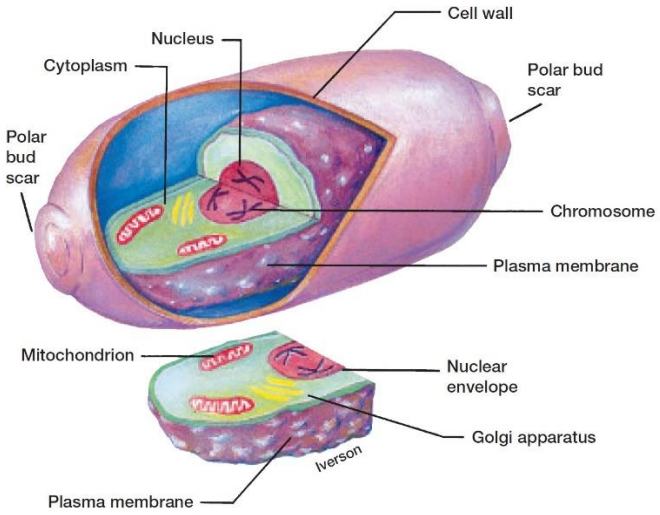

Figure 2.3 Diagrammatic drawing of a yeast cell displaying the typical morphology. For clarity, the plasma membrane was drawn separated from the cell wall, but in a living cell, it adheres tightly to the wall [9].

With the increase of the knowledge of this unicellular eukaryote, *S. cerevisiae* became a well-established model system which is used nowadays to understand fundamental cellular processes in higher eukaryotes, mammalian and plant cells. Because it can be grown at almost any scale from laboratory cultures in shaking flasks to some hundred litters in industrial fermenters, yeast is viewed as a relevant tool both in academic and industrial fields. It can also be easily genetically manipulated, for example by nutritional means, supressing or inducing certain biochemical pathways for a desired application, since it was the first eukaryotic organism whose genome was sequenced. Thus, *S. cerevisiae* is considered as an attractive organism for several biotechnological purposes [5,10,11].

2.1.1. Baker's yeast production

For many years, *S. cerevisiae* strains have been selected by their dough-leavening characteristics. During the baker's yeast fermentation, ethanol and carbon dioxide (CO_2) are produced, where the last compound determines the dough leavening and contributes to the flavour and crumb structure of the bread. This gas production rate entirely depends on specific fermentation parameters and especially on intrinsic characteristics of baker's yeast. However, the role of baker's yeast production goes further than just gas production in bread making. Today, with the rapidly growing population, the whole world is facing low agricultural production, which led to an economic targeting towards for the production of food-grade yeasts and make them more acceptable for being used as food supplements [12,13].

The most important requisites in a commercial production of baker's yeast are rapid growth and high biomass yield, which are achieved by the use of a well-established feed-batch fermentation method with sequential stages differing in fermenter size, feeding and aeration conditions (Figure 2.4) [12].

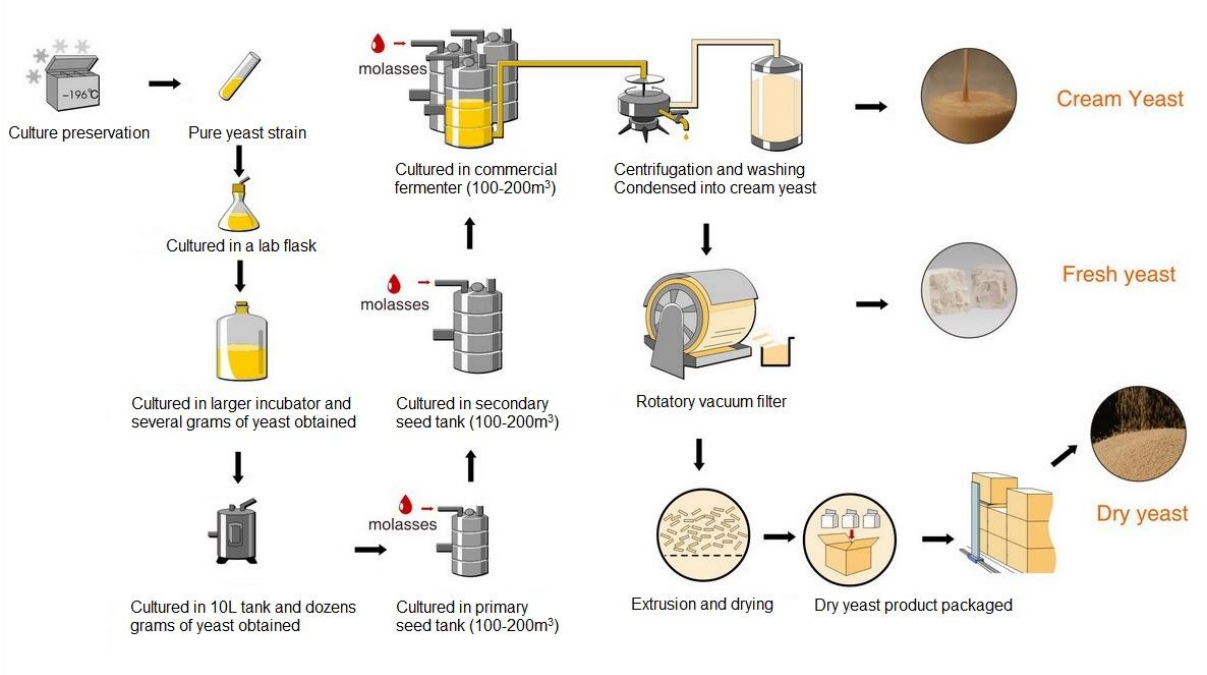

Figure 2.4 Exemplified scheme of a commercial yeast production process (edited) [14].

The main substrate used in yeast production is the cane molasses, which is abundantly available and cheap since it is a waste product from sugar refineries. Molasses contain a high sugar concentration (65-75%), wherein the predominant sugar is sucrose, which yeasts can extracellularly hydrolyse into monosaccharides, namely fructose and glucose, and metabolise them afterwards. However, their sugar composition may vary significantly from batch to batch and sucrose-refining procedures, but it also depends on weather conditions, which makes molasses a non-ideal growth media. Moreover, molasses are deficient in essential compounds for yeast growth, i.e. minerals (nitrogen, potassium, phosphate, magnesium and calcium), trace elements (iron, zinc, copper, manganese and molybdenum), salts and vitamins, which have to additional supplemented in the culture medium to improve the yeast growth [12,15,16].

The initial stage takes place in the laboratory, wherein a selected pure yeast strain is inoculated in a sterilized flask containing molasses as growth media. The entire content of this flask is used to inoculate the first fermenter in the pure culture stage. Generally, in this stage of process are used one or two fermenters which operate in batch mode and with aeration, whereby ethanol will be formed [12,16].

Afterwards, the fed-batch stages are performed using full aeration and incremental feeding of culture medium (molasses and other required nutrients). Consequently, the content of each fermenter is pumped to the flowing one, as such in each stage the size of the fermenters and the amount of yeast growth increase. Furthermore, the sequence of the fermentation stages varies among manufacturers [12,16].

After all of the required molasses has been fed into the final fermentation stage, the fermenter is still under aeration, which allows yeast cells maturation. Thus, the cells completely assimilate the substrate and accumulate reserve carbohydrate, increasing their stability and prevention to autolysis [15,16].

Finally, the yeast cells are recovered from the final fermenter by centrifugal separators and following a washing step, obtaining in the end the yeast cream which may be subjected to downstream processing steps like cell disruption, protein extraction and purification [13,16]. Thereafter, yeast extracts, yeast autolysates and dried yeast preparations are produced, which are the most commercially significant products derived from yeasts and are widely used in the food industry as flavour ingredients, providing a source of savoury, roasted, nutty, cheesy, meaty and chicken flavours [4].

Additionally, the centrifuged yeast cells can be subjected to a rotatory vacuum filter forming a fresh yeast cake usually sold to bakery manufacturers. From a subsequently additional step of extrusion and drying, a dry yeast is produced and also sold to bakery manufacturers [16].

2.2. Yeast extract production

According to the Food Chemical Codex, yeast extract (YE) is defined as the water-soluble components of the yeast cell, composed primarily of amino acids, peptides, carbohydrates and salts. YE is considered as a flavour product enriched in amino acids, such as glutamic acid in the free acid form or as monosodium glutamate, which provides a brothy taste to food. Therefore, it's mainly used in the food industry as a flavouring agent to improve and enhance the flavour in soup, sauces, gravies, stews, snack food and canned food, as well as in pet foods. Nevertheless, YE can also be used in other applications, such as vitamin and protein supplements in health foods and as a source of nutrients in microbiological media [4,17,18].

As mentioned previously (see 2.1.1), the YE is manufactured from the yeast cells in the yeast cream, which are subjected to a specific treatment such that the cells are disrupted due to the hydrolysis of peptide bonds and its content is released. This sort of treatment is generally done by two different processes, autolysis or hydrolysis, resulting in yeast extracts with different product and flavour attributes. Moreover, the final flavour profile and extract yield in a well-controlled process is largely dependent on temperature, pH, reaction time, viability, enzyme activity, solubilizing aids and concentration of yeast [4,17,19].

After the autolysis or hydrolysis process, a suspension (autolysate or hydrolysate, respectively) is obtained, which is then separated from the soluble cell components (YE) and the insoluble yeast cell wall (YCW) fraction (Figure 2.5) [17]. The insoluble autolysed YCW fraction was studied in detail in this thesis as the starting material.

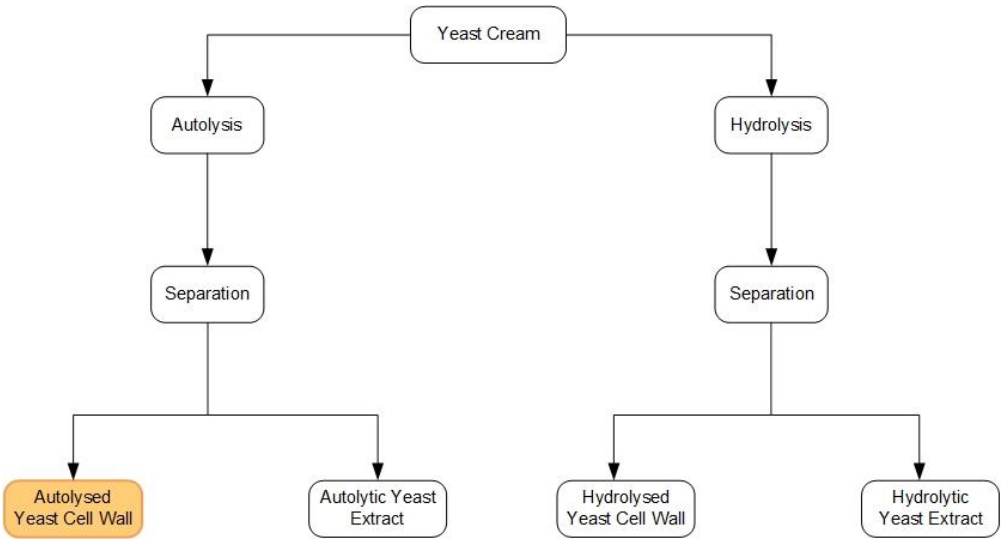

Figure 2.5 Scheme of a yeast extract production.

2.2.1. Autolysis process

Autolysis is a degradation process performed by activating yeast's own degradative enzymes, i.e. endogenous enzymes, which occurs naturally in yeasts when the cell growth cycle is completed and the death phase is initiated [18,20].

The reaction conditions play an important role in the autolysis, more precisely, the first phase of the process is performed at a particular pH range combined with a certain temperature. Initiation of autolysis is stimulated and accelerated by addition of plasmolyzing agents, such as sodium chloride and/or altering the permeability of the cell with ethyl acetate or chloroform. A typical process is executed at a temperature range of 50–55°C, which kills the cells but promotes high enzymatic activity to the native yeast enzymes [17,21].

The degradative enzymes, which are located in the general matrix of the cell, are responsible for damaging and/or partially disrupting the cell wall, leading to a partial open of the cells and the release of its content. These hydrolytic enzymes, particularly proteases and nucleases, also break down the cell content, i.e. insoluble macromolecules like proteins and nucleic acids to soluble products of peptides, amino acids (mainly glutamate), nucleotides and amino acid derivatives, causing its release and contributing significantly to a powerful savoury flavour to YE [4,17,20].

2.2.2. Hydrolysis process

Contrary to autolysis, through a hydrolysis process, the enzymatic degradation to the disruption of the yeast cell wall and digestion (lysis) of the cell content is carried out by exogenous enzymes. Generally, these employed enzyme preparations have one or more of the following activities: proteolytic

activity (proteases or peptidases), RNA degrading activity converting it to 5'-ribonucleotides (nucleases), lipolytic activity (lipases or phospholipases) or deaminase activity (deaminases) [17,18].

Since this process involves the addition of exogenous enzyme preparations, one of the steps of the process consists the inactivation of the yeast's own enzymes, usually by a heat shock, to ensure the activity of the added enzyme preparations to the yeast cells [17].

Through an enzymatic yeast hydrolysis a better control of the process can thereby be achieved. However, this process depends upon various factors with regards the selection of the enzyme preparations, such as the source of raw materials and methods used in the extraction and purification process. Furthermore, the reaction conditions are also very important and dependent on the type of enzyme used [17,22].

2.2.3. High nucleotide YE production

The production of food-grade yeast extracts is a promising approach, for instance, to obtain flavouring foodstuff since baker's yeast cells are a rich source in ribonucleic acid (RNA). The RNA content in *S. cerevisiae* is typically in the order of 7-12% (w/w) depending on the specific growth rate as shown [8,23].

Ribonucleotides such as 5'-Guanosine monophosphate (5'-GMP) and 5'-Inosine monophosphate (5'-IMP) are important natural flavour enhancer components of yeast extracts which have been shown great importance in the food sector. These 5'-ribonucleotides are characterized due to the demonstrated improvement the taste of food and the ability to promote a synergistic effect together with the amino acid glutamate or glutamic acid, also an important flavour enhancer [4,22].

During the autolysis process, the yeast's own enzymes break down the cells content, meaning that the native RNA loses its native folding which is not degradable into 5'-ribonucleotides. Therefore, the yeast extracts rich with a high nucleotide content are typically produced by enzymatic hydrolysis. After the hydrolytic yeast extract production, the process is further extended by addition of a nuclease, usually a 5'-Phosphodiesterase (5'-Fdase), to convert RNA into 5'-ribonucleotides. 5'-Fdase is generally obtained from a microbial (from *Penicillium citrinum* or *Streptomyces aureus*) or a vegetable source (from malt root extract). Only 5'-GMP has taste-enhancing properties, while the other 5'-ribonucleotides generated as by-products, such as A/C/UMP, are taste-neutral. However, with the addition of an adenyl-deaminase enzyme, the 5'-AMP is converted into 5'-IMP which also has taste-enhancing properties [17,23,24].

2.3. Yeast cell wall

The *S. cerevisiae* cell wall represents a dynamic structure which determines the cell shape and integrity of the organism during growth and cell division. The yeast cell wall (YCW) is a robust and elastic

structure providing physical protection and osmotic support, which presents a remarkably thick envelope (100–200 nm) [25–29].

The YCW represents about 15-30% of the dry weight (DW) of the cell and has a bi-layered structure composed mainly by polysaccharides (~85%), such as β-glucans, mannan and a small amount of chitin, proteins (~15%) and lipids (Figure 2.6) [4,30].

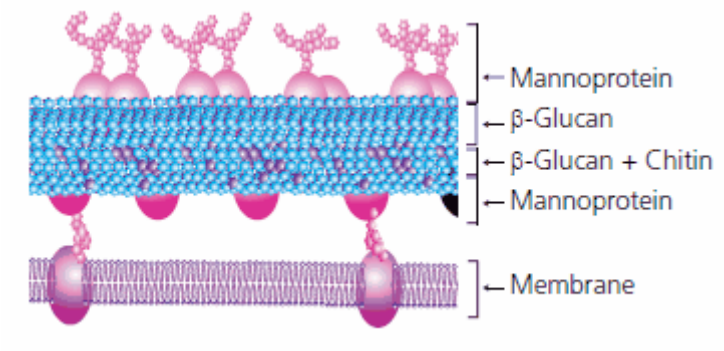

Figure 2.6 Structure of the YCW. The wall is primarily composed of mannoproteins and β-glucan that is linked (1→3) and (1→6) [31].

The inner layer is essentially composed by β-glucans, linked between β-(1,3) bonds, and chitin, it is largely responsible for the mechanical strength of the wall and also affords the attachment sites for the proteins in the outer layer of the wall. Consequently, the outer layer constitute a heavily glycosylated mannoproteins emerging from the cell surface, which are highly involved in cell-cell recognition proceedings. Their function consists to limit the accessibility to the inner layer of the wall and the plasma membrane, shielding it against attacks by foreign enzymes as well as membrane-perturbing compounds. The mannose polysaccharides side chains contain multiple phosphodiester bridges, resulting in numerous negative charges in the cell surface at physiological pH values. Therefore, they are responsible for the hydrolytic properties of the wall, promoting water retention and drought protection. The cell wall proteins (CWP) are covalently linked to the β-(1,3)-glucan-chitin network either directly or indirectly through a β-(1,6)-glucan side chain, which stabilizes the cell wall [25,26,28,32].

Table 2.1 demonstrates the polysaccharides composition of the YCW in percentage of wall DW as well as the degree of polymerisation and branching of each constituent.

As previously mentioned (see 2.2), the inner part of the yeast cells are isolated and used to produce YE, while the outer part (YCW) remains as waste, for which no commercial use has been established, except as a supplement for animal feed [33].

The motivation of this thesis was to study the YCW for possible valuable applications since it is a by-product of the YE production. The YCW structure exhibits a great source of health-promoting activity, where polysaccharides and mannoproteins reveal a range of bioactive properties in humans and animals. Consequently, β-glucans and mannoproteins have been recently reported for their potential flavour applications as food fibre additives and for their emulsifying and health improvement properties [4,34].

Table 2.1 Macromolecules composition of the *S. cerevisiae* cell wall [26].

Macromolecule	Wall DW (%)	DP	Branching
Mannoproteins	35 - 40	200	Highly branched
β-(1,6)-Glucan	5 - 10	140	Highly branched
β-(1,3)-Glucan	50 - 55	1500	Moderated branched
Chitin	1 - 2	190	Linear

The cell wall components are presented in the order in which they are found in the cell wall from the outside to the inside. DP, degree of polymerisation; DW, dry weight. Note that the data presented may vary depending upon the conditions and stage of growth.

2.3.1. β-glucans

As stated before, the most abundant component in the YCW is β-glucan. This natural polysaccharide is found in cereal, plant, algae, bacteria, fungi and yeast sources, which exhibits different macromolecular structures depending on the source. Yeast β-glucans are a highly branched glucose oligosaccharide consisting of a main chain of β-(1,3)-D-glucose residues, along which are randomly dispersed side chains of β-(1,3)-D-glucose units attached by β-(1,6) linkages. Their constitution is rearranged by a random coil or more organized conformations, such as a network composed of single helix chains associated in tripe helix, which are stabilized through inter- and intra-hydrogen bonds [35,36].

Due to the highly branched composition, yeast β-glucans are insoluble in water, but through an enzymatic hydrolysis or other chemical methods it is possible to produce soluble glucans with a low content of glucosyl branches, which facilitates their use to different applications for food and pharmaceutical areas. Figure 2.7 illustrates the molecular structure of soluble (slightly branched) and insoluble (highly branched) yeast glucans [37].

Yeast β-glucans have attracted attention due to their physico-chemical and bioactive properties. They have demonstrated potential in improving the physical properties of food products as gelling, thickening, water-holding and oil-binding agents as well as emulsifying stabilizer. Table 2.2 lists the properties of yeast β-glucans compared with other food yeast products. Regarding the biological properties, they have been especially attractive due to their immune-stimulant activity, which activate the white blood cells in response against infections and support the repair of damaged tissues in the body by stimulation of collagen biosynthesis. Moreover, they also act as cholesterol and glycaemic reducing agents and reveal anti-tumorigenic activity and anti-inflammatory effect in animals and humans [4,34–36,38–41].

Besides the different chemical structures from different sources, β-glucans also vary in solubility, degree of branching and polymerisation, molecular weight (MW), viscosity and solution conformation. These structural and physicochemical properties have a large influence on sort of potential application which might be applied. For instance, the ability of β-(1,3/1,6)-glucans to activate the innate immune

cells depends on its branching. Consequently, a β-(1,3)-glucan without any side chains (branches) does not activate white blood cells, meaning that the β-glucans from mushrooms which only have one single glucose residue in the side chain present a lower immune-stimulant activity than YCW β-glucans [42,43].

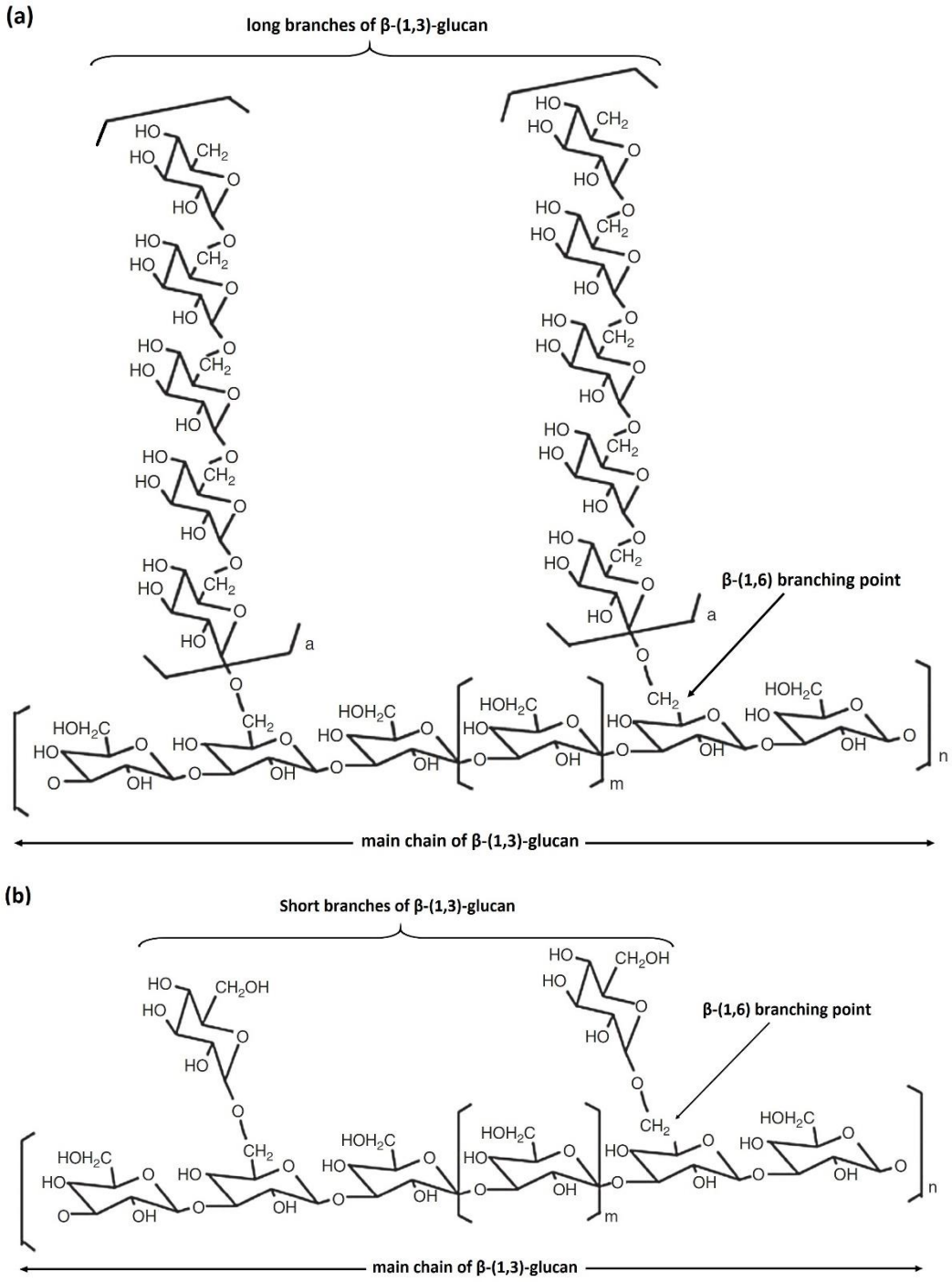

Figure 2.7 An example of the molecular structure of insoluble **(a)** and soluble **(b)** yeast β-glucans (edited) [37].

Table 2.2 Physico-chemical characteristics of dried yeast and yeast derivates [38].

Physico-chemical characteristics	Dried yeast	Yeast proteins	Yeast β-glucan	Yeast extract
Water absorption	+	+	++	
Fat binding		+	+	
Dispersibility		++	+	
Gelling properties		+	+	
Texture improving		+		
Forming fibbers		+		
Thickener			++	
Neutral flavour		+	+	
Flavour	+			+++
Flavour enhancer	+			+++

+ slightly, ++ very well, +++ excellent.

The solubility of yeast β-glucans also reveals an important aspect to be considered, since soluble and insoluble glucans stimulate the immune system through different biological pathways. Therefore, their combination may be a powerful immune-stimulate tool [43].

In addition, the chain length is an essential factor which determines the β-glucan application. Generally, a medium or large MW is related to a higher immune-stimulant activity, whereas a lower one is usually associated to food applications since they are more soluble and easier to disperse in a food matrix. A biological activity also exists in low MW glucans, although it is very low and limited. To highlight, it was shown high biological activity in fractions with a high MW, between 100 to 200 kDa, which exhibit a single and/or triple helix conformations, while a fraction from the same source with a low MW, in the range 5 to 10 kDa demonstrated a limited activity. Therefore, β-glucans which have the same MW differed significantly in their biological potency depending on their conformation [36,37].

The viscosity is directly proportional to the quantity of MW. The solubility and concentration are other parameters which affect the viscous nature of the β-glucans. From the technological point of view, β-glucans with a MW up to 3 x 10^3 kDa are larger molecules which have tendency to introduce high viscosity in the products. High viscosity is attributed to very strong intra- or intermolecular associations between the own molecule or other components of the product, respectively. Thus, the application of β-glucans with low viscosity and MW (~ 9 kDa) is more suitable for food preparations, as they can easily rearrange themselves due to less linkages [44].

Lastly, the isolation or extraction procedures (enzymatic digestion or chemical methods) of yeast glucans also have a great impact on their MW, degree of branching and polymerization, viscosity, composition and functionality [36,37].

2.3.2. Mannoproteins

Yeast cell wall mannoproteins are large mannose polysaccharide-protein complexes, containing about 95% (w/w) carbohydrate. These mannose polysaccharides are usually named as mannan-oligosaccharides (MOS) and are composed through several single residues of α-D-mannose (Figure 2.8), which are linked to protein in two different ways, as *N*- or *O*-glycosidic bonds. N-glycans consist of up to 50 α-D-mannose units composed by a long α-(1,6)-linked main chain, with short α-(1,2)- and α-(1,3)-linked side chains, forming a highly branched structure which contain as many as 200 mannose residues. This mannan oligosaccharide fraction is linked through an *N*-glycosidic bond between an *N*-acetylglucosamine (GlcNAc) and an asparagine (Asn) residue. Although, *O*-glycans comprise short chains of up to five α-D-mannose units, which are linked through an α-mannosyl bond with the hydroxyl side chains of serine (Ser) or threonine (Thr) residues [30,45–47].

Regarding the cell wall structure, mannoproteins can be linked either to β-(1,3)-glucan or to β-(1,6)-glucan through a glycosylphosphatidylinositol (GPI) anchor. The GPI anchor, made of a lipid and oligosaccharide units, is an important key mechanism to select proteins that contain a C-terminal signal sequence and serves to direct and localize these proteins to plasma membrane and cell wall [30,45,48].

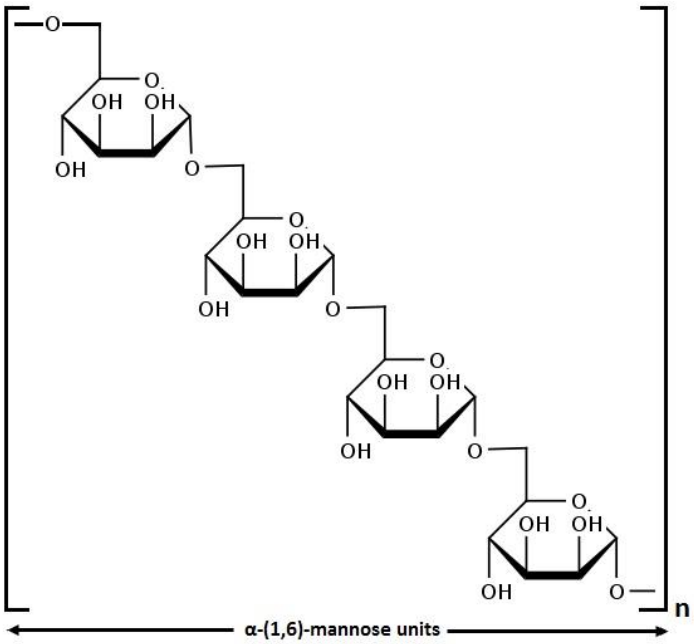

Figure 2.8 Struture of mannose units linked through α-(1,6) bonds (edited) [49].

The MOS composition is the responsible for the immune modulation activity, where each single unit of mannose represents a potential binding site for pathogenic bacteria, such as *Escherichia coli* and *Salmonella*, and for possible toxins produced from them. Thus, MOS have the capacity to block them and prevent their adhesion and colonization to the gastrointestinal tract, enhancing the growth rate, feed efficiency and live ability in animals. Apart from these positive properties, anti-oxidant, anti-mutagenic and anti-cancer activities were also demonstrated [39,50–52].

2.3.3. Chitin

Chitin is a linear polysaccharide of β-(1,4)-linked GlcNAc which accounts for only a small amount of the yeast cell wall by DW (1 to 2%) (Figure 2.9). The chitin chain length in the YCW is approximately 100 to 190 GlcNAc units, wherein about 40 to 50% of the chains are linked to non-reducing ends of β-(1,3)-glucan through a β-(1,4) bond which allows the connection between the reducing end of the chitin polymer [28,30,48].

The linear chains associate into microfibrils by forming hydrogen bonds between residues of adjacent residues. This leads to a crystalline structure which confers a stretching resistance to the cell wall, contributing to its overall integrity. Thus, a disruption of the chitin synthesis may lead to a disorderly of the cell wall which causes a deformation and osmotic instability to the yeast cells [30,47,48].

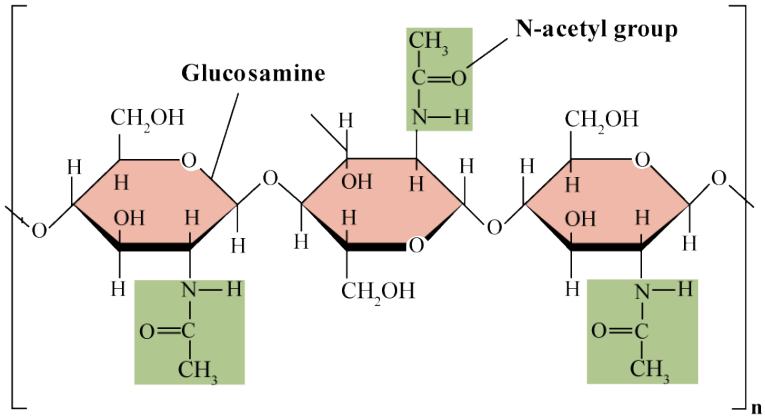

Figure 2.9 Molecular structure of chitin [53].

2.4. Ohly's products generated from the yeast cell walls

Ohly GmbH supplies YCWs as natural components derived from *S. Cerevisiae* and Torula (*Candida utilis*) yeasts for a wide range of customers in nutraceuticals, dietary, clinic, animal feed markets [1].

Auxoferm HCT is an Ohly's YCW product rich in a high molecular content of polysaccharides, such as glucan and mannan from the pure culture yeast of the species *S. cerevisiae*. After YE production through a gentle autolysis process, the cell wall fraction is purified and finally dried, obtaining a fine powder which presents a light beige appearance and is characterized by its distinctive yeast flavour [1]. The Auxoferm HCT specification sheets are attached in Appendix A.

The incorporation of animal feed products with Auxoferm HCT is recommended for aquaculture and young animals, where an activation of the immune system and improvement of the intestinal tract is required for growth and weight gain. Therefore, the YCWs are proper alternatives to antibiotics when these are not allowed or intended [1].

YCWs can either be use based on the immune effect or based on its functional properties. The latter allows applications as an agent for wine treatment, encapsulation of flavours and water-binding properties in different food systems. Moreover, Auxoferm HCT stands out due to the high content of the active component which also has a positive impact on the skin through cell regeneration and protection against the negative effect of UV exposition [1]

YCW products are usually sold for a low profit margin, therefore there is an opportunity to alter the YCW composition or structure in order to obtain new products with innovative applications. The main aim of the work described in this document was to use the Auxoferm HCT as the starting material for enzymatic reactions and altering its composition and structure, thereby understating what sorts of reactions were taking place and what types of unique hydrolysates could be obtain.

2.5. Carbohydrate-active enzymes

The carbohydrate-active enzyme (CAZyme) families constitute a group of enzymes which are able to breakdown or synthesize glycosidic bonds in complex carbohydrates and glyco-conjugates with high specificity. CAZymes are classified by different groups according the enzyme activities:

- *Glycoside hydrolases (GHs):* responsible for the hydrolysis of the glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety.
- *Glycosyltransferases (GTs):* involved in the biosynthesis of glycosidic bonds from phosphor-activated sugar donors, forming disaccharides, oligosaccharides and polysaccharides.
- *Polysaccharide lyases (PLs):* group of enzymes that cleave uronic acid-containing polysaccharide chains by a β-elimination mechanism to generate an unsaturated hexenuronic acid residue and a new reducing end [54–57].

In this thesis, the main used enzymes are included in the GHs and PLs classes and present different activities and side activities namely glucanases, mannanases, chitinases, proteases, pectinases, cellulases, amylases, hemicellulases, xylanases, lipases, galactosidases, cellobiases, glucoamylases, galacturonases, galactomannanases, glycosidases and galactosidases. The most efficient enzyme classes reported for YCW degradation are glucanase, peptidase, lipase and glucosidase [58].

In general, enzymes are proficient catalysts which have a myriad of industrial and biotechnological applications in textiles, pulp and paper, cosmetic, food processing, fine and bulk chemical and pharmaceutical areas. Enzymes present several unique properties, such as high substrate specificity (recognition of a particular substrate), high rate of catalysis, ability to perform with improved yield and reduced waste generation. Moreover, they are usually user-friendly as they catalyse reactions under mild conditions (e.g. temperature, pH and pressure), do not require protecting the functional

groups of the substrate, present longer half-life and higher enantioselectivity, which yields stereo- and regio-selective products [59,60].

In the food industry, enzymes have been used for the production of raw materials and subsequent final products enabling a solubility improvement, clarification and debranching of the end products [59,60].

2.6. Degree of polymerisation

In biochemical research on carbohydrates, namely oligo- and polysaccharides, the determination of the number-average molecular weight (MW_n) and degree of polymerisation (DP) is generally required. The MW_n provides a prediction of the colligative properties such as boiling and freezing temperature, whereas the DP is related with the polymer chain mobility, where larger chains have limited movement. The average length of β-glucans and mannans can be estimated through the DP, which is known as a key parameter to determine the average number of glucose and mannose for each β-glucan and mannan chain, respectively [61,62].

The number-average DP (DP_n) can be measured by membrane osmometry, cryoscopy, ebullioscopy, vapour pressure oscomometry, size exclusion chromatography or determination of reducing-end concentration. These established methods demand a long time for sample preparation, relatively large sample size, usage of volatile and/or toxic reagents as well as expensive and specialized instruments. Thus, an alternative approach is to divide the number of glucose and mannose residues by the number of reducing-ends. However, the assays required to determine these parameters present some disadvantages such as the destruction of the monomers if not well controlled temperature and duration and incomplete accessibility of chain ends [63,64].

β-glucans with a high DP (DP > 100), related with the content of side substituted chains, are completely insoluble in water. However, β-glucans with identical DP can differ significantly in the frequency and content of side branches, meaning that they present different solution conformations. A higher frequency of β-(1,6) branching points with low content of β-(1,3) side chains favours polysaccharide-solvent interactions and consequent hydration of molecules, therefore soluble in water (DP ~ 20). Thus, to improve β-glucans solubility and reduce their MW, DP and viscosity, a number of methods have been applied [36]. In this thesis, an enzymatic hydrolysis method was employed.

2.7. Potential applications derived from the yeast cell walls

The relation between health and nutrition has become an important and attractive field of research. Since there is a high competition in the food industry, manufacturers are always looking to develop new ingredients to decrease the cost of raw materials and, in addition, to offer new products in order to satisfy consumer tendencies. Currently, there has been interest in natural and healthy foods which contain low amounts of calories and fat, but with high dietary fibbers [38,65].

Some functional food products containing yeast β-glucans are already commercially introduced to the market (Table 2.3) and their number will progressively increase in the near future, since the USA's FDA has given yeast β-glucans a GRAS status [38,44].

Table 2.3 Commercially available yeast β-glucans [35].

Product name	Manufacturer	MW (kDa)
Glucan 300	Southeastern Pharmaceutical Company	-
Biorigin	Brazil Betamune Company	-
MacroGard	Norway Biotec-Mackyzmal Company	-
Immunowall	Brazil Betamune Company	-
β-Glucan	Sigma-Aldrich Company	-
Zymosan	Sigma-Aldrich Company	-
Zymosan A	BioParticles Company	-
Brewer's yeast extract	Cosfa International Trading Co., Ltd.	0.2
Yeast extract E100	Shanghai Youthretain Comapny	0.2
Natriance Brightener yeast extract	Vincience Company	0.04
Carboxymethly β-glucan C90	Angel yeast Co., Ltd.	404
Beta-glucan G70	Angel yeast Co., Ltd.	1000

As mentioned before, β-glucans have applications in human and veterinary medicine, feed production and pharmaceutical, cosmetic and chemical industries and food and feed production [35,38]. Table 2.4 summarizes the various applications of β-glucans.

Prebiotics are non-digestible dietary ingredients that beneficially affect the host by selectively stimulating the growth and/or activating the metabolism of health-promoting bacteria in the intestinal tract. These prebiotic agents are consisting of oligosaccharides which include MOS. Since the European legislation has forbidden all antibiotic growth promotors (AGP) in livestock feeds, MOS are an attractive alternative to antibiotics for improved growth and intestinal health in aquaculture, broiler chickens, turkeys and weanling pigs [8,50,66–68].

In addition, mannoproteins present a very essential role in wine characteristics and processing, whereby they enhance and interact with aroma compounds, providing the change of the sensory properties of wine. They act as natural inhibitors of potassium hydrogen tartrate crystallization, preventing the occurrence of precipitates in wine and thus potentially protect wine from protein haze. Moreover, mannoproteins stimulate the growth of lactic acid bacteria, which will support in the timely completion of malolactic fermentation [69–71].

Lastly, the glucans and mannans from *S. cerevisiae* as a potential infinite supply will dominate the market for the foreseeable future to satisfy the consumer trends with new innovative applications [36].

Table 2.4 Potential and current applications of β-glucans in different areas [35,38].

Area of application	Products	Functionality
Food	Prebiotic sausage formulation	Noticeable effect on physical (water-holding) and sensory properties
	Gluten-free bread	Acceptable results of sensory analysis
	Dairy products	Calorie-reduced and cholesterol-lowering
	Yogurts	Faster proteolysis, lower release of large peptides and a higher proportion of free amino acids
	Extruded ready-to-eat snacks	Manipulate the glycaemic response
	Beverages, including juices and dairy drinks	Control food intake and reduce 24h energy intake
	Cakes	Good quality attributes
	Salad dressings (creamy, vinegar, mayonnaise)	Calorie-reduced, cholesterol-lowering and emulsifying stabilizer
	Soups and sauces	Food thickeners with neutral flavour, characterized by a smooth and creamy mouthfeel, as fat replacers, dietary fibbers, emulsifiers and films
Medicine	Wound dressing material	Layer inner cavity diameter
	Transparent wound dressing sheet	Therapeutic efficacies comparable or superior to a commercial wound dressing
	Curing partial-thickness burns	Decrease post injury pain
	Curing burn-induced remote organ injury	Be effective against burn-induced oxidative tissue damage
	Poly-membranes	Accelerated wound healing effects
	Bone-substituting material	Easy manipulation and good adaptation to the shape and dimensions of even large bone defects
	Vaccine delivery platform	Can be exploited for vaccine development
Cosmetic	Film-forming moisturizer	Efficacy for reducing fine-lines and wrinkles
	Skin and dermatological compositions	Moisturization of skin or mucosa and anti-aging and revitalizing effect on skin
	Cosmetic product	Defer skin aging, impart skin whitening effect and cure skin damage effectively
	Cosmetic product	Treat collagen loss in aging skin
	Cosmetic product	Enhance ulcer healing and increase epithelia hyperplasia
Animal feed	Animal feed additive	Enhance immunity and as a potential antitumor agent
	Fish feed additive	Increase the number of specific antibody secreting cells and specific Ig levels in serum
Other health products	Personal care compositions	Hair care additives
	Novel prebiotics	Health-promoting property
	β-Glucan nanoparticles	Exhibit antifungal activity against *Pythium aphanidermatum*

CHAPTER 3

3. Enzyme screening of the yeast cell walls

3.1. Materials and Methods

3.1.1. Materials

Table 3.1, Table 3.2 and Table 3.3 list all the materials, reagents and equipment used for the analytical methods preformed as well as their respective manufacturers.

Table 3.1 List of materials, and respective manufacturers, used for the anlytical methods.

Material	Manufacturer
250mL Baffled conical flasks	Schott AG, DE
250mL Beakers	Schott AG, DE
50mL Conical centrifuge tubes	Nerbe plus GmbH, DE
1.5 and 2.5mL Disposable cuvettes	Brand GmbH + Co. KG, DE
2.5mL UV cuvettes	Brand GmbH + Co. KG, DE
1.5 and 2mL Reaction tubes	Brand GmbH + Co. KG, DE
100 and 500 mL Volumetric flasks	Brand GmbH + Co. KG, DE
96-welll sterilize plates	VWR International, LLC., USA
0.45µm sterile filters	Wicom Germany GmbH, DE
1.5mL vials	Wicom Germany GmbH, DE
10mL Pyrex screw cap tubes	--
20 and 100mL Duran bottles	Schott AG, DE
0.2µm sterile filters	Pall Corporation, USA

DE, Germany. USA, United States of America.

Table 3.2 List of reagents, and respective manufacturers, used for the anlytical methods.

Reagent	Manufacturer
2.5M Sulphuric acid	Carl Roth GmbH + Co. KG, DE
α-D-Glucose	Merck KGaA, DE
30% Potassium-sodium tartrate tetrahydrate	Sigma-Aldrich Co. LLC, USA
3,5-Dinitrosalicylic acid	Sigma-Aldrich Co. LLC, USA
D-Glucose kit	R-Biopharm AG, DE
D-Mannose, D-Fructose and D-Glucose kit	Megazyme, IE
Disodium hydrogen phosphate dihydrate	Merck KGaA, DE
Magnesium chloride hexahydrate	Carl Roth GmbH + Co. KG, DE
1M Sodium hydroxide	Carl Roth GmbH + Co. KG, DE
Glucose oxidase/catalase mixture (E-GOXCA)	Megazyme, IE
Pullulan (Shodex Standard P-82)	Showa Denko K. K., JP
β-(1,3)-laminaripentaose	Megazyme, IE
α-D-Mannose	Merck KGaA, DE
37% hydrochloric acid	Merck KGaA, DE

DE, Germany. IE, Ireland. USA, United States of America.

Table 3.3 List of equipment, and respective manufacturers, used for the analytical methods.

Equipment	Model/Version	Manufacturer
Magnetic stirrer	MR2002	Heidolph Instruments, DE
Moisture analyser	HB54-S Halogen	Mettler-Toledo AG, CH
pH Meter	pH-Meter 766	Knick, DE
Water baths	GFL 1012, 1086 and 1092	GFL Gesellschaft für Labortechnik mbH, DE
Precision scale	Quintix 5102-1CEU (d=10mg)	Sartorius Lab Instruments GmbH & Co. KG, DE
Heating immersion circulator	Julabo ED	Julabo GmbH, DE
Viscometer	Haake ViscoTester VT5^{R}	Thermo Fisher Scientific, USA
Centrifuge	Heraeus Multifuge X3R	Thermo Fisher Scientific, USA
Digital refractometer	--	TEC++ Dr. Volker Schmidt GmbH, DE
UV-VIS Spectrophotometer	DR 6000 and DR 2800	Hach, USA
Analytical scale	AX224 (d=0.1mg)	Sartorius Lab Instruments GmbH & Co. KG, DE
Vortex	--	VWR International, LLC., USA
Microplate reader	Epoch 2	BioTek Instruments, Inc., USA
Shaking incubator	INCU-Line ILS4	VWR International, LLC., USA
HPLC-SEC	Ultimate 3000	Thermo Fisher Scientific, USA
RI detector	L-2490	VWR International, LLC., USA
PROTEEMA SEC-column	--	PSS Polymer Standards Service GmbH, DE
Chromeleon software	6.8	Thermo Fisher Scientific, USA
Autoclave	VARIOKLAV Classic 400	H+P Labortechnik GmbH, DE
HPAE-PAD	DIONEX DX-120 Ion Chromatography	Thermo Fisher Scientific, USA
Dionex CarboPac PA100 column	--	Thermo Fisher Scientific, USA

CH, Switzerland. DE, Germany. USA, United States of America.

3.1.2. Methods

3.1.2.1. Enzymatic reactions

The YCWs produced from the autolysis process were used as the starting material or substrate for the enzymatic assays in the work described in this thesis. All the enzymatic assays were performed using the same batch of autolysed YCW product in powder form, Auxoferm HCT (see Appendix A for the product specification sheets).

A collection of 56 enzymes, mostly carbohydrases, proteases and lipases, were screened in this thesis (see Appendix B for the list of the screened enzymes).

In order to perform the enzymatic reactions, the substrate in powder form was dissolved with distilled water to 12% dry matter (DM) by using a magnetic stirrer. The DM of the starting material was confirmed by using a moisture analyser. Afterwards, pH was adjusted to 4.5 with 2.5M sulphuric acid by using a pH meter.

The enzymatic assays were developed in lab-scale by standardized parameters such as dry matter of the substrate, enzyme dosage, temperature, pH, shaking and reaction time. The pH and temperature values were selected according to the stability and activity profile of the enzymes screened.

All the enzymatic reactions were conducted with a total reaction weight of 200g in baffled conical flasks and an enzyme dosage of 1% (w/w) per substrate DM at 50°C and 120 rpm in a water bath. All the experiments were done in duplicates and 20g of representative samples were weighed in a precision scale and collected for further characterization at time intervals of 3 and 24h. The enzymatic reactions were stopped by heat inactivation at 80°C for 30min in a water bath. Table 3.4 lists all the variables and intervals tested for the YCW enzymatic screening.

Table 3.4 Enzymatic reaction conditions.

Dry matter	12%
Weight	200 g
Temperature	50°C
pH	4.5
Time	3 – 24h
Shaking	120 rpm
Dosage enzyme	1% (w/w) per substrate DM

3.1.2.2. Hydrolysates characterization

At the end of the enzymatic conversion, the hydrolysate products were characterized qualitatively, analytically and chemically in terms of viscosity, solubilisation and phase separation, turbidity, reducing and total sugars, free glucose and mannose contents and molecular weight (MW)

profiles. The following sub-sections will describe in detail the hydrolysates characterization methods performed.

3.1.2.2.1. Viscosity

After the 24h reaction and subsequently enzyme inactivation, the hydrolysate suspensions were transferred to a beaker, since a sample volume of approximately 200mL was needed to measure the viscosity.

The viscosity was measured for all the enzymatic reactions at 25°C in a water bath with a heating immersion circulator by using a rotational viscometer. Measurements were performed at a rotational speed range between 50 to 200 rpm, depending on the viscosity of the fluid, and with a coaxial spindle number R-2 which was immersed into the hydrolysate suspension in order to measure its resistance against the rotational speed assigned. The resulting torque or resistance measures the viscosity of the fluid in mPa·s. Readings were taken for every hydrolysate suspension until the indicator reached a constant value.

3.1.2.2.2. Solubilisation and phase separation

The solubilisation and phase separation were evaluated from 20g of representative samples collected at 3h and 24h reactions time. Thus, these samples were centrifuged at 4,700rpm, 25°C and 9 acceleration and braking curves for 20min. Afterwards, the pellet form was visually characterized in terms of solid or loose as well as the turbidity of the supernatant (SN). In addition, the DM of the SN was measured by using a digital refractometer. The volume of the SN was compared to the volume of the pellet and in between of the different points of the reaction, to evaluate the enzyme activity in relation to the hydrolysed amount released to the SN. The latter was taken to conduct further experiments on.

3.1.2.2.3. Turbidity

The turbidity of the SN samples was measured through a spectrophotometer at a wavelength (λ) of 660nm. All the SN samples were diluted to 1% DM with double-distilled water (ddH_2O) in order to obtain comparable absorbance values.

3.1.2.2.4. Reducing sugars content

The concentration of reducing sugars (RS) in the enzymatic hydrolysates was determined by the dinitrosalicylic acid (DNS) colorimetric method.

Calibration curve construction

A calibration curve was created using glucose as the standard sugar. Table 3.5 shows the different volumes of ddH_2O and 1% (w/v) glucose stock solution required for the construction of the calibration curve which were added in different reaction tubes. The sugar stock solution was prepared from 1g of α-D-glucose to 100mL of ddH_2O in a volumetric flask. Calibration samples were thereafter handled the same way as the hydrolysis samples.

Table 3.5 Different sugar concentrations for the construction of the calibration curve.

	Blank	**Calibration solutions**				
Vol. 1% (w/v) glucose stock solution	0.0	0.05	0.10	0.15	0.20	0.25
Vol. ddH_2O (mL)	1.0	0.95	0.90	0.85	0.80	0.75
Approx. sugar conc. (g/L)	0.0	0.5	1.0	1.5	2.0	2.5

Vol., volume. Approx., approximately. Conc., concentration.

Appendix C shows the various calibration curves used for the determination of RS, since a DNS solution was freshly prepared every time.

Preparation of the DNS solution

100mL of 2M sodium hydroxide solution was previously prepared in order to dissolve 30% potassium-sodium tartrate tetrahydrate by using a magnetic stirrer. 5g of 3,5-DNS was weighed by using an analytical scale and added to a 500mL volumetric flask. The solution previously prepared was added to the volumetric flask, which was then filled up with ddH_2O.

The solution was stirred overnight and, in case of the reagent was not completely dissolved on the next day, it was slightly heated up. The DNS reagent was stored in the freezer which allows a stability of approximately six months.

Measurement procedure

The concentration of RS was measured for the supernatant of the hydrolysate products in order to measure the amount of solubilised sugars.

Blank

For the DNS reaction, a blank was prepared by adding 0.2mL of ddH_2O and 0.5mL of DNS reagent in a reaction tube.

Reference samples

In order to avoid matrix effects caused by the turbidity of the samples, prior to DNS reaction, 0.2mL of each sample+DNS (without heating) was added to a disposable cuvette for later absorbance measurement.

Hydrolysis samples

SN samples from the hydrolysates were either undiluted or diluted to 1:2, 1:5 or 1:10 with ddH_2O. Afterwards, 0.2mL of the diluted sample and 0.5mL of DNS reagent were introduced in a reaction tube and mixed using a vortex. The reaction tubes were boiled for 5min at 100°C in a water bath and then cooled down in cool water for up to 10-15min. Afterwards, the samples were centrifuged at 3,000rpm, 25°C and 7 acceleration and braking curves for 5min in order also to avoid matrix effects caused by the turbidity of the samples. 0.2mL of each reaction tube was added in disposable cuvettes. The absorbance measurements were done to 1:10 dilution. Therefore, 1.8 mL of ddH_2O was added to each cuvette which contained the blank, hydrolysate and reference samples. The spectrophotometer was zeroed with the blank and the absorbance was measured at 530nm for all the samples. Double determinations were carried out in each sample and the measured values were within the range of the calibration curve. In order to remove the turbidity effect, the absorbance values of each sample were subtracted to its respective absorbance value of the reference.

3.1.2.2.5. Free glucose and mannose contents

Free glucose measurement

The free glucose content was estimated for the SN of the hydrolysate products by using an enzyme kit. This spectrophotometric method consists of a couple of enzymatic reactions.

The measurements were done following the procedure provided from the manufacturer. The absorbance was measured at 340nm either in UV cuvettes by using a spectrophotometer or in a microplate.

For the measurements done in a microplate, a calibration curve construction was required. Thus, a standard stock solution of 2.3g/L α-D-glucose was previously prepared. In different reaction tubes were introduced different volumes of the stock solution and ddH_2O. These different reaction tubes constitute the different standard concentrations needed for the construction of the calibration curve (Table 3.6).

Table 3.6 Different glucose concentrations for the construction of the calibration curve.

Standard	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Vol. ddH_2O (µL)	800	825	870	910	955	980	990	1000
Vol. stock solution (µL)	200	175	130	90	45	20	10	0.0
Approx. glucose conc. (g/L)	0.5	0.4	0.3	0.2	0.1	0.05	0.02	0.0

Vol., volume. Approx., approximately. Conc., concentration.

Subsequently, the enzyme suspension provided from the kit was diluted to 1:5 due to the small scale used for the measurements and to avoid absorbance values above 1. In addition, depending on the enzyme activity and the reaction time sample, the SN samples were undiluted or diluted to 1:2, 1:5, 1:10 or 1:20.

70µL of the buffer (provided from the kit), 110µL of ddH_2O and 10µL of the standard or diluted sample were pipetted in triplicates into each well of a 96-welll sterilize plate. Afterwards, the plate was introduced to a microplate reader (MPR) which already had a pre-set measurement program. The pre-set program consists in measure for 5min six kinetic cycles with shaking in the beginning for 120s and between cycles for 10s. Finished the first measured absorbance values (A_0), in each well was added 10µL of the previously diluted enzyme suspension and once again, the plate was introduced to the MPR. These last absorbance measurements (A_1) comprised 30min of 31 kinetic cycles with shaking in the beginning for 120s and between cycles for 10s.

Finally, all the data was exported and treated in an Excel file, where the concentration of free glucose was calculated based on the average of 10 stable cycles in each well for the A_0 and A_1 of each standard or diluted sample. According the Lambert-Beer's Law, the subtraction between A_1 and A_0 is proportional to concentration of free glucose.

Free mannose measurement

The free mannose content was also determined for the SN of the enzymatic hydrolysates with the use of an enzyme kit. This spectrophotometric method comprises several enzymatic reaction steps.

The absorbance readings were done at a λ of 340nm in disposable cuvettes by using a spectrophotometer.

The measurements were executed following the procedure provided from the manufacturer which, according to it, the differences between the absorbance values from the reaction steps should, as a rule, be at least 0.100 absorbance units to obtain reliable values. Since the SN of the hydrolysate products presented a higher concentration of free glucose than free mannose, this fact was not complied. Therefore, in order to achieve accurate results, an additional step of free glucose reduction was required before measuring the free mannose content.

Free glucose reduction

The previously additional step consisted to reduce the free glucose content of the SN samples using glucose oxidase/catalase reagent in the presence of atmospheric oxygen (O_2). The procedure method was based on the Megazyme D-Fructose/D-Glucose kit instructions, however some alterations were required due to the limited SN samples size.

The procedure began with the preparation of 250mL sodium phosphate buffer (300mM pH 7.6) plus 5mM $MgCl_2$. 13.36g of disodium hydrogen phosphate dihydrate was dissolved by stirring with 150mL of ddH_2O. 0.255g of magnesium chloride hexahydrate was added and dissolved in the previous solution. Afterwards, the pH was adjusted to 7.6 with 1M sodium hydroxide and the solution's total volume was filled up with ddH_2O.

Subsequently, the content of one vial of glucose oxidase/catalase mixture was dissolved in 20mL of the buffer previously prepared, which was then divided into 2mL aliquots and stored in the freezer. 0.5mL of the SN sample, 0.5mL of the buffer and 0.02mL of the enzyme solution were introduced in a reaction tube. As the atmospheric O_2 is important for the reaction, the reaction tubes were filled only half of their volume. All the reaction tubes were incubated at 25°C and 170rpm for 4h in a shaking incubator. Afterwards, to inactivate the glucose/catalase mixture, the reaction tubes were boiled for 10 min in a water bath.

Finally, according to the enzyme activity and reaction time sample, the reduced glucose samples were either undiluted or diluted to 1:5, in order to measure the free mannose using the enzyme kit.

3.1.2.2.6. Total sugars content

The total sugars content of the hydrolysate products was estimated by conducting an acid hydrolysis, which cleaves all glycosidic bonds in the oligosaccharides and releases D-glucose and D-mannose as single sugars (monosaccharides).

In order to determine the single sugars of the acid hydrolysed SN samples, a preparation of a mixture standard of D-glucose and D-mannose was required to identify them.

The acid hydrolysis method is applied for samples in solid form. Since the SN samples were in liquid form, they were prepared in order to have an end solution which corresponds to 100mg of 100% DM. Thus, some calculations were done for all the analysed samples applying the equation:

$$C_1 \times m_1 = C_2 \times m_2 \Leftrightarrow SN\ DM \times m_{SN} = 100\% \times 100mg \qquad \text{(Equation 1)}$$

where $SN\ DM$ represents the DM of the each SN sample and m_{SN} the needed weight to measure each SN sample in order to perform the acid hydrolysis reactions. Since D-glucose and D-mannose standards were in solid form (100% DM), this means that the standard mixture was constituted of 50mg from each standard sugars.

Both SN samples and standard mixture were weighed in duplicates in a 10mL Pyrex screw cap tubes. Subsequently, 1.5mL of 37% hydrochloric acid was pipetted into each tube, which were then

mixed with a vortex. The sample tubes were incubated at 30°C for 45min in a water bath and mixed once again in intervals of 15min by a vortex. Afterwards, content of each tube was transferred to a 100mL Duran bottle and all the tubes were washed with 40mL ddH_2O. The Duran bottles were placed in an autoclave and were autoclaved at 128°C for 60min. After cooling, the content of each Duran bottle was transferred to a 100mL volumetric flask and the bottles were washed with ddH_2O. The volume of the volumetric flasks was adjusted with ddH_2O. These volumetric flasks constituted the stock solutions of each SN sample acid hydrolysed.

In order to measure the total amounts of glucose and mannose from the acid hydrolysed samples, the standard mixture were diluted to 1:200 and the SN samples were diluted to 1:100 in 20mL volumetric flasks. Each resulting solution was filtered through a 0.2µm sterile filter to a vial. The vials were introduced in a high performance anion-exchange chromatography combined with pulsed amperometric detection (HPAE-PAD) and analysed by a carbohydrate method developed at Ohly GmbH. Carbohydrates were separated with a Dionex CarboPac PA100 column and the resulting chromatograms were analysed with the Chromeleon 6.8 software.

3.1.2.2.7. Estimation of the number-average degree of polymerisation (DP_n)

Once the RS, free glucose and mannose contents as well as the total glucose and mannose contents were determined, an estimation of the DP_n was performed. The difference between the total glucose content and the RS and free glucose contents and between the total mannose and the RS and free mannose contents represents the total oligo glucan and oligo mannan contents, respectively:

$$Total\ oligo\ glucan = Total\ glucose - RS - free\ glucose \quad \text{(Equation 2)}$$

$$Total\ oligo\ mannan = Total\ mannose - RS - free\ mannose \quad \text{(Equation 3)}$$

Since there was not an available equipment or method to specify the RS content in mannose and glucose, the estimated DP_n was considered as a conjugation of oligo-glucans and mannans. Therefore, through the following ratio, DP_n was estimated for the SN of the enzymatic hydrolysates:

$$DP_n = \frac{Total\ oligo\ glucan + Total\ oligo\ mannan}{RS - free\ glucose - free\ mannose} \quad \text{(Equation 4)}$$

3.1.2.2.8. Molecular weight analysis

The MW profiles were analysed for the SN of the enzymatic hydrolysates through a high performance liquid chromatography with size exclusion chromatography (HPLC-SEC) and a refractive index (RI) detector.

Each SN sample was diluted to 1:10 and filtered through a 0.45µm sterile filter to a vial, which was introduced in the HPLC-SEC. The carbohydrates separation was carried out by using a PROTEEMA SEC-column. The resulting chromatograms were analysed with the Chromeleon 6.8 software. In order to estimate the MW fraction of the samples, 1g/L of pullulan and 0.1g/L of β-(1,3)-laminaripentaose (MW=990.80 Da) standards were previously prepared and analysed on the HPLC-SEC (Table 3.7).

Table 3.7 Molecular weight and weight distribution of each grade of the pullulan standards.

Grade	MW x 10^{-4} [Da]	M_w/M_n
P-200	21.2	1.13
P-100	11.2	1.12
P-50	4.73	1.06
P-20	2.28	1.07
P-10	1.18	1.10
P-5	0.59	1.09

MW, molecular weight. M_n, number-average molecular weight.

Through the establish of MW and retention time (RT) intervals of the pullulan and β-(1,3)-laminaripentaose standards, the MW fraction was determined, based on the peak area in those intervals, of the SN samples.

3.2. Results

3.2.1. Viscosity

The viscosity values of the enzymatic hydrolysates from the screening shown to be in the range of 28 – 917 mPa·s (Figure 3.1).

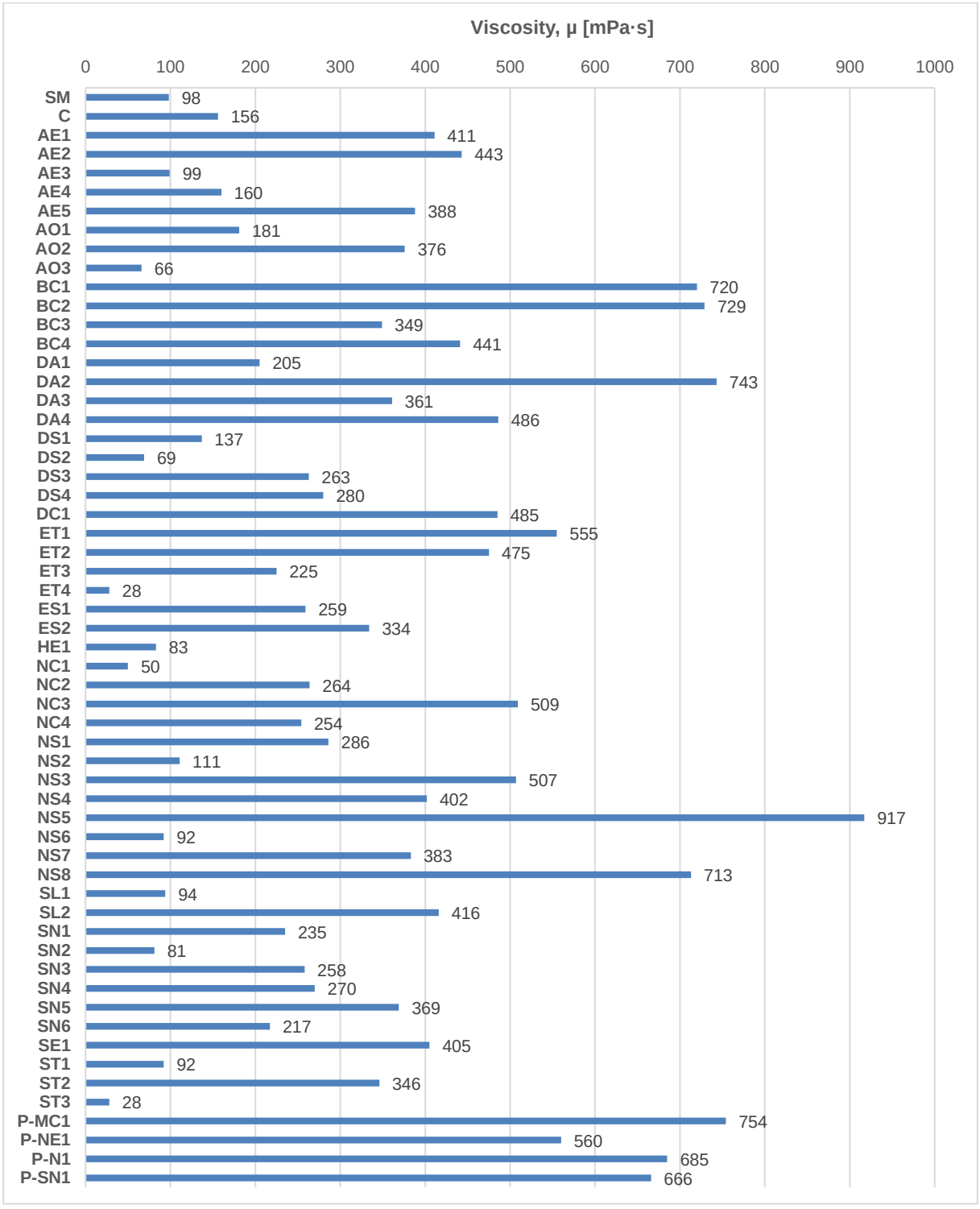

Figure 3.1 Viscosity profile of the enzymatic hydrolysates from the screening.

Comparing the obtained results for the starting material, SM, without incubation and the control sample, C, which was incubated without enzyme addition and with the enzymatic reactions, an increase of the viscosity value of the control sample was observed.

Another important observation is the increase and decrease of the viscosity of the enzymatic hydrolysates when compared with the SM or control. Lower viscosity values (28 – 137 mPa·s) were verified for some enzymatic hydrolysates, however most of the hydrolysate products displayed higher viscosity values (160 – 910 mPa·s).

3.2.2. Solubilisation and phase separation

A comparison between the volumes of the pellet and supernatant as well as determination of the dry matter (DM) of the supernatant (SN) and analysis of the pellet form were performed after separation of the 20g representative samples of the hydrolysates products (Table 3.8). In order to simplify, only the relevant enzymatic hydrolysates will be analysed in this section and the results of the remaining samples are attached in Appendix D.

Table 3.8 Comparison between the volumes of the pellet and supernatant after separation as well as the pellet form and dry matter of the supernant of the relevant enzymatic hydrolysates.

Enzymatic hydrolysate	Pellet form	Vol. Pellet [% v/v]	Vol. SN [% v/v]	SN DM [% w/w]	Pellet form	Vol. Pellet [% v/v]	Vol. SN [% v/v]	SN DM [% w/w]
SM	S	50	50	1.8	--	--	--	--
	3h				**24h**			
C	S	55	45	1.7	S	55	45	1.9
AE3	S	60	40	4.4	L	55	45	7
AO3	S	60	40	4	S	37.5	62.5	6
ET3	S	47.5	52.5	3.8	S	50	50	6.1
ET4	L	22.5	77.5	6.9	L	22.5	77.5	7.6
HE1	S	47.5	52.5	3.6	S	45	55	5.8
NC1	L	15	85	7.5	L	10	90	8.3
NS6	S	50	50	5.2	L	40	60	6.9
SL1	S	45	55	4.2	S	37.5	62.5	6.3
SN2	S	47.5	52.5	5.8	S	37.5	62.5	7.6
ST3	L	25	75	7.6	L	25	75	8.2

C, control. DM, dry matter. L, loose. S, solid. SM, starting material. SN, supernatant. Vol., volume.

Both starting material, SM, and control, C, presented similar values of DM of the SN as well as pellet form and volumes of the SN and pellet. The control sample showed a DM of the SN of 1.7% (w/w) and remained constant over reaction time. When compared with the enzymatic hydrolysates, an increase of the DM of the SN was observed.

The pellet form was classified as solid or loose, wherein the latter refers to unstable and liquefied pellet. Most of the enzymatic hydrolysates presented a solid pellet. After 24h of enzyme treatment, AE3 and NS6 presented a loose pellet. Also three of the hydrolysate products presented in the previous table (ET4, NC1 and ST3) exhibited a loose pellet from 3h to 24h reaction time and a high volume and DM of the SN which remained mostly constant over reaction time.

Usually, in most of the hydrolysates products, an increase of the volume of the SN was observed, which leads to an increase of solubilised content and also of the DM of the SN.

Figure 3.2 shows the 20g of representative samples of the enzymatic hydrolysates after phase separation. From 3h to 24h of each hydrolysate, some differences in the turbidity and colour intensity of the SN were observed. The hydrolysates AE3, AO3, ET3, NS6 and SL1 presented a light and non-turbid SN as the control at 3h, however at 24h reaction time a completely turbid SN was visualised. A colour intensity of the SN was observed after 24h reaction time for the hydrolysates HE1 and SN2. In the case of the three hydrolysates previous referred (ET4, NC1 and ST3), a distinctive and different turbidity and colour of the SN were verified. The SN presented a milky colour, the volume of the pellet was very minimal and difficult to distinguish it when compared with control or other hydrolysates.

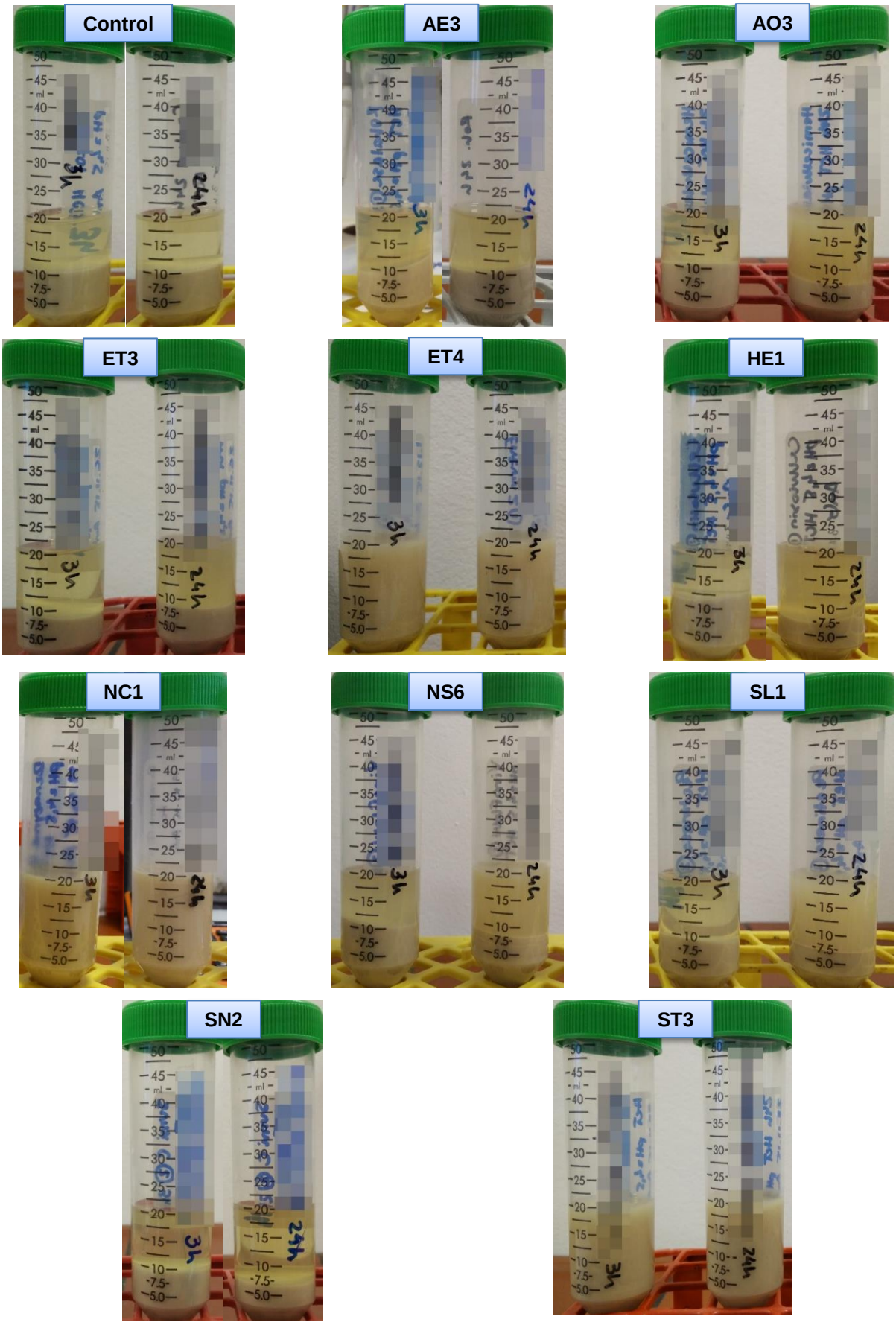

Figure 3.2 Phase separation of the 20g representative samples of relevant enzymatic hydrolysates.

3.2.3. Turbidity

As stated before, some SNs of the obtained hydrolysates showed to be turbid. Thus, Table 3.9 presents the turbidity, in absorbance values, of the SN of the hydrolysate products.

Table 3.9 Absorbance values at 660nm, corresponding to the turbidity of the supernatant of the hydrolysate products.

Enzymatic hydrolysate	SN DM [%]	Abs. (λ = 660nm)	
		3h	24h
C	1	0.072	0.082
Usual turbidity		[0.063, 0.099]	[0.063, 0.082]
AE3		0.070	0.120
AO3		0.080	0.341
ET3		0.058	0.270
NS6		0.097	0.899
SL1		0.068	0.656
ET4	0.25	0.776	0.916
ST3	0.1	0.705	0.947

Abs., absorbance. DM, dry matter. SN, supernatant. Vol., volume.

The turbidity of the SN was measured for the control, as a base of comparison, and for most of the samples, which showed a similar turbidity to the control, the absorbance values were in the range of 0.063 – 0.099 for 3h and 0.063 – 0.082 for 24h.

From the turbid SN samples mentioned before (AE3, AO3, ET3, NS6 and SL1), the hydrolysates NS6 and SL1 shown the most turbid SNs both at 3h and 24h, when compared with the other hydrolysates at 1% of SN DM. The hydrolysates which presented a milky SN (ET4, NC1 and ST3), an absorbance value above 1 for 1% of SN DM was observed. In order to obtain absorbance values over 1, the SNs of these hydrolysates were diluted to 0.1 and 0.25% of DM. Since the absorbance values were similar between the hydrolysates ET4 and ST3 at 3h and 24h reaction time, the turbidity of the hydrolysate NC1 was not measured as the same results were expected.

3.2.4. Total reducing sugars, free glucose and mannose contents

Once the RS, free glucose and mannose contents were measured for all the SNs of the enzymatic hydrolysates at time intervals of 3h and 24h, the results were plotted in the following Figure 3.3 and Figure 3.4, in percentage of amount of total sugar in the SM. The total glucose and mannose contents in the SM was previously determined through acid hydrolysis, corresponding to 23.9% (w/w) and 29.5% (w/w), respectively. The respective standard deviation results of the RS, free glucose and mannose measurements are attached in Appendix E.

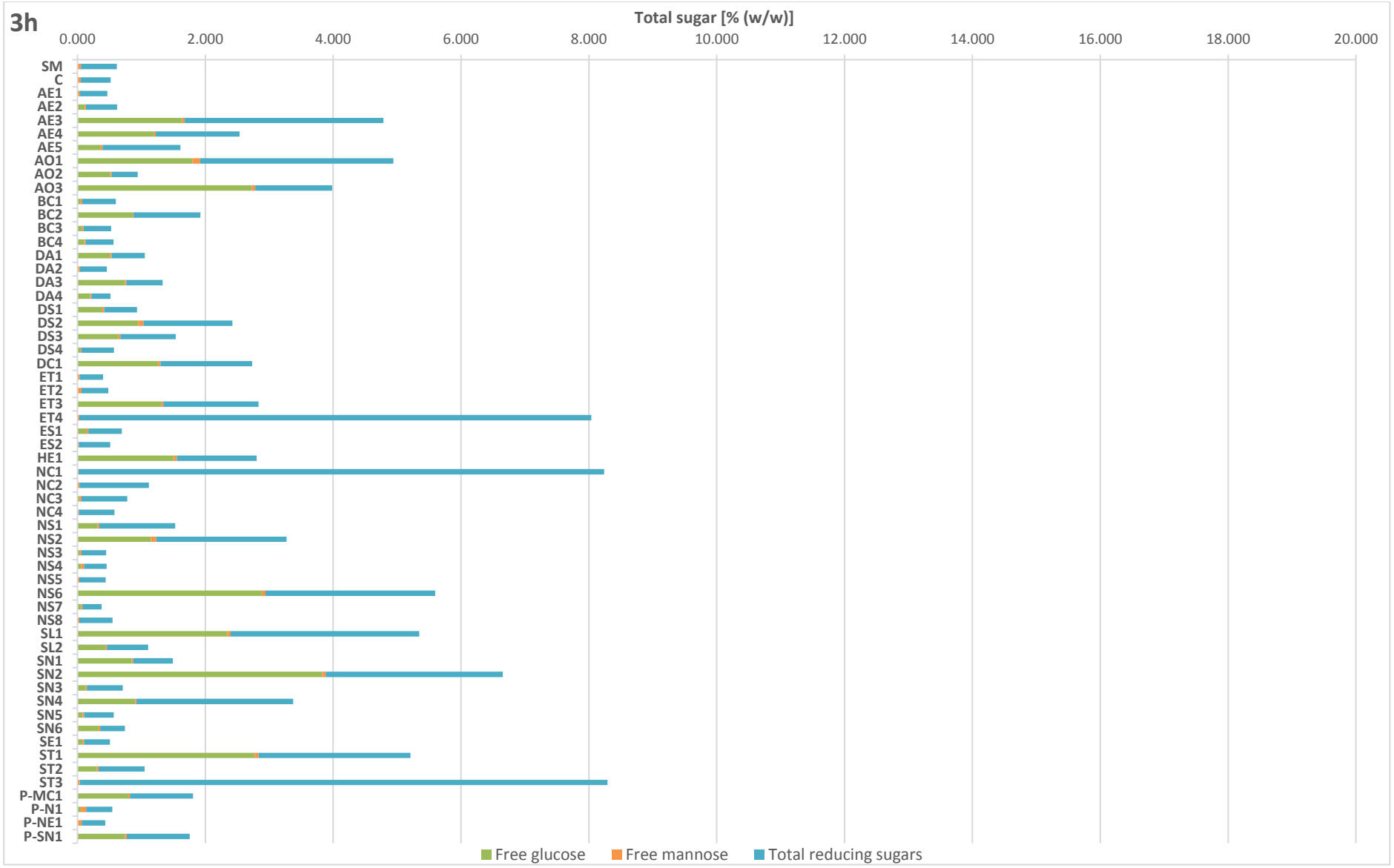

Figure 3.3 Reducing sugars, free glucose and mannose contents in the supernatant of the enzymatic hydrolysates at 3h reaction time.

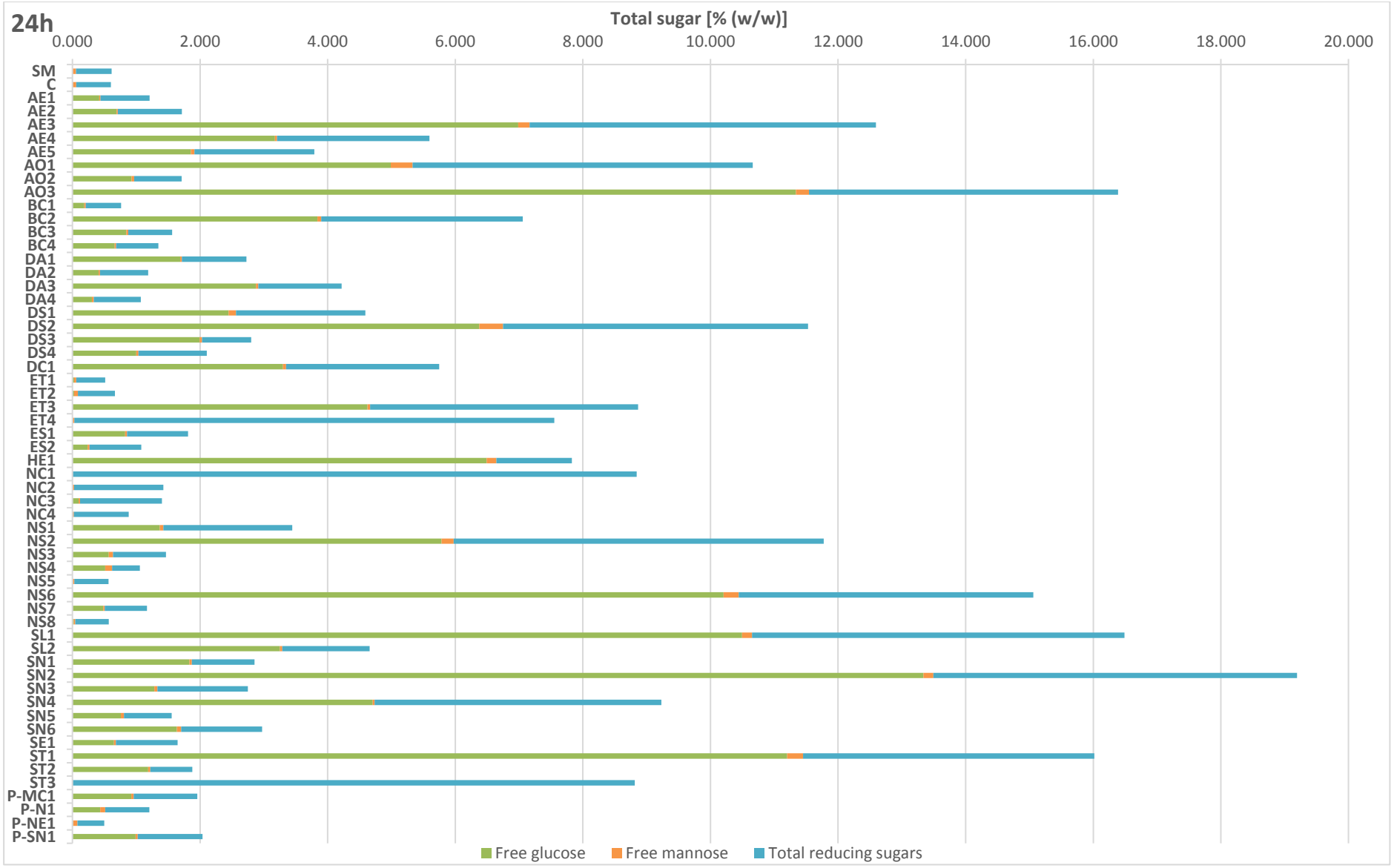

Figure 3.4 Reducing sugars, free glucose and mannose contents in the supernatant of the enzymatic hydrolysates at 24h reaction time.

The sugar release at 3h reaction time was shown to be in the range of 0 – 8.5 % (w/w) and at 24h between 0 – 19.5% (w/w).

The control sample, C, which was incubated without enzyme addition but under the same conditions as the other hydrolysate samples, presented a constant release of RS and free mannose in the SN as well as the starting material, SM, which was not submitted to incubation.

Three hydrolysate products, ET4, NC1 and ST3, stand out of the rest of the hydrolysates in their ability to non-release of free glucose, presenting a release of RS of 8% (w/w) at 3h and between 7.5 to 9% (w/w) in total sugars at 24h reaction time. The hydrolysates AE3, AO1, AO3, DS2, ET3, NS2, NS6, SL1, SN2, SN4 and ST1 demonstrated a high release of RS and free glucose of around 2.5 to 7% (w/w) at 3h and 9 to 19% (w/w) in total sugars at 24h reaction time. However, more than half of the enzymatic hydrolysates displayed a release of RS and free glucose equal or less of 2% (w/w) in total sugars both at 3h and 24h. For both 3h and 24h reaction time, all the enzymatic hydrolysates exhibited a reduced release of free mannose.

3.2.5. Total sugars: oligo-glucan and mannan contents

Through acid hydrolysis of the SNs of the enzymatic hydrolysates and analysis by HPAE-PAD, the total sugar, oligo-glucan and mannan contents, at reaction time of 3h and 24h were determined as in percentage of amount per DM, wherein the results are presented in Figure 3.5, Figure 3.6, Figure 3.7 and Figure 3.8. Appendix F contains the standard deviations of the total glucose and mannose results obtained by HPAE-PAD.

The acid hydrolysis was performed for mostly of the hydrolysates, since only six hydrolysates (AO2, ET1, ET2, NC4, NS5 and P-NE1) did not present any release of sugars or RS.

Since from the last results, the control at 3h and 24h presented similar results, the determination of oligo-glucan and mannan was only executed for the control at 24h reaction time. The starting material, SM, and the control sample, C, displayed similar values of oligo-glucan released in the SN. However, the oligo-mannan content released was higher for the control at 24h which was incubated together with the other enzymatic reactions.

All the hydrolysates, both at 3h and 24h reaction time, presented always a high oligo-mannan content than oligo-glucan released. The oligo-glucan content increased over reaction time from 3h to 24h. The oligo-mannan content also increased over reaction time, however, for AE3, BC2, BC3, DS2, ET3, ET4, ES2, HE1, NC1, NC2, NS1, NS3, NS6, SL2, SE1, ST3 and P-MC1 hydrolysates remained mostly constant and for AE4, AO1, AO3, NS2, SL1, SN2, SN4 and ST1 hydrolysates decreased over reaction time.

The ET4, NC1 and ST3 showed the highest oligo-glucan content which stayed constant over reaction time.

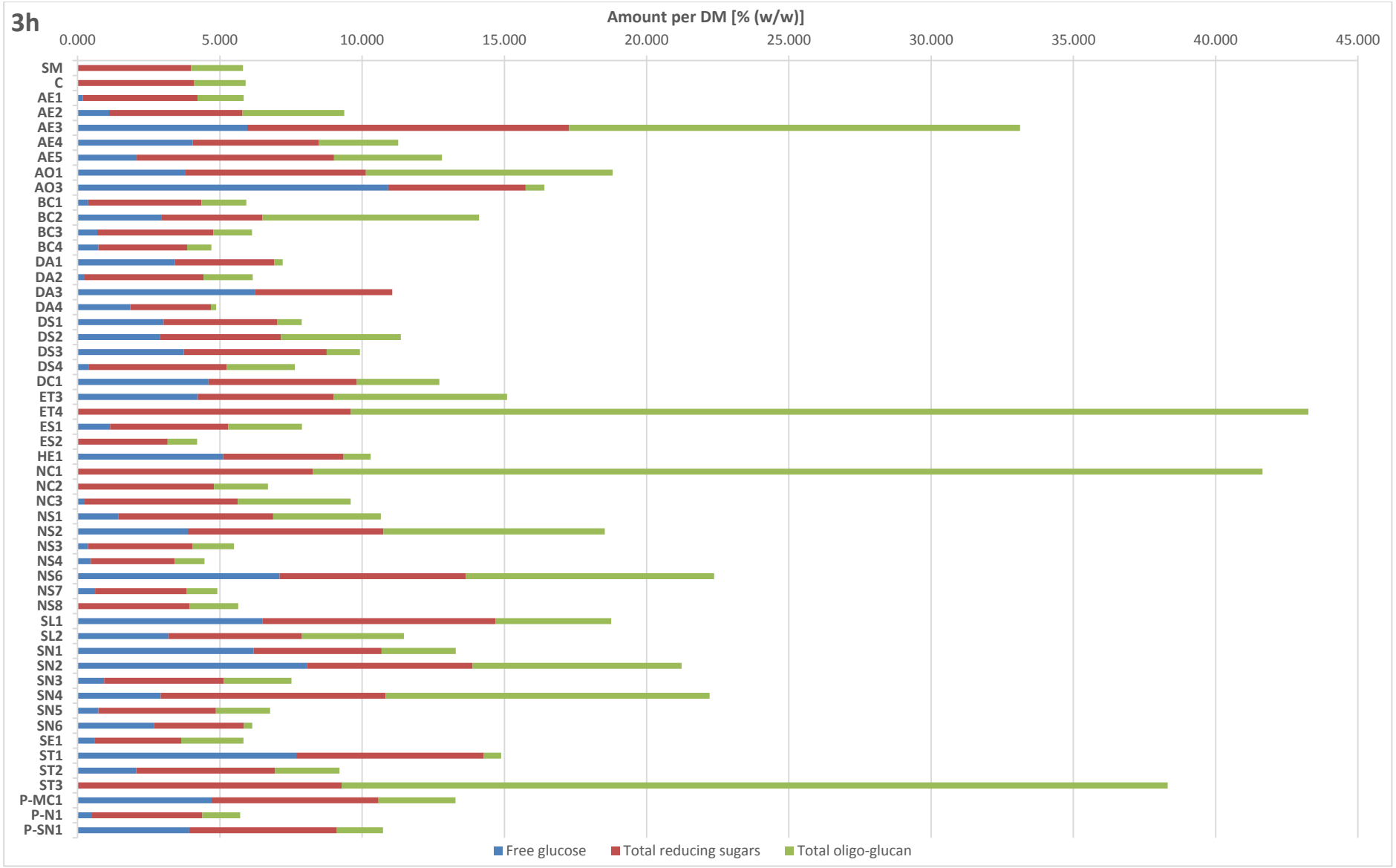

Figure 3.5 Comparison between the total RS, free glucose and total oligo-glucan contents at 3h reaction time.

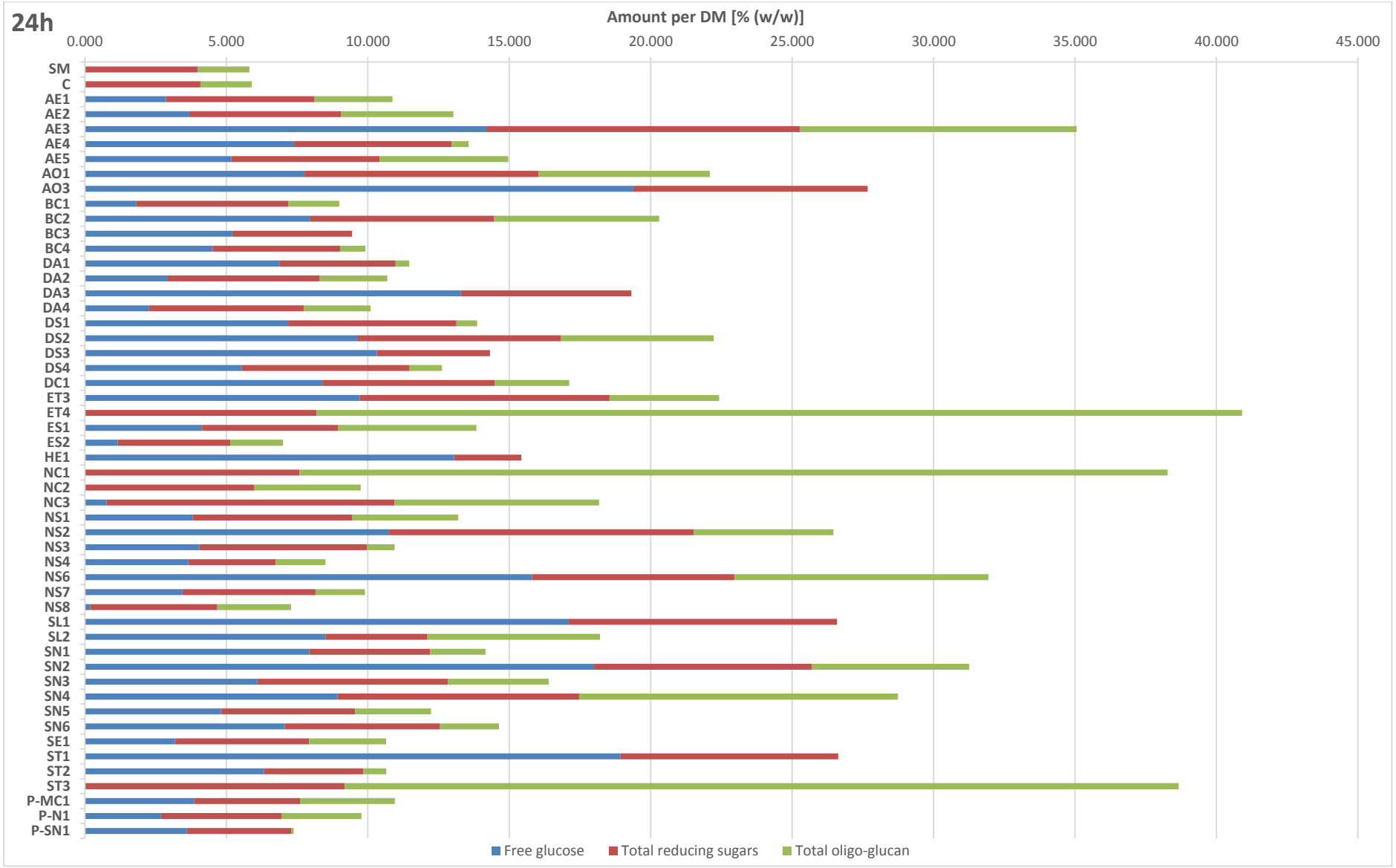

Figure 3.6 Comparison between the total RS, free glucose and total oligo-glucan contents at 24h reaction time.

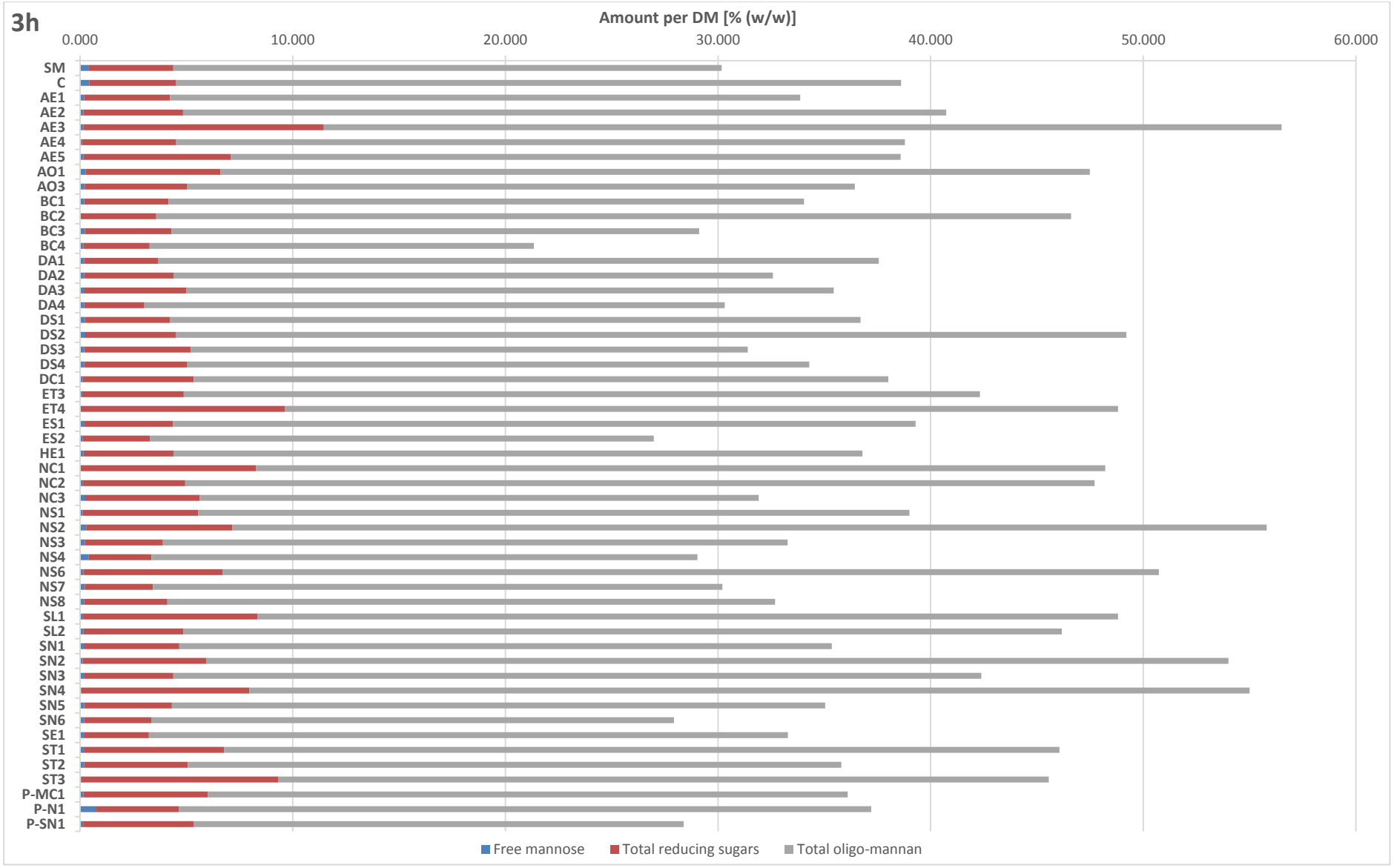

Figure 3.7 Comparison between the total RS, free glucose and total oligo-mannan contents at 3h reaction time.

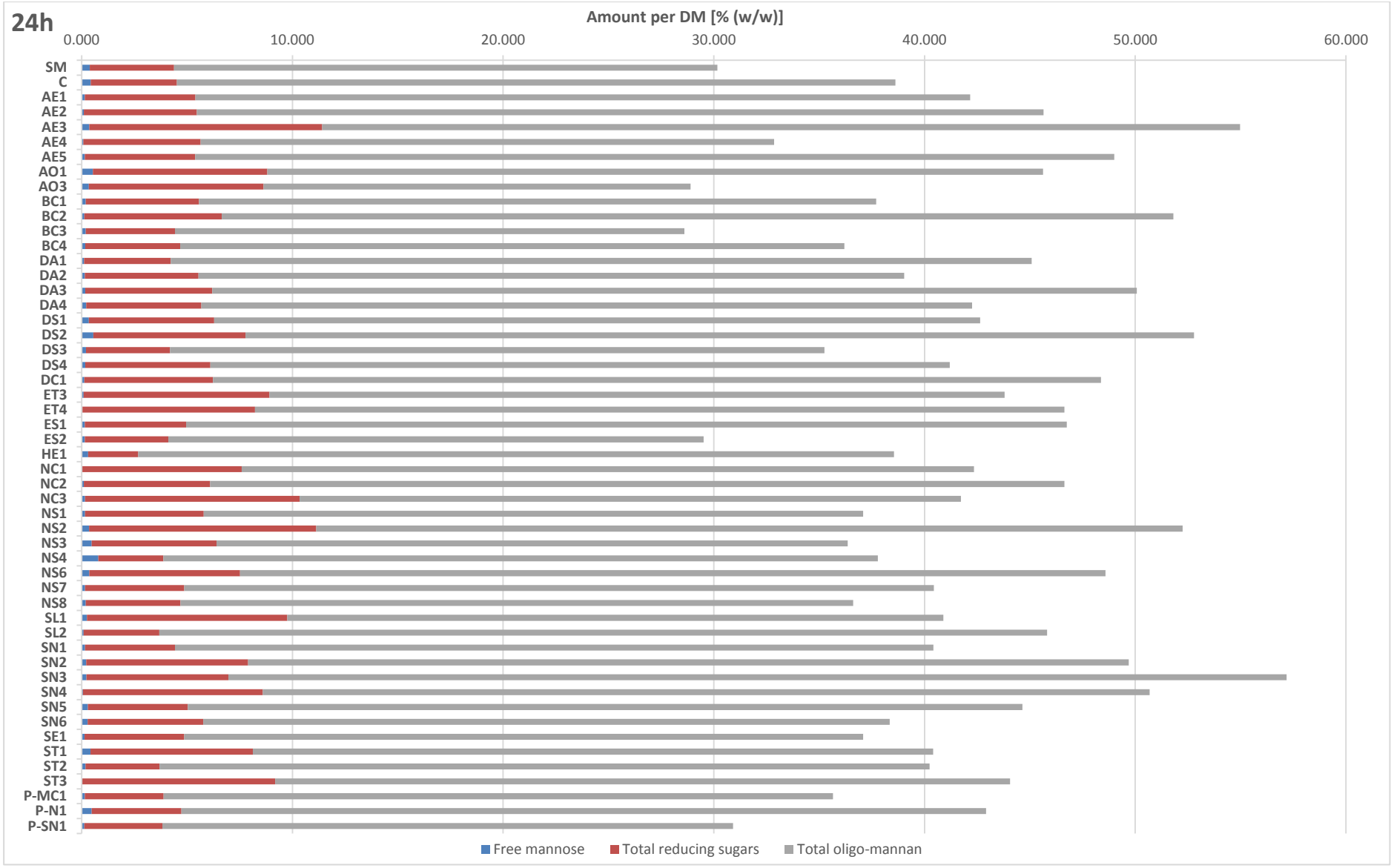

Figure 3.8 Comparison between the total RS, free glucose and total oligo-mannan contents at 24h reaction time.

3.2.6. Number-average degree of polymerisation (DP_n)

From the previous results, oligo-glucan and mannan contents released in the SN of the enzymatic hydrolysates at time intervals of 3h and 24h, the DP_n was estimated through the Equation 4 (Table 3.10). To simplify, only the hydrolysate products with a relevant release of oligo-glucan, which were also submitted to a molecular weight analysis, will be review in this section. Appendix G contains the DP_n values of the remaining hydrolysates.

Table 3.10 DP_n values for the SN of the enzymatic hydrolysates at 3h and 24h reaction times.

Enzymatic hydrolysate	DP_n	
	3h	24h
AE3	5.38	4.83
AE5	5.09	9.19
AO1	7.80	5.18
BC2	14.26	7.81
DS2	11.52	6.99
ET3	9.11	4.39
ET4	7.58	8.69
NC1	8.87	8.63
NS1	6.85	6.22
NS2	8.21	4.27
NS6	8.07	7.00
SL2	9.55	13.44
SN2	9.50	6.16
SN4	7.39	6.25
ST3	7.03	7.01

DP_n, number-average degree of polymerisation.

The DP_n values decreased over reaction time from 3h to 24h of most. However, for AE3, ET4, NC1, NS1, NS6, SN4 and ST3 hydrolysates the DP_n maintained constant and for AE5 and SL2 hydrolysates increased over reaction time.

3.2.7. Molecular weight analysis

The molecular weight (MW) for the SN of the hydrolysates, which were previously determined the DP_n, was analysed by HPLC-SEC. The following figures present the resulting chromatograms for the hydrolysates as well as the pullulan and β-laminaripentaose standards and the respective MW fraction results.

All the samples present a similar separation profile between 5 to 8.5min and from 12 to 14min retention time (RT). Through the comparison of the RT of the standards, all the samples exhibited peaks in a range of 212 to 23kDa and soon after the β-laminaripentaose peak which presents a MW of 0.99kDa. Thus, the peak after β-laminaripentaose may corresponds to free glucose released. The peaks

in the range of 7 to 8min and from 12 to 14min displayed a low height, which suggest to a not well separation of the components as compared with the first peak at around 6 to 7min.

Since the chromatograms were evaluated based on the area of the peaks, the resulting peaks will be analysed and compared in terms of area. Relative to the first peak, the area increases over reaction time in most of the hydrolysates. However, in the AE3, BC2, NS2, NS6, SN2 and SN4 hydrolysates, the area of this peak increases slightly over reaction time. The ET4, NC1 and ST3 hydrolysates presented similar area results of the first peak as well as an identical separation profile. In addition, the peaks at 7 to 8min with a low height observed in the other chromatograms, were not verified and from 12 to 14min, two well defined peaks were verified, wherein the first come out peak presented a similar RT as the β-laminaripenatose standard. All the referred evaluations are also confirmed by the analysis of the graphical representations of the MW fraction results.

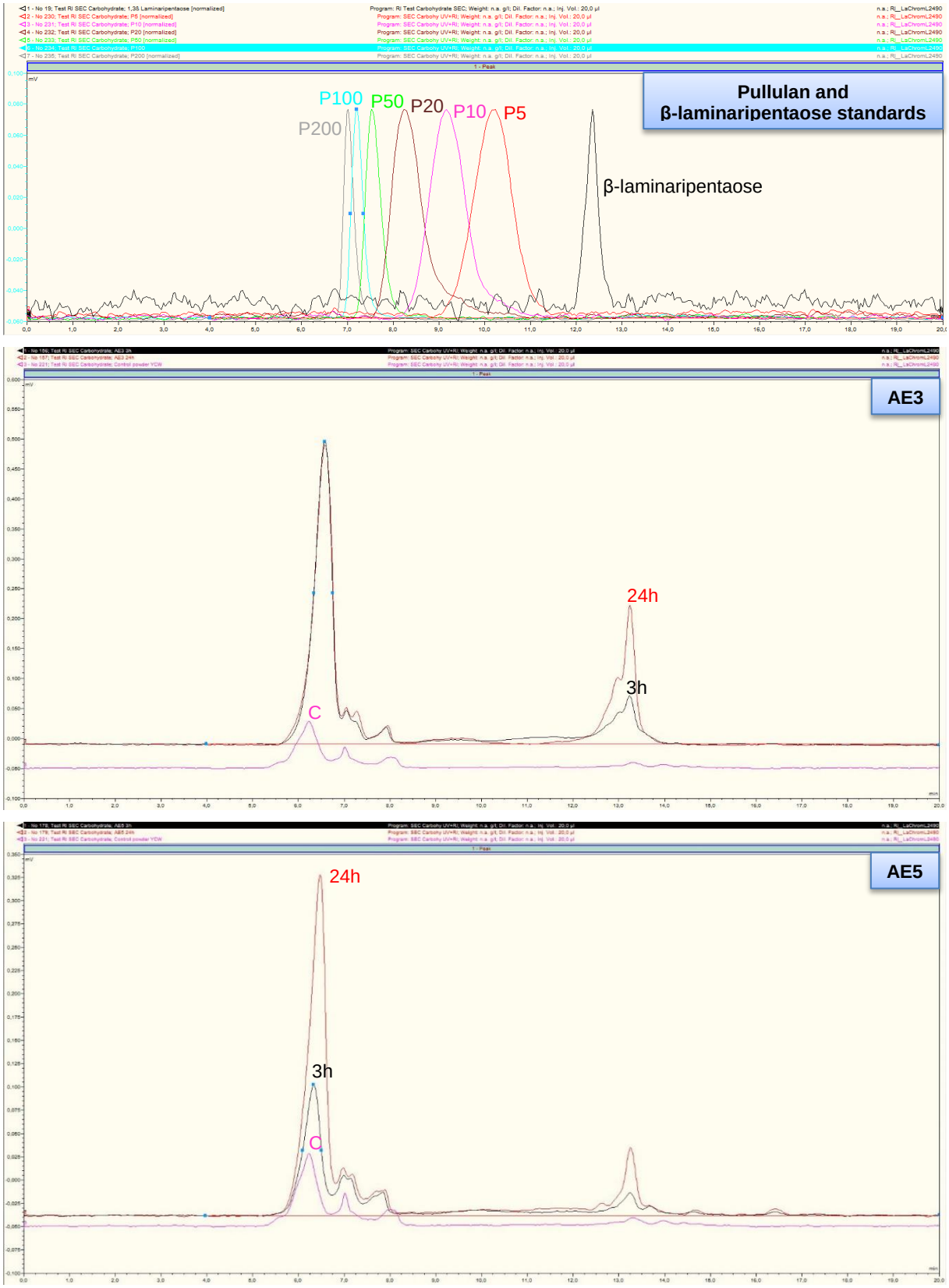

Figure 3.9 Chromatograms of the pullulan and β-laminaripentaose stardards, AE3 and AE5 hydrolysates over reaction time and comparison with the control sample, C.

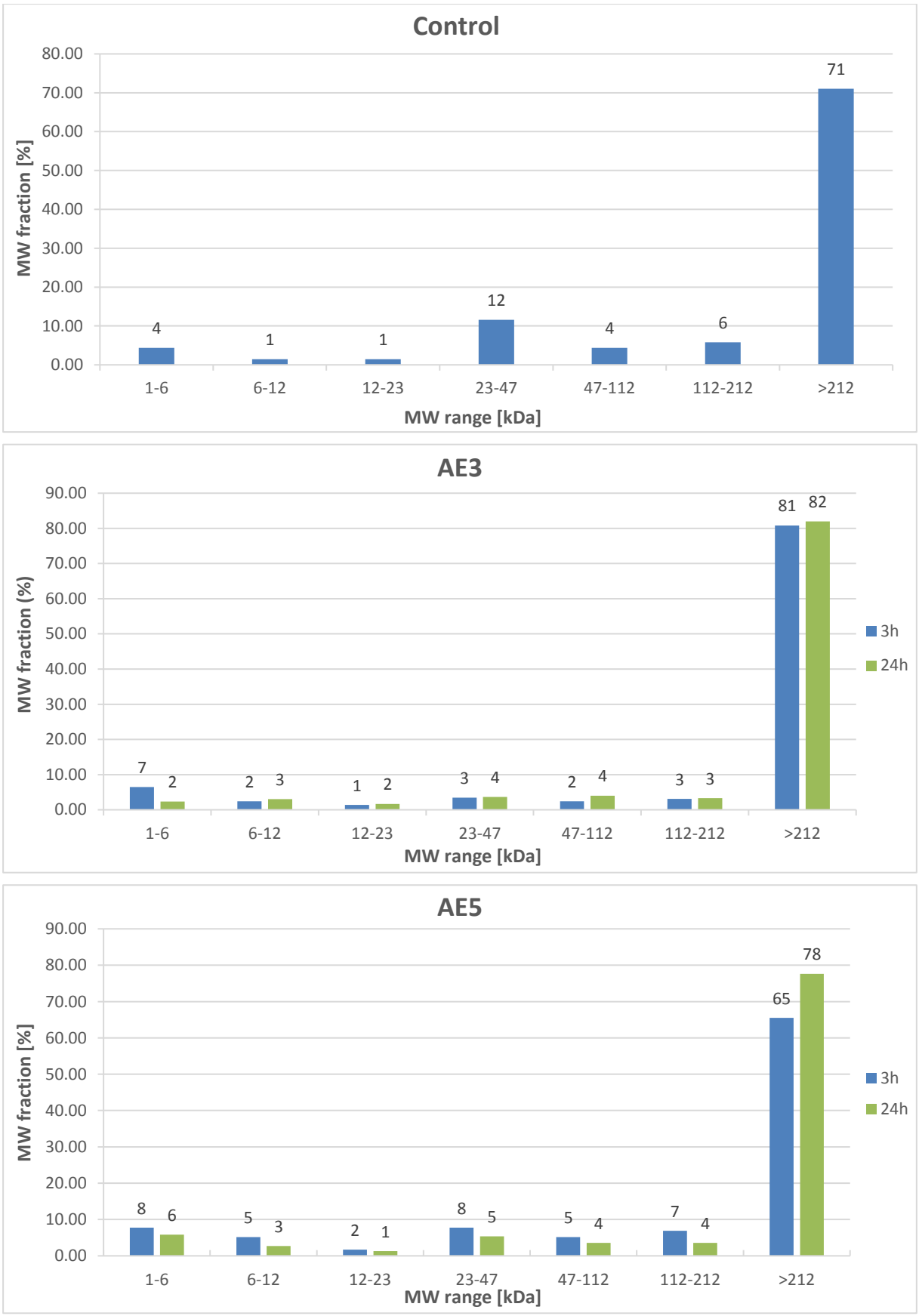

Figure 3.10 Molecular weight (MW) fraction results in the MW range of the standards for the control, AE3 and AE5 hydrolysates over reaction time.

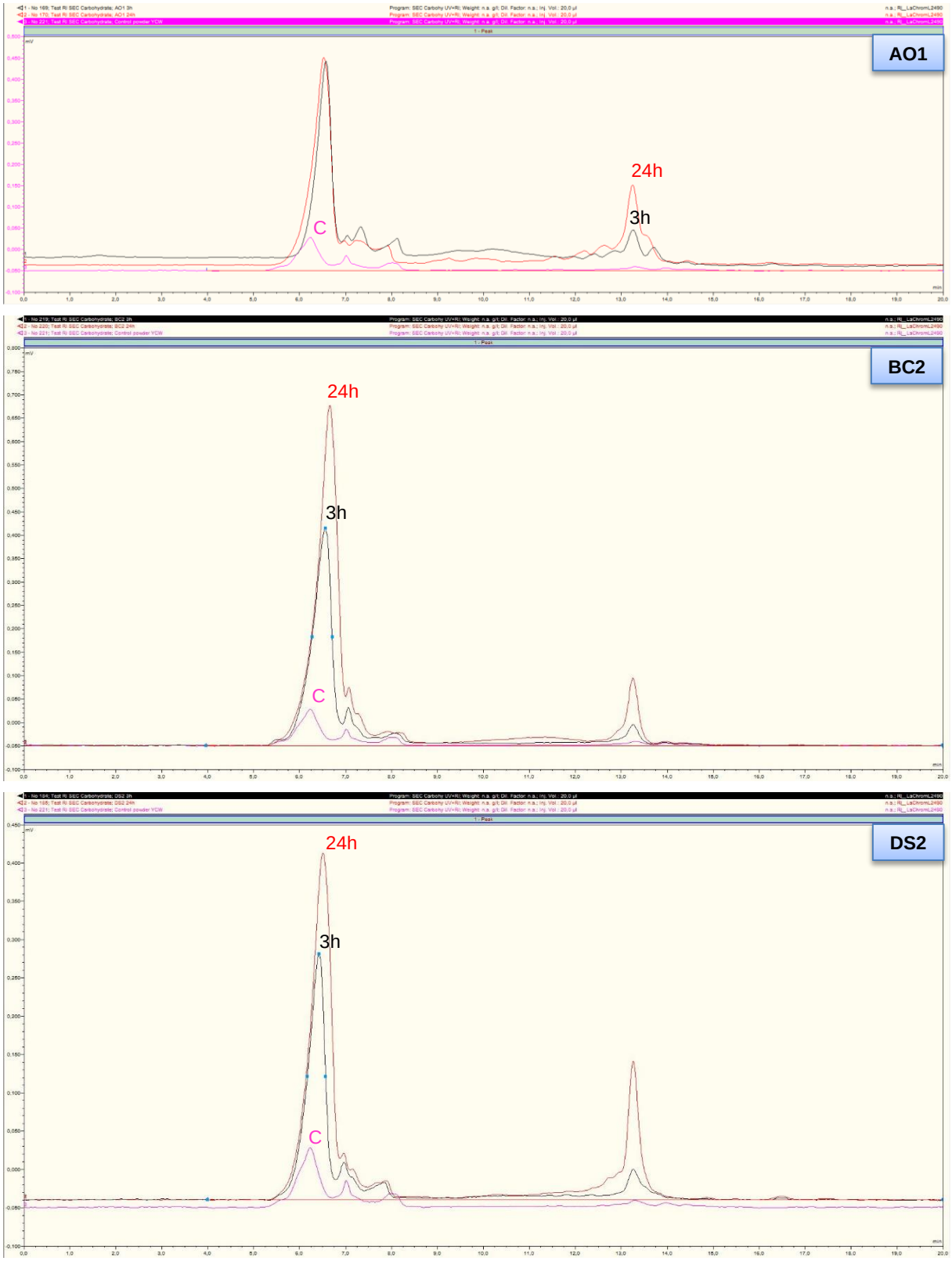

Figure 3.11 Chromatograms of the AO1, BC2 and DS2 hydrolysates over reaction time and comparison with the control sample, C.

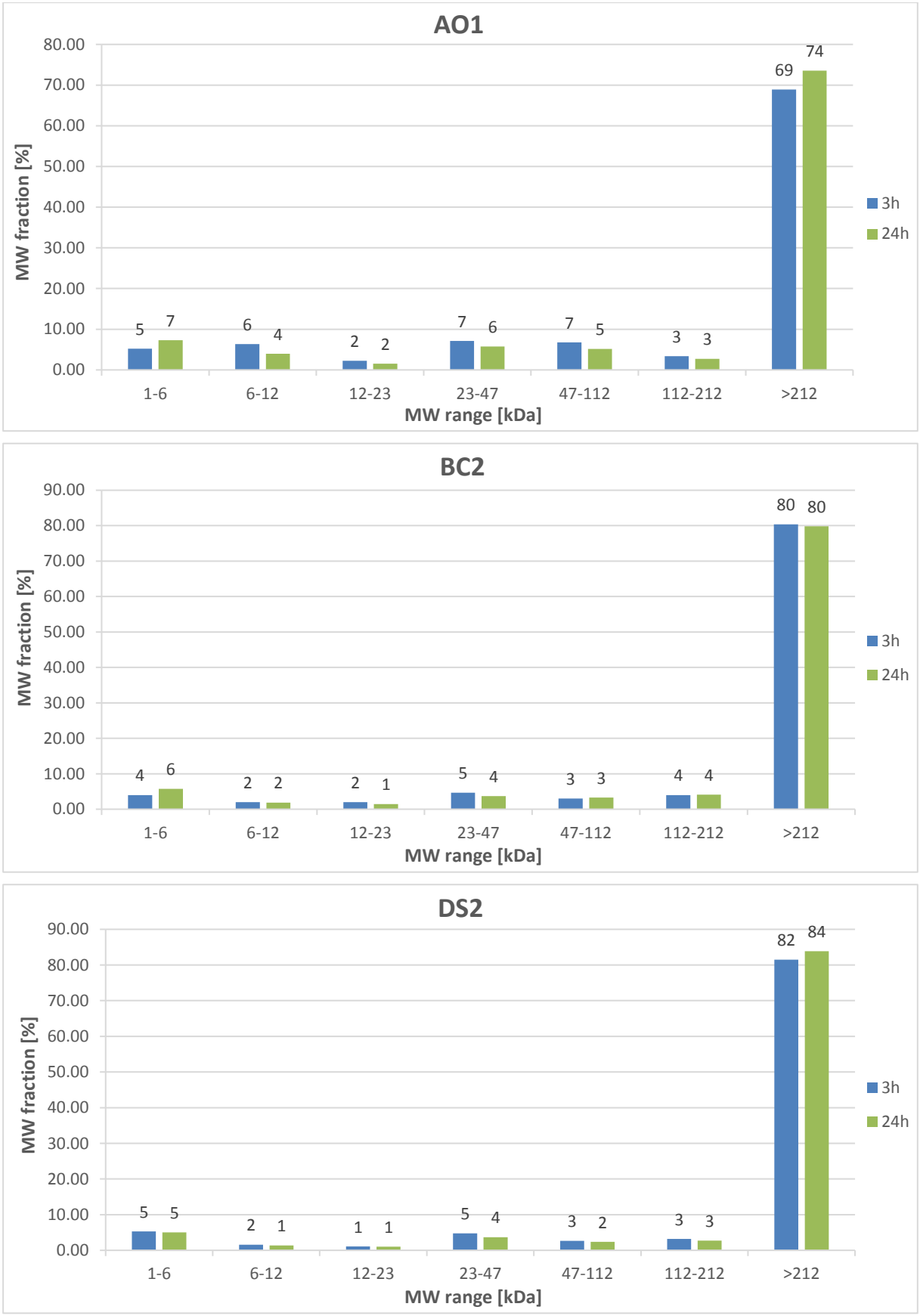

Figure 3.12 Molecular weight (MW) fraction results in the MW range of the AO1, BC2 and DS2 hydrolysates over reaction time.

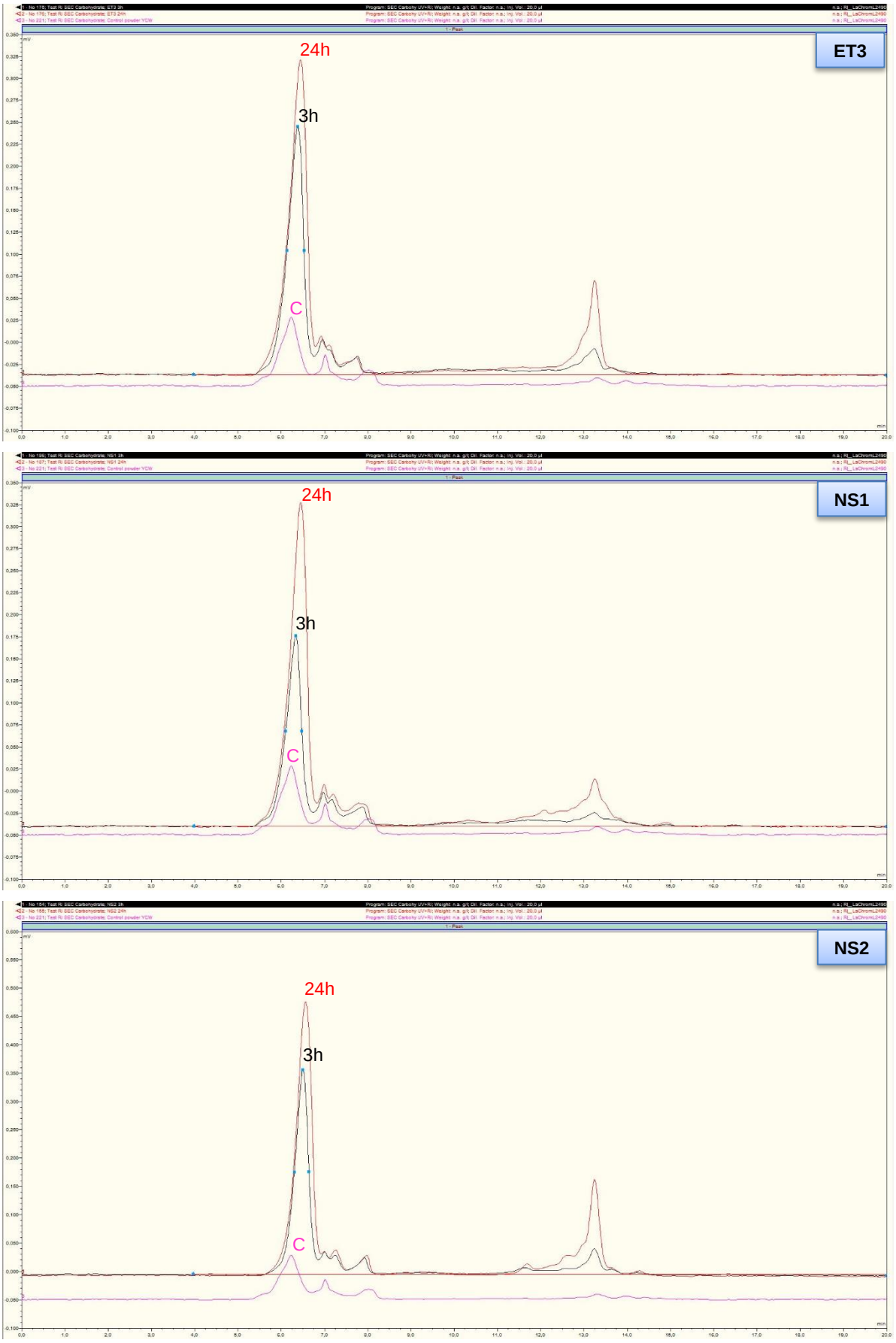

Figure 3.13 Chromatograms of the ET3, NS1 and NS2 hydrolysates over reaction time and comparison with the control sample, C.

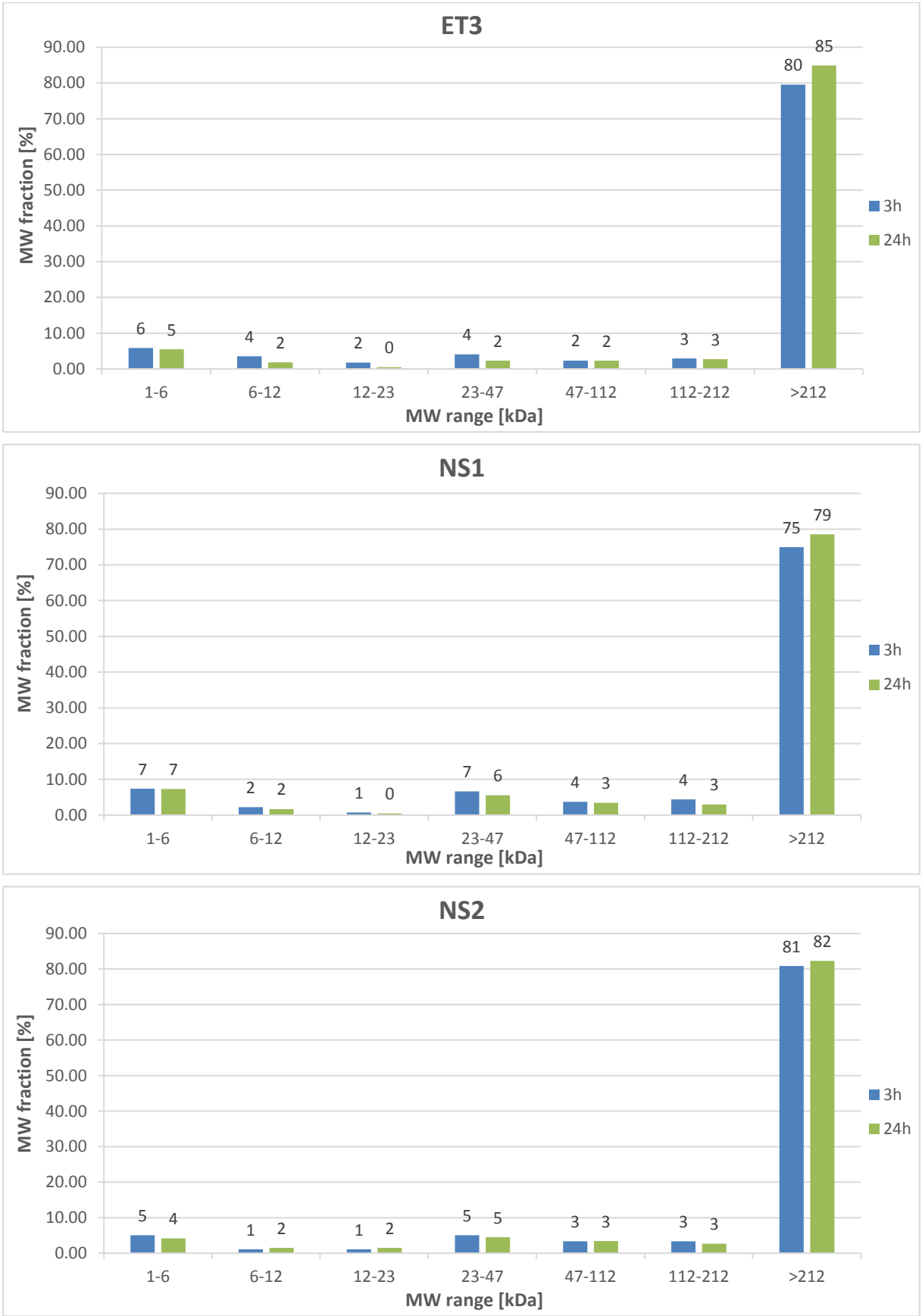

Figure 3.14 Molecular weight (MW) fraction results in the MW range of the ET3, NS1 and NS2 hydrolysates over reaction time.

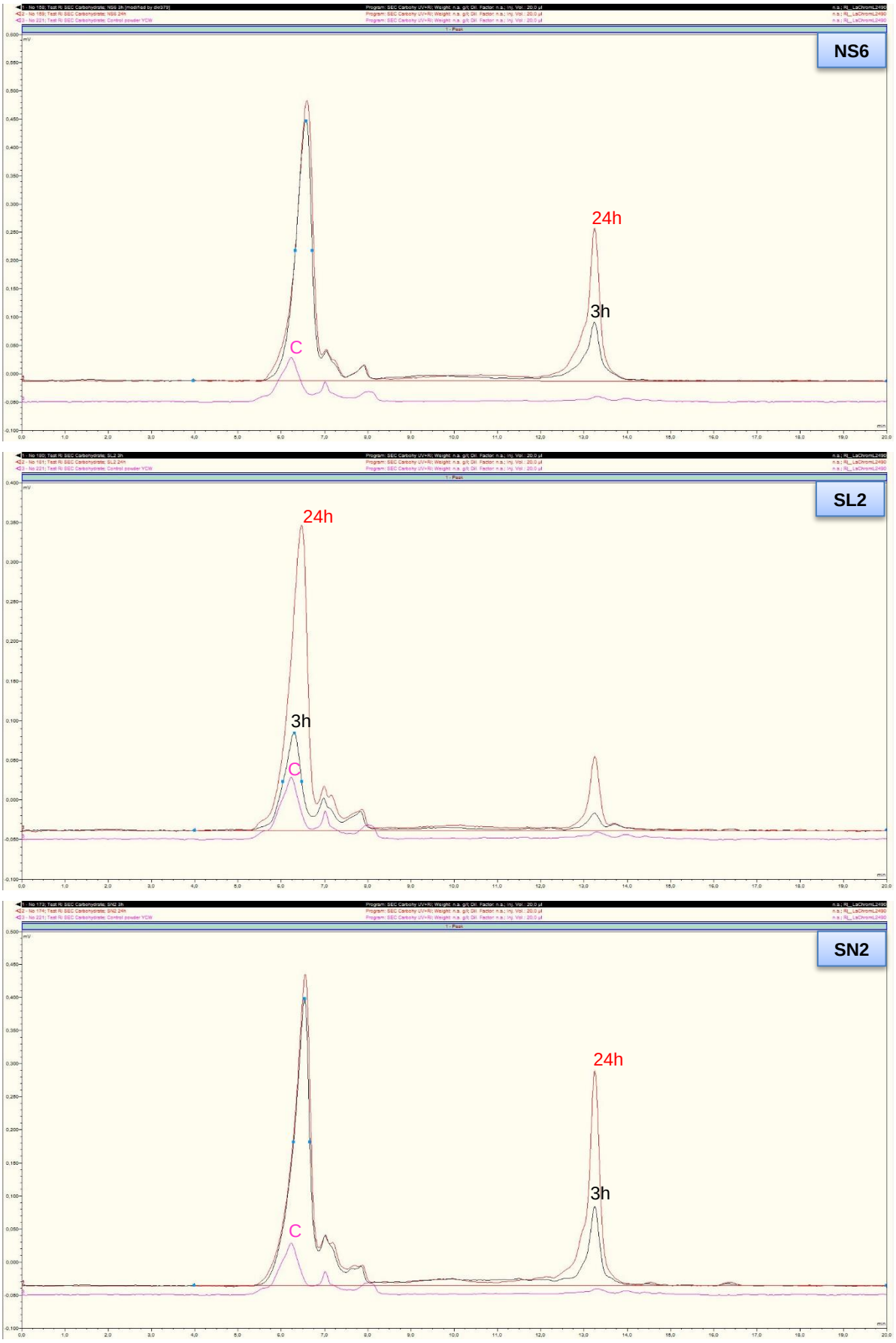

Figure 3.15 Chromatograms of the NS6, SL2 and SN2 hydrolysates over reaction time and comparison with the control sample, C.

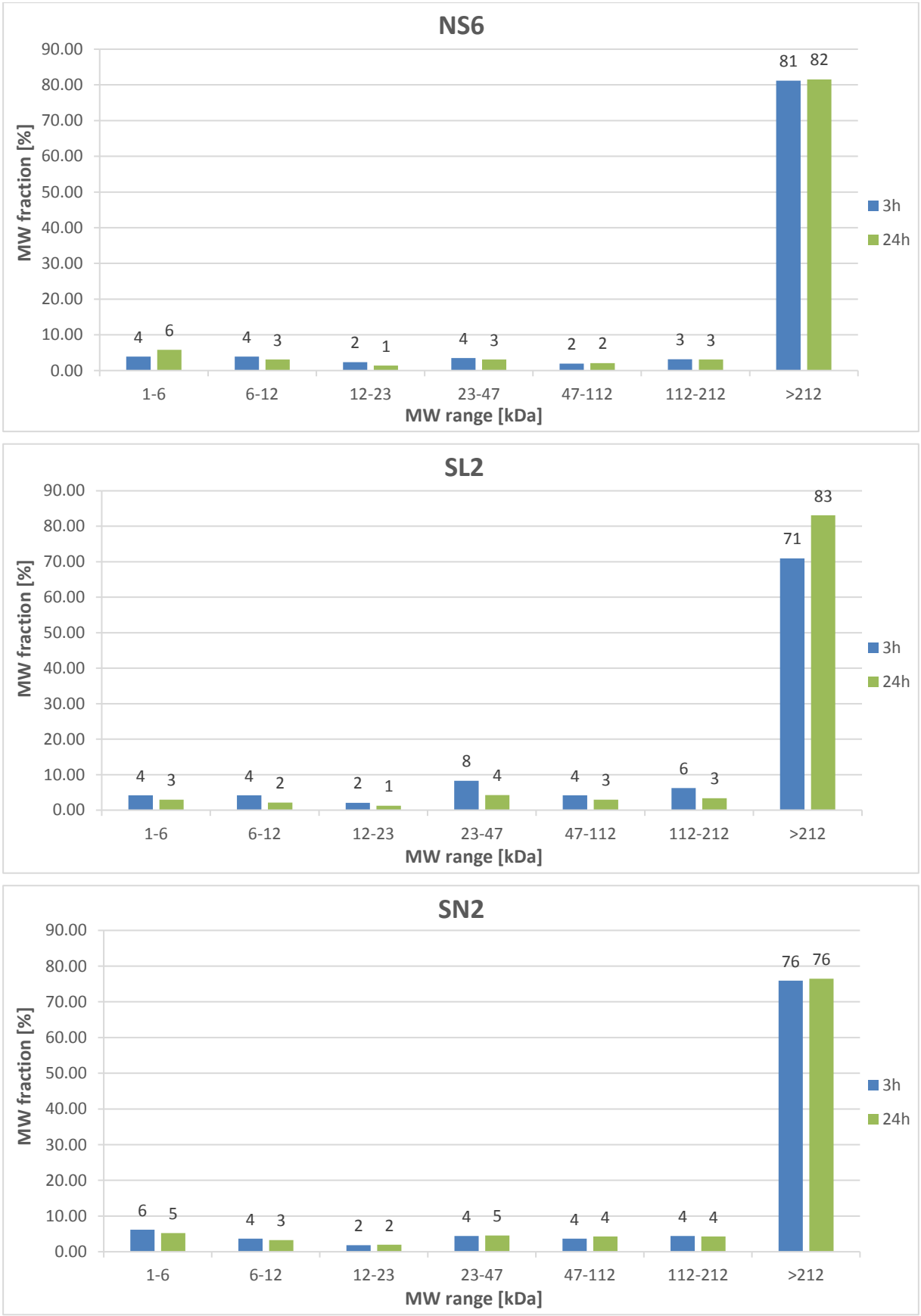

Figure 3.16 Molecular weight (MW) fraction results in the MW range of the NS6, SL2 and SN2 hydrolysates over reaction time.

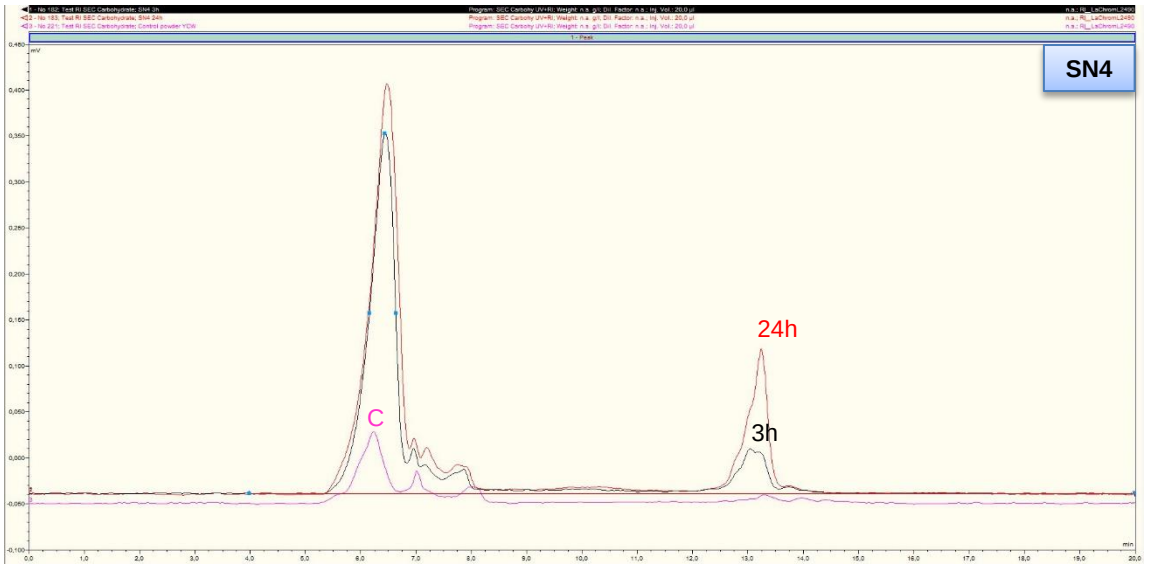

Figure 3.17 Chromatogram of the SN4 hydrolysate over reaction time and comparison with the control sample, C.

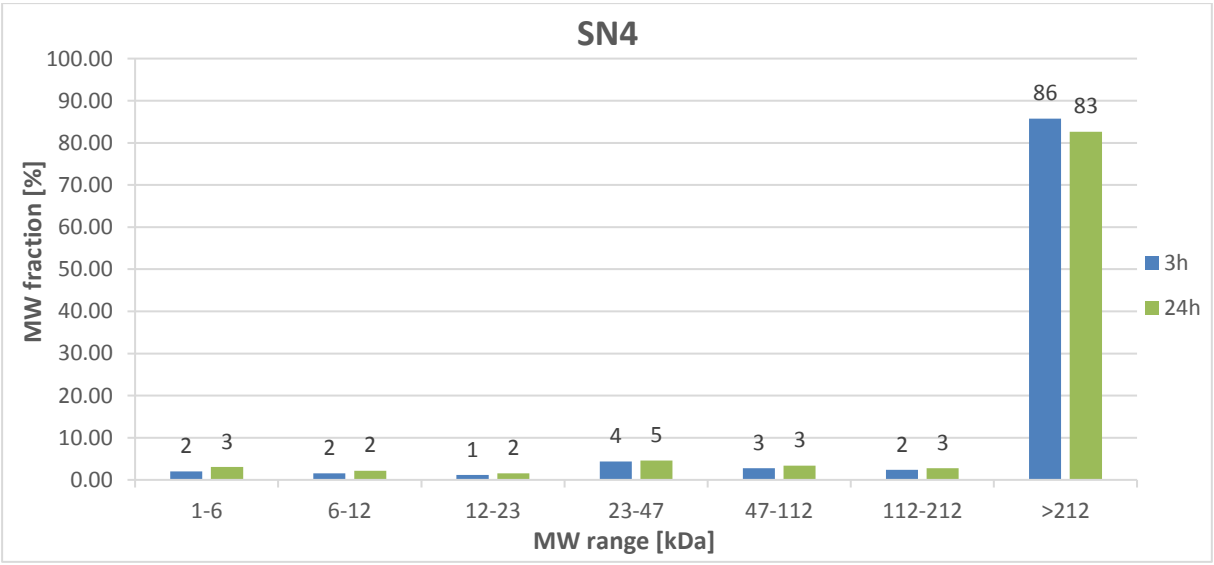

Figure 3.18 Molecular weight (MW) fraction results in the MW range of the SN4 hydrolysate over reaction time.

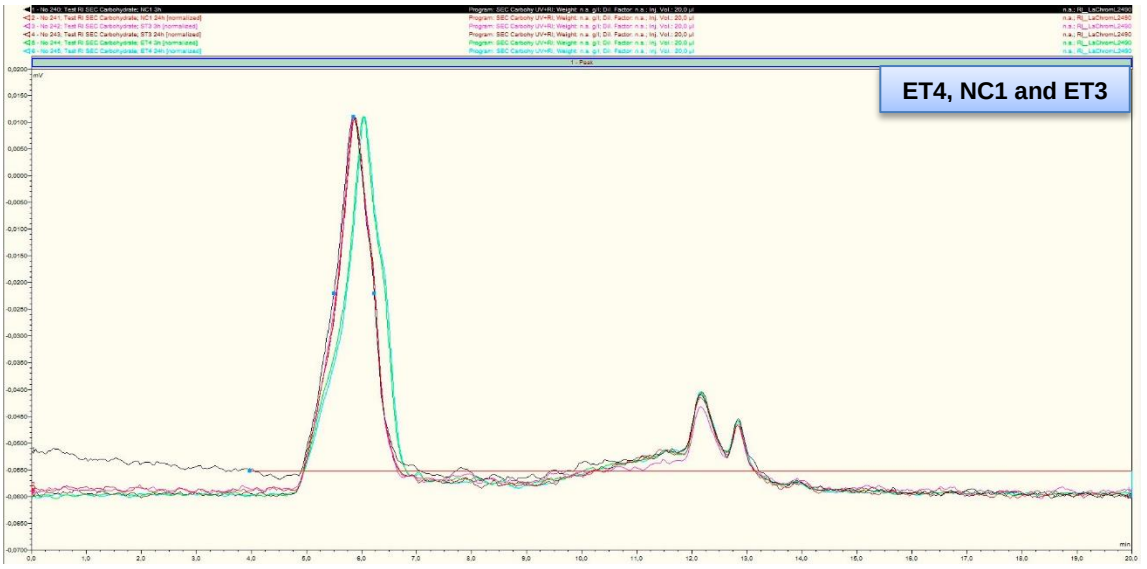

Figure 3.19 Chromatograms of the the comparison between ET4, NC1 and ET3 hydrolysates over reaction time.

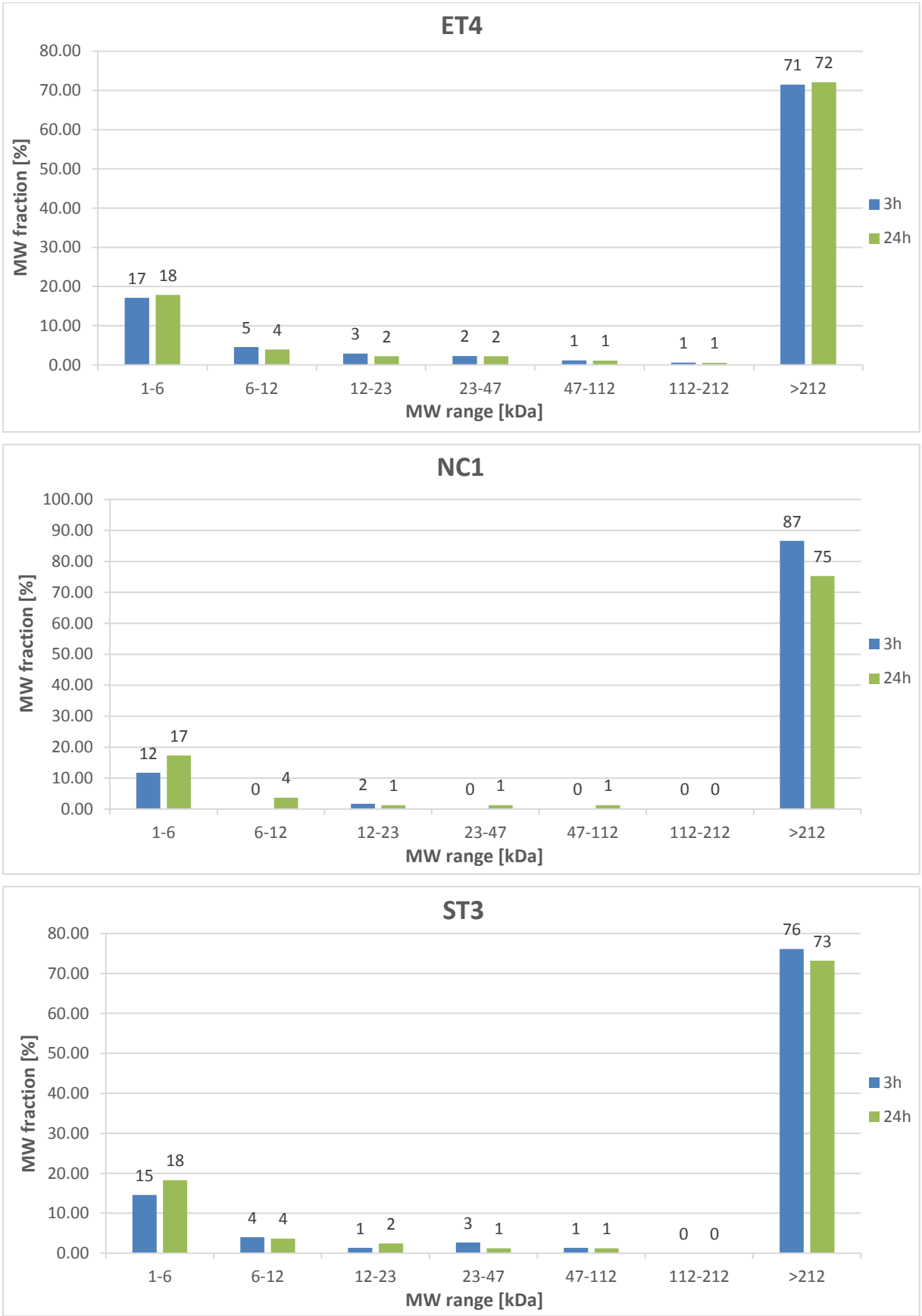

Figure 3.20 Molecular weight (MW) fraction results in the MW range of the ET4, NC1 and ST3 hydrolysates over reaction time.

3.3. Discussion and Conclusion

3.3.1. Comparison of physical properties and release of sugars

The viscosity values provide information related to the molecular weight (MW) of the oligosaccharides content in qualitative terms. In general, high MW and concentration correspond to high viscosity. Moreover, the viscosity is highly dependent of the concentration, solubility and conformational structure of the oligosaccharides [35,44,72].

Through the comparison of the starting material (without incubation) and control sample (incubated without enzyme addition and together with the enzymatic reactions), an increase of the viscosity from 98 to 156mPa·s was observed. In general, viscosity decreases reversibility with increase in temperature [72]. However, Borchani et al. (2016) reported in his studies that temperature and pH affect the viscosity. In their studies, comparing a native YCW fraction and YCW after hot treatment, a significant increase in the viscosity was observed for the latter [73]. This effect could be due to structural changes of polysaccharides, which cause an increase in viscosity [74]. Moreover, since the pH was adjusted for the control sample, according to the literature, the pH can cause an effect on the viscosity due to the sensitivity of the β-(1,3) bond in the β-glucan fraction, which may conduct to an increase of the viscosity [35,73].

Since the viscosity is dependent on the oligosaccharides content, the content of mainly oligo-glucan content obtained in the different hydrolysates will be compared, as the amount of released oligo-mannan was very similar in the different samples. It is also important to refer that the viscosity was measured for the entire suspension and the oligo-glucan content for the supernatant (SN) of the hydrolysates. Therefore, the determined oligo-glucan is not directly related to the measured viscosity, i.e. high oligo-glucan content in the SN may corresponds to a high enzyme activity and degradation of the cell wall (CW), leading to low viscosity values.

Starting with the hydrolysates which presented higher release of glucan-oligosaccharides at 24h reaction, namely AE3, AO3 DS2, ET4, NC1, NC3, NS6, SL1, SN4 and ST3, most of them exhibited low viscosity values from around 28 to 94mPa·s, which is also associated to a high enzyme activity and cell wall (CW) degradation, leading to oligosaccharides of low MW. For instance, Khan et al. (2016) reported a decrease in viscosity due to the degradation of yeast β-D-glucan [40]. Only the SN4 hydrolysate presented the highest viscosity as 270mPa·s, which suggest oligosaccharides with higher MW or hydrolysed macromolecules in linear chains were released. In general, high viscosity is attributed to very strong intramolecular association within the same molecule or with intermolecular association with other constituents in solution, resulting in aggregates [44,75]. Doublier and Wood (1995) studied the rheological properties of β-glucans derived from oats, reporting that short chain molecules with low MW present more mobility, allowing them to approach other compounds to form aggregates/agglomerates [75–77]. Therefore, this is a fair assumption which can support the high viscosity in the entire suspension and high oligo-glucan in the SN for the SN4 hydrolysate.

The AE1, AE2, AE5, BC1, DA2, DA4, DC1, ES2, NS4, NS7, NS8, SE1, P-MC1 and P-NE1 hydrolysates exhibited the lowest release of oligo-glucan to the SN and the highest viscosity values, between 334 to 754mPa·s. Therefore, it was possible to conclude that the enzymes presented a low activity on digestion of the CW, whereby the polysaccharides remained as non-degradable as well as the CW, conferring a high viscosity to the suspension. On the other hand, the ET3, NC2, NS1, SN1 and SN3 hydrolysates displayed viscosity values in the range of 225 to 286mPa·s and an oligo-glucan content also fairly reduced, when compared with the latter hydrolysates. As relatively low viscosity values were verified, a possible assumption to consider would be the attainment of di or tri-saccharides, since a high release of free glucose and reducing sugars (RS) was observed.

The AO1, BC2, NS2 and SL2 hydrolysates exhibited an intermediate oligo-glucan content released, when compared with the latter hydrolysates, and the viscosity values corresponded from around 111 to 729mPa·s, wherein the SL2 and BC2 hydrolysates presented the highest values, 416 and 729mPa·s, respectively. This discordance of the results may correspond to different structures of hydrolysates. Since SL2 and BC2 presented the highest viscosity values, the enzymes may digested the CW into big chunks of oligosaccharides, however in low concentration. According to the literature, even at low concentration, the β-glucan fraction tends to form high viscosity [72].

Finally, it is also important to refer that the conformational structure of the hydrolysate oligosaccharides, as well as the content of side branches play an important role in the viscosity values [35], whereby the last conclusions represent fairly assumptions based on obtained results.

In this dissertation, enzymatic digestion was applied to depolymerize the insoluble macromolecular structures in the YCW, in order to improve the solubility of its content and produce soluble polysaccharides [36].

After phase separation of the enzymatic hydrolysates, the water-soluble fraction, SN, contained the hydrolysed content (reducing and free sugars), while in the non-water-soluble fraction, pellet, remained the non-hydrolysed content, which is mainly the majority of the CW. Therefore, through the dry matter (DM) of the SN was possible to quantify the solubilised content and sugar released over reaction time [75].

The control sample showed a SN DM of 1.7% (w/w) and remained constant over reaction time. When compared with the enzymatic hydrolysates (Table 3.8 and Appendix D), an increase of the SN DM was observed approximately in most, which is associated to a higher solubilisation of the components to the SN over reaction time. Additionally, both SN volume and reducing and free sugars released (Figure 3.3 and Figure 3.4) also rose over reaction time, promoting a well-grounded support on the last assumption.

However, some hydrolysates displayed a decrease of the SN volume from around 2 to 13% and an increase of the DM SN and sugars released, namely DA1, DA3, DS3 and DC1, which may indicate that the SN volume is not a valid parameter to relate the release of sugars and the enzyme activity. According to the literature, polysaccharides insoluble in water can absorb and retain water, which contributes to their swelling [73]. Thus, a fair assumption to the increase of the pellet volume, over

reaction time for these hydrolysates, could be its capacity to swelling. For the AE1, BC4, DA4, ES2, SN5, SE1, ST2 and P-SN1 hydrolysates the same observations were verified, although the increase of SN DM and sugars released were lowest. Additionally, the ET1, ET2, NC2, NC3, NC4, NS5 and NS8 hydrolysates showed the same decreasing of SN volume, however the SN DM and release of sugars were nearly constant over reaction time, proving that the SN volume may not be directly related to the enzyme activity or does not present a great impact on it. Lastly, the AE4, ET3, NS1, NS3, SN1 and SN3 hydrolysates presented a constant SN volume over reaction, but a rise of the SN DM and sugars release, supporting once more the latter conclusion.

Some discrepancies in the SN DM results were observed by the comparison with the reducing and free sugars released (Figure 3.3 and Figure 3.4). However, the highest fluctuations were verified for the hydrolysates which presented a turbid SN. Since the SN DM was measured by using a digital refractometer, the SN turbidity may affect the measurement, as was also reported by Carpenter and Deitz (1963) [78]. Moreover, the refractometer does not represent an accurate instrument to measure the SN DM as, for instance, a moisture analyser. As the SN samples size was reduced and in order to perform all the experiments, the digital refractometer was the only possible equipment to measure the DM.

A loose pellet was observed on the AE3 and NS6 hydrolysates after 24h reaction. These hydrolysates also presented a high release of free glucose and RS and a low viscosity value in the entire suspension, which means that most of the CW was digested. Thus, the non-hydrolysed content contain a less concentration of polysaccharides or small oligosaccharide chains, which induces to a lower viscosity or more fluid pellet [72,78].

Three of the hydrolysate products (ET4, NC1 and ST3) exhibited a loose pellet from 3h to 24h reaction time and a high SN volume. However, they presented a high SN DM which remained mostly constant over reaction time, meaning that at 3h reaction the enzymes hydrolysed all the oligosaccharides which are specific for the CW. This evidence can also be supported through the analysis of the highest RS and oligo-glucan contents, at 3h and 24h reaction, by comparison with the other hydrolysates.

In summary, the sort of enzyme and its activity present a high influence in the phase separation as well as in turbidity and colour intensity of the SN. Furthermore, according to the literature, the solubility of the polysaccharides depends on the length and frequency of side branches as well as the degree of polymerisation (DP) [36,79]. It is also known that a β-glucan fraction with a high DP (> 100) is insoluble in water and the removal of constituents residues of the side branches may causes the precipitation of the polysaccharides [36,79]. Through comparison of the estimated number-average DP (DP_n) for the solubilised content (SN) of the hydrolysates (Table 3.10 and Appendix G), the values were less than 100 (from around 5 to 15), which are according to the reported. However, from the obtained results it was not possible to conclude about the degree of branching.

The turbidity, also referred to as cloudiness or haziness, is generally caused by suspended particles in a liquid. This parameter presents a great importance in a product characterization, since the

presence of a turbid or darker colour of the raw ingredient when incorporated to light or transparent food products, would give to these products a spoilt or darker appearance, which is not desired [17,73].

The AE3, AO3, ET3, NS6 and SL1 hydrolysates presented a turbid SN after 24h reaction, whereas ET4, NC1 and ST3 displayed the highest SN turbidity levels both at 3h and 24h reaction time (Table 3.9). Speers at el. (2003) investigated how β-glucans MWs and concentration affect the turbidity in wort and beer produced by *S. cerevisiae*, as well as the effect of pH [80]. They concluded that the turbidity increased in beer containing high MW β-glucans (250, 337 and 443kDa) at concentrations higher than 400mg/L, which may be caused due to polymer aggregation and larger particle sizes. In wort, they obtained a highest turbidity level at pH 4.0, which may be related to the electrostatic properties of the proteins, possessing a high net positive charge that caused a high intermolecular electrostatic repulsion. As wort also contained high MW β-glucans, the increased haze was hypothesised to be derived from both proteins and β-glucans. However, as stated before, about the rheological studies of oat β-glucans from Doublier and Wood (1995), they reported that short chain molecules with low MW present more mobility, allowing them to form aggregates [75–77], causing a contradiction of conclusions, which does not mean that are incorrect since both studies were done from different starting materials. Analysing the AE3, ET3, ET4, NC1, NS6 and ST3 hydrolysates, they presented a turbid SN, low viscosity values and high sugars and oligo-glucan and mannan released. Assuming both conclusions from Speers at el. (2003) and Doublier and Wood (1995), it may be formed aggregates but with low MW polysaccharides. In addition, the reaction pH 4.5 may be the cause for the SN turbid [80]. Contrary to the AO3 and SL1 hydrolysates, they did not show oligo-glucan release, but only high oligo-mannan was released. As in the CW the mannan-oligosaccharides are directly linked to the proteins, a fair assumption may also be the release of proteins to the SN which were affected by reaction pH and caused the haze SN [25,80].

In summary, through the physical properties previously analysed may attain an idea and some knowledge about the constitution of the obtained hydrolysates products. Therefore, the viscosity, phase separation and solubilisation as well as turbidity of the supernatant represented to be relevant properties to the characterization of the hydrolysates.

3.3.2. Enzyme activity on release of sugars

First of all, to provide a better understanding, the following descriptions display the theoretical background behind the methods used to measure the reducing sugars (RS), free glucose and mannose contents.

RS content

The DNS method allows the estimation of the amount of sugar in a sample and even, although indirectly, the enzyme activity responsible for the hydrolysis of polysaccharides. The DNS reaction takes

place through the reduction of 3,5-DNS which reacts with the free carbonyl (aldehyde group) of the RS under alkaline conditions. Thus, 3-amino-5-nitrosalicylic acid is formed and the aldehyde group of sugars is oxidised into the respective carboxylic acid (Figure 3.21). Since 3,5-DNS presents a distinctive yellow colour, the reaction between it and the RS will lead to the conversion of the aromatic compound in its reduced form (3-amino-5-nitrosalicylic acid). Its formation will provide a formation of an orange-red colour with a maximum absorption at 530nm, which allows to a quantitative spectrophotometric measurement of the amount of RS [81,82].

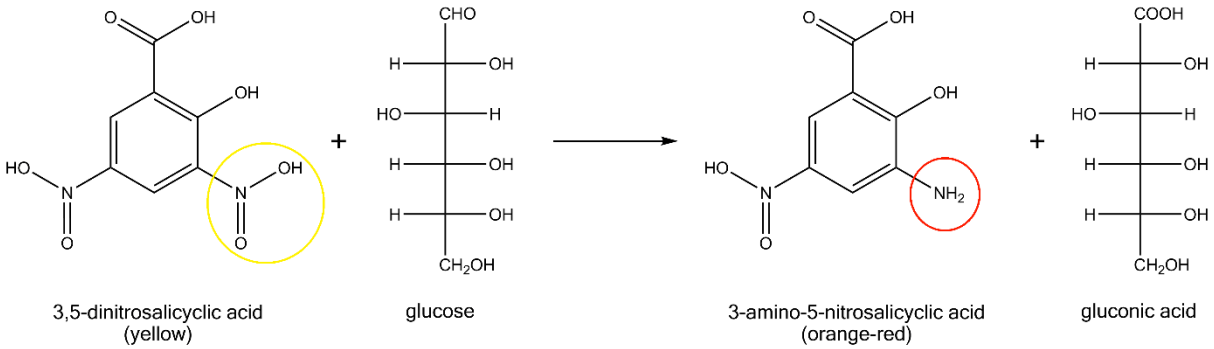

Figure 3.21 Conversion of 3,5-DNS into 3-amino-5-nitrosalicyclic acid by oxidation of glucose into gluconic acid.

Free glucose content

The free D-glucose present in the SN of the hydrolysates is phosphorylated to D-glucose-6-phosphate (G-6-P) through the catalytic action of the enzyme hexokinase (HK) and by adenosine-5´-triphosphate (ATP), with the simultaneous formation of adenosine-5'-diphospahte (ADP) (Equation 5) [83].

$$D - Glucose + ATP \xrightarrow{HK} G - 6 - P + ATP \qquad \text{(Equation 5)}$$

Subsequently, G-6-P is oxidised in the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH) by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate, with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NAPH) (Equation 6) [83].

$$G - 6 - P + NADP^{+} \xrightarrow{G6P-DH} D - gluconate - 6 - phosphate + NADPH + H^{+} \qquad \text{(Equation 6)}$$

The amount of NADPH formed is stoichiometric to the amount of free D-glucose and is measured by means of its light absorbance at 340nm [83].

Free mannose content

Since the SN of the enzymatic hydrolysates presented a high content of free glucose, the latter was reduced prior to the mannose measurement.

Free glucose reduction

Through the action of glucose oxidase (GOD) and atmospheric oxygen (O_2), free D-glucose is oxidised to D-gluconate (Equation 7) [84].

$$D - glucose + O_2 + H_2O \xrightarrow{GOD} D - gluconate + H_2O_2 \quad \text{(Equation 7)}$$

Consequently, the hydrogen peroxide (H_2O_2) formed is decomposed by catalase (Equation 8) [84].

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2 \quad \text{(Equation 8)}$$

Mannose measurement

Once the free glucose was reduced, the mannose measurement was proceeded through the following enzymatic reactions.

In the presence of D-glucose, D-fructose and D-mannose, enzyme HK and ATP phosphorylate them to G-6-P, fructose-6-phosphate (F-6-P) and mannose-6-phosphate (M-6-P), respectively, with the simultaneous formation of ADP (Equations 9-11) [85].

$$D - glucose + ATP \xrightarrow{HK} G - 6 - P + ADP \quad \text{(Equation 9)}$$

$$D - fructose + ATP \xrightarrow{HK} F - 6 - P + ADP \quad \text{(Equation 10)}$$

$$D - mannose + ATP \xrightarrow{HK} M - 6 - P + ADP \quad \text{(Equation 11)}$$

As stated before, G-6-P is converted to gluconate-6-phosphate through the Equation 6 and the amount of formed NADPH is stoichiometric with the amount of D-glucose. On the completion of this reaction, F-6-P is converted to G-6-P by phosphoglucose isomerase (PGI) (Equation 12) [85].

$$F - 6 - P \xleftrightarrow{PGI} G - 6 - P \quad \text{(Equation 12)}$$

G-6-P reacts again through the Equation 6, conducting to a further rise in absorbance which is stoichiometric with the amount of D-fructose. Finally, M-6-P is converted to F-6-P by phosphomannose isomerase (PMI) (Equation 13), which is then immediately converted to G-6-P by PGI in the reaction mixture, leading to another further rise in absorbance at 340nm that is stoichiometric with the amount of D-mannose present in the SN of the hydrolysates [85].

$$M - 6 - P \xleftrightarrow{PMI} F - 6 - P \quad \text{(Equation 13)}$$

Comparing the sugars and oligosaccharides contents released (from Figure 3.3 to Figure 3.8) in the SN of the starting material (SM) and control (C), similar results were observed, however only the mannan-oligo content was approximately 10% (w/w) per DM higher on the latter. It is also important to refer that the SM SN presented a highest release of oligo-mannan of around 25% (w/w) per DM, when compared with the RS, free mannose and oligo-glucan released. Thus, without any treatment the yeast cell wall (YCW) exhibited a high amount of mannan-oligosaccharides solubilised in the SN. As mentioned before, the control sample suffered a pre-heating as it was incubated together with the enzymatic reactions, as well as the same enzyme inactivation procedure (80°C for 30min). According to the literature, high temperatures (90°C) can cause degradation or depolymerisation of oligosaccharides/glycoproteins and their solubilisation to the SN [86,87]. Therefore, a fair assumption may be the a slight depolymerisation of the mannoproteins content, which are more accessible as they are present in the outer layer of the CW, altering their concentration and probably impair their behaviour [25,86,87].

Regarding the enzymatic hydrolysates, the AE3, AO1, AO3, BC2, DS2, ET3, ET4, HE1, NC1, NS2, NS6, SL1, SN2, SN4, ST1 and ST3 displayed the highest release of reducing and free sugars over reaction time, expect for BC2, DS2, ET3, HE1 and NS2 which only exhibited at 24h reaction. Comparing the AE3 and SN4 enzyme activities, both present β-(1,3/1,6)-glucanase as well as protease activities, although in AE3 is a side activity and SN4 is a endo-β-glucanase. Both presented a similar release of sugars profile over reaction time, however at 3h and 24h the release of RS and free glucose were lowest for SN4. This observation may be explained by the endo activity from SN4, which attacks the linkages at intermediate points of the β-(1,3)-glucans chain, releasing a mixture of oligosaccharides with reducing and free sugars as minor products [47]. Scott and Schekman (1980) used a yeast lytic enzyme (“lyticase”), composed by a endo-β-(1,3)-glucanase and protease activities, in order to lyse the YCW [88]. From their results, they stated that the glucanase attacked β-(1,3)-glucans in a random endolytic fashion releasing oligosaccharides, while the protease promoted the CW lysis by attack on the protein portion of the mannoprotein content. As the β-glucan layer of the CW is covered by a layer of mannoprotein, they concluded that the latter must be modified before a lytic glucanase can reach its substrate, namely β-(1,3)-glucans, promoting cell lysis by the synergistic effect of glucanase and protease activities. In addition, it was also reported that endo-β-(1,3/1,6)-glucanases are only capable to digest linear structures and branch points are resistant to their action [32]. Therefore, the latter reported conclusions may substantiate the high activity observed by AE3 and SN4 enzymes [47,88]. The AO1 enzyme also present protease activity, although with many unknown side activities. When compared with AE3, both presented a similar profile on sugars released, indicating that probably AO1 also contains a β-(1,3/1,6)-glucanase activity. The ET3 and NS6 enzymes exhibit an exo-β-(1,3)-glucanase activity, though in the former as a side activity. Regarding the release of sugars, both showed a similar profile, however ET3 displayed a lowest release which could be due to the expression of the exo-β-glucanase as a side activity or the presence of a different pattern of activity and specificity by the exo-β-glucanase [89]. Fontaine et al. (1997) studied two exo-β-(1,3)-glucanases activities isolated from the cell wall of *Aspergillus fumigatus* [89]. They concluded that the two exo-β-glucanases exhibited

different specificities as well as different catalytic mechanisms of the enzyme reaction, wherein one was able to degrade the β-(1,6)-glucan branch points on linear β-(1,3)-glucan, corresponding to a β-D-glucoside-glucohydrolase (EC 3.2.1.21), whereas the other was blocked by the β-(1,6)-glucan branch points, which is denominated as a β-(1,3)-glucan-glucohydrolase (EC 3.2.1.58) [89]. Therefore, the ET3 may present an enzyme specificity which is blocked by the branching points in the β-(1,3)-glucan chains [89]. After 24h reaction, both presented a higher free glucose content released than RS, which may be explained by the exo-glucanase activity [47]. According to the literature, the exo-(1,3)-β-glucanases account for the greater part of total glucanase activity in yeasts and hydrolyse the β-*O*-glycosidic linkages at the non-reducing end to the β-(1,3)-glucan chain, resulting in high release of free glucose [47]. Furthermore, they are not particularly specific as they can also present the activity to act on β-(1,6) linkages, although with less efficiency [47]. Both BC2 and ST1 enzymes show a β-glucanase activity, which is not specified as neither endo- or exo-glucanase or what sort of linkages is specific to hydrolysis. In contrast to BC2, ST1 additionally display galactomannanase, cellulase and β-glucosidase activities. At 3h and 24h reaction, ST1 exhibited the double of the sugars content released with a higher release of free glucose when compared with BC2. These differences could be explained by the additional β-glucosidase activity, which promotes the release of higher free glucose and RS contents, as it hydrolyses the non-reducing β-D-glucosyl residues ends with release of β-D-glucose [54,55,57]. For instance, Magnelli, Cipollo and Abeijon (2002) used an enzymatic method to determine the *S. cerevisiae* cell wall composition, wherein the final step involved using laminarinase and β-glucosidase in order to hydrolyse a remaining β-(1,3)-glucan fraction to solely glucose [90]. The ET4, NC1 and ST3 are denominated as purified β-glucanases and were produced from the same source, namely *Streptomyces* sp. These carbohydrases stood out due to the non-release of free glucose, solely release of RS and presenting the highest content of oligo-glucan released. Shrestha et al. (2011), Nishimura et al. (2001) and Wu et al. (2009) characterized the structure and studied the mechanism of action of a laminaripentaose-producing β-(1,3)-glucanase (LPHase), which was first purified from *Streptomyces matensis* DIC-108 and the corresponding gene was further cloned (accession no. BAA34349 in GenBank) [91–93]. The LPHase presents a particular product specificity, since it acts on the β-(1,3)-glycosidic bonds to release/produce exclusively pentasaccharide oligomers from β-(1,3)-glucan (laminaripentaose) as the predominant product, not having the release of mono- and disaccharides [91–93]. They concluded and proved that LPHase is an inverting glycosidase, which follows a one-step single-displacement mechanism with the assistance of a general acid and base. The general base polarizes a water molecule to develop a stronger nucleophile to attack the anomeric carbon (C-1), while the general acid protonates the glycosidic oxygen to accelerate the reaction [91–93]. Therefore, the ET4, NC1 and ST3 enzymes are possible candidates as LPHases, considering the obtained sugars released [91–93]. Regarding DS2 and NS2 enzymes, both are endo-β-(1,3/1,4)-glucanases, although NS2 presents xylanase and hemicellulase as side activities. As the CW does not exhibit β-(1,4) bonds [25], the latter enzyme mixtures solely may display the endo-β-(1,3)-glucanase activity to digest the YCW. In terms of sugars released, either at 3h or 24h reaction, NS2 showed a highest release of free glucose and RS, where the former was higher over reaction time. These differences could be explained by the fact that both enzymes were produced from different organisms, namely DS2 from *Talaromyces*

emersonii and NS2 from *Aspergillus aculeatus*, which also demonstrate a great impact and influence on the formed oligosaccharides as well as sugars released [94]. This can also be evident for example, by comparing the DS1 and DS2 which display the same enzyme activity. However, the endo-β-(1,3/1,4)-glucanase activity of DS1 was obtained from two different sources (*Talaromyces emersonii* and *Trichoderma longibrachiatum*) than DS2, constituting an enzyme mixture, where the release of sugars was much lower [94]. Finally, AO3, HE1, SL1 and SN2 enzymes exhibited xylanase, galactomannanase, cellulase, pectinase and hemicellulase activities, which should be specific to degrade plant cell walls and not YCW [95,96]. However, these enzymes displayed a high release of free glucose and RS, over reaction time, whereby the unique possible conclusion to do is these enzyme mixtures present side activities which are unknown or not reported. This conclusion can be supported for example by the comparison with AE1, AE2, BC1, BC3, DA4, ET1, ET2, ES1, NC3, NS3, NS4, NS8, SN5, and ST2 which display enzyme activities specific to digest plant cell walls and presented a sugars released content less than 2% (w/w) in total sugars at 3h and 24h reaction time.

Half of the enzymatic hydrolysates displayed a release of RS and free glucose equal or less of 2% (w/w) in total sugars both at 3h and 24h, meaning that the respective enzyme mixtures were not suitable to digest the CW as they presented a low activity. As stated before, this may be associated due to the fact that most of these enzyme mixtures exhibit enzyme activities specific to degrade plant cell walls, such as cellulase, hemicellulase, glucoamylase, xylanase and pectinase activities [95,96]. However, some of them are chitinase (NC4), lipases or phospholipases (P-MC1, P-NE1, P-N1 and P-SN1) and mannosidases (AO2 and NS5). In order to hydrolyse the CW, generally some authors used lipases or chitinases combined with β-glucanases, since they are only specific to degrade the phospholipid and β-(1,4)-GlcNAc linkages, respectively [90,97]. For instance, as reported from Mauch et al. (1988) they tested a purified chitinase, a purified β-(1,3)-glucanase and a combination of both to digest a fungi CW and concluded that only with the chitinase, a low content of glucan oligosaccharides were solubilised, whereas a combination of both promoted a synergistic effect on the degradation of the CW, releasing both GlcNAc and glucan oligomers [97]. Since chitin and β-(1,3)-glucan are directly attached by glycosidic linkages, thus chitin is entrapped in the β-glucan layer and also as the lipid content (membrane) is covered by the CW, it was understandable the non-release of sugars by enzymes with lipase and chitinase activities [25,98]. Related the mannosidase enzymes, it was noticeable that both exhibit a β-mannosidase activity. In the YCW, the mannan-oligosaccharides (MOS) are composed by a main chain of α-D-(1,6)-mannose with short α-(1,2)- and α-(1,3)-linked side chains [30]. On the basis of knowledge of MOS structure, one might expect exo- and endo-α-(1,6)-mannanase to be involved in the hydrolysis of MOS and an endo-β-*N*-acetylglucosaminidase to cleave the connection between MOS and protein [47]. Therefore, a β-mannosidase may not display any degradative activity on MOS as it was observed and predicted.

By the comparison of the oligo-glucan and mannan contents for all the hydrolysates, the latter presented a high released amount. However, it is also important to refer that the SM, without any treatment, displayed a release of oligo-mannan of around 25% (w/w) per DM and the control sample, which was incubated with the enzymatic reaction, showed a highest release of approximately 35% (w/w) per DM. Related to the oligo-glucan, both SM and control sample exhibited a release of oligo-glucan of

roughly 2% (w/w) per DM. Therefore, the oligo-glucan and mannan contents from the enzymatic hydrolysates will be discussed considering a release of oligosaccharides above of the amounts previously mentioned of the SM and control sample.

Starting with the oligo-mannan content release over reaction time, the hydrolysates which presented a high release of free glucose and RS, namely AE3, AO1, AO3, BC2, DS2, ET3, ET4, HE1, NC1, NS2, NS6, SL1, SN2, SN4, ST1 and ST3, additional displayed a higher release of oligo-mannan than the SM and control sample. Most of them present β-(1,3/1,6)-glucanase or protease or a combination of both activities. From Caro et al. (1997) results and literature, mannoproteins can be released from the CW by β-(1,3/1,6)-glucanases, since mannoproteins can be linked either to β-(1,3)-glucan or to β-(1,6)-glucan through a glycosylphosphatidylinositol (GPI) anchor [30,48,99]. According to the literature, mannoproteins can be divided into two groups, the sodium dodecyl sulphate (SDS)-extractable mannoproteins and the glucanase-extractable mannoproteins, which are solubilised by glucanase digestion of the glucan layer [100]. The glucanase-extractable mannoproteins have been identified and present two characteristics in common: their C-terminal are rich in serine (Ser) and threonine (Thr) and they all contain a glycosylphosphatidylinositol (GPI) attachment signal [100]. Therefore, the oligo-mannan content solubilised to the SN by these enzymes, may be the glucanase-extractable mannoproteins [99,100]. However, in case of AO3, HE1 and SL1 hydrolysates, the oligo-mannan content released was approximately equal as amount released from the control sample. Therefore, the same conclusion mentioned before for the high oligo-mannan released when compared with the SM, may explain the same oligo-mannan amount released as the control sample, which could be due to the reaction or inactivation temperature, wherein the enzymatic reactions were submitted, promoting a degradation or depolymerisation of the oligosaccharides/glycoproteins and, consequently, their solubilisation to the SN [86,87]. Additionally, these enzymes present declared activities as xylanase, galactomannanase, cellulase and pectinase, whereby as it was stated before, they are specific to digest plant cell walls [95,96] and due to the high reducing and free sugars released, it was possible to conclude that these enzymes present other unknown side activities not reported.

The enzymatic hydrolysates which showed a release of RS and free glucose equal or less of 2% (w/w) in total sugars both at 3h and 24h, displayed a release of oligo-mannan content similar to the SM and the control sample. However, only the NC2 hydrolysate exhibited a higher release of oligo-mannan when compared with the SM and the control sample. This may be explained by its β-N-acetylglucosaminidase activity, which presents the particular cleavage of the connection between MOS and protein, releasing the intact MOS [47].

In addition, a reduced free mannose released was observed for all the enzymatic hydrolysates. Therefore, since the enzyme mixtures does not present a α-mannanase activity, it is understandable the non-release of free mannose, as the oligo-mannan content released was due to either the reaction or inactivation temperature or the combined effect of β-(1,3/1,6)-glucanases and proteases, as it was previously concluded [86,99].

Finally, regarding the oligo-glucan content released, most of the enzymatic hydrolysates exhibited a higher oligo-glucan content released over reaction time than the SM and control sample,

wherein the AE3, AO1, BC2, DS2, ET3, ET4, NC1, NS2, NS6, SN2, SN4 and ST3 hydrolysates displayed the highest amounts as well as the reducing and free sugars. As stated before, these enzymes present mainly β-(1,3/1,6)-glucanase or protease or a combination of both activities, whereby as previously concluded, these enzyme activities promote the release of glucan oligosaccharides as well as a combination of both, which exhibit a synergistic effect on CW lysis from the glucanase and proteases activities [88]. Moreover, as also mentioned before, an endo- or exo-glucanase present different mechanisms of action, affecting the chain length of the oligo-glucan produced [47]. The ET4, NC1 and ST3 hydrolysates exhibited the highest release of oligo-glucan. As previously concluded, these enzymes may be classified as LPHase, since they solely release RS and do not present free glucose [91–93]. Therefore, the high oligo-glucan released also supports this assumption, whereby their composition may be as pentasaccharide oligomers [91–93].

The AO3, HE1, SL1 and ST1 hydrolysates showed a lowest or almost null oligo-glucan released over reaction time. These enzymes also exhibited a high reducing and free sugars released and present enzyme activities specific to the plant cell walls, such as xylanase, galactomannanse, cellulase and pectinase, whereby it was latter concluded that they may contain unknown side activities not reported. As the oligo-glucan content was reduced, a possible assumption could be that these enzymes only release di- and monosaccharides, which is associated, for instance, to an exo-glucanase activity [47].

Furthermore, some fluctuations on the oligo-glucan and mannan results were observed over reaction time, which could be due to the acid hydrolysis performed. According to several authors, acid hydrolysis is a chemical method rather harsh and may destroy the monomers, hence underestimate the content of the corresponding polysaccharides in the CW, if not well controlled in terms of duration and temperature [64,101]. For instance, Dallies et al. (1998) studied different acid hydrolysis procedures of CW polymers, where they used 72% (w/w) sulphuric acid, 2N trifluoroacetic acid and 2N hydrochloric acid. From their results, they concluded that the sulphuric acid hydrolysis method appeared to be much more reliable than hydrolysis with trifluoroacetic and hydrochloric acids, as the yields of monosaccharides released were lower [101]. Therefore, based on the latter statements, a fair assumption may be the monosaccharides destruction by the harsh acid hydrolysis and/or the inefficient/incomplete hydrolysis due to the use of hydrochloric acid [64,101,102].

As expected and observed from the previously discussed results, the solubilisation of sugars and CW degradation increased over reaction time, which are mainly dependent on it [75]. In addition, the screened enzymes from different sources formed different hydrolysates with different sugar released as predicted, as well as their side activities, which in some enzyme mixtures are not reported, present a great influence in the results [75,94].

3.3.3. Relation between DP_n and possible applications

Through the analysis of the DP_n results (Table 3.10), over reaction time, for the SN of the enzymatic hydrolysates, it was possible to conclude, select and differentiate the hydrolysates according

to possible applications. Therefore, in order to select and discuss possible applications, the obtained DP_n values from the solubilised hydrolysates will be compared with the respective viscosity values measured in the entire suspension, as this physical parameter provides information related to the MW of the oligosaccharides content [44]. Additionally, it is important to refer that the DP_n values represent a number-average in conjugation of oligo-glucan and mannan contents, as it was not possible to quantify the amount of RS as free mannose or glucose.

Regarding the latter conclusions related to the ET4, NC1 and ST3 hydrolysates, they presented DP_n values in the same order, from between 7 to 9, which did not vary over reaction time. This goes according which was concluded before, wherein the SN SM, reducing and free sugars as well as the oligo-glucan and mannan contents also did not exhibited variations over reaction time. From the obtained DP_n values, it is also possible to confirm that these enzymes released pentasaccharide oligomers and thus, are classified as LPHases [91–93].

The AE3, AE5, AO1, ET3, ET4, NC1, NS1, NS2, NS6, SN4 and ST3 hydrolysates exhibited DP_n values from approximately 5 to 9 over reaction time. Since they presented low DP_n values, they appear interesting to be used for food applications, whereby low MW polymers are generally more soluble and easier to disperse in a food matrix [37]. According to the literature, low MW β-glucans (9kDa) are appropriate to manufacture soft gels as they are easily to rearrange due to the less linkages [44]. Naumann et al. (2006) incorporated β-glucan with low MW (80kDa) into a fruit drink and studied its effect on serum concentrations of total LDL cholesterol in human volunteers [103]. They effectively concluded that low MW β-glucans can reduce the serum cholesterol concentration when consumed in beverages [103]. Therefore, as AE3, ET4, NC1, NS2, NS6 and ST3 hydrolysates presented low viscosity values, from around 30 to 110mPa·s, they may be interesting to be used for this application and among others, such as soft brined cheeses, yogurts and other soft milk products, soft doughs [38,103]. For instance, since the AE5, AO1, ET3, NS1 and SN4 hydrolysates exhibited high viscosity values, in the range of 220 to 390mPa·s, they could be suitable to improve water-holding and oil-binding capacities, gelling or thickener characteristics and emulsion stabilization in food products, such as low fat ice-cream, low fat cheese, low fat sausages, soups, sauces, salad toppings (dressings), frozen desserts, panning doughs and conditioners [38]. Moreover, as these hydrolysates released a high amount of RS, they may be also interesting to be used as an ingredient for an application through a Maillard reaction, which is used in savoury and meat flavour applications [104,105].

Related to the BC2, DS2, SL2 and SN2 hydrolysates, the obtained DP_n values were in the range of 6 to 14 over reaction time. The highest values corresponded to 14 for BC2 at 3h, followed by 13 and 12 for SL2 at 24h and DS2 at 3h, respectively. The SN2 displayed the lowest DP_n value of 10 at 3h reaction time. Therefore, these hydrolysates appeared to be interesting for an immune-stimulant application, since a oligosaccharide chain length of 7 to 15 glucose units is required for binding in an immune recognition, as it was reported by Hanashima et al. (2013) and Adams et al. (2008) [43,106,107]. However, according to the literature, the polymer length (degree of polymerisation), degree of branching and conformational structure also influence their ability to modulate the immune system, wherein high MW glucans present higher affinity interaction with the immune recognition

receptors [106,107]. From Adams et al. (2008) results they concluded that for an immune recognition of glucan ligands is required a backbone chain length of at least 7 glucose units and at least one glucose side chain branch, which means that the presence of β-(1,6)-linked side chain branches increases the affinity to pattern recognition for glucan [106]. Moreover, they also stated that a helical nature of glucan polymers may also facilitate the interactions of glucans with the immune recognition receptors. It is also important to refer that SL2 and SN2 hydrolysates exhibited the highest free glucose released. Therefore, these hydrolysates may not be great candidates as an immune-stimulant application, since from Ilyas et al. (2011) results, they concluded that a high concentration of free glucose may disrupts the oligosaccharide recognition function [108]. Additionally, even that the hydrolysates contain both oligo-glucan and mannan contents, according to Santin et al. (2001) and Zhang et al. (2005) results, when feed was supplemented with YCW, the improvement of broiler chicken productivity as well as the stimulation of the gut-associated immune system and growth body weight gain was observed [109,110].

Since β-glucans exhibit the ability to activate the immune system, other interesting application could be their use as carriers for drug delivery in form of micro- or nanoparticles. β-glucans have also been used as structural units for encapsulating drugs, serving as a specific target to enhancing the uptake by immune cells. Therefore, the micro- and nanoparticles based on β-glucans provide promising opportunities for drug delivery as structural units and as targeting ligands, wherein the combination of both promote a drug delivery vehicle with enhanced potency [111].

In some enzymatic hydrolysates, an increase of the DP_n value over reaction time was observed, which is the case of the SL2 hydrolysate. However, as stated before, the DP_n values are a number-average combined with both oligo-glucan and mannan contents. Therefore, in order to confirm the reliability of the obtained DP_n values, the SN of the enzymatic hydrolysates were analysed by HPLC-SEC. This technique is a pore size limited and diffusion controlled which separates macromolecules based on their size in solution. The separation was carried out through a column packed with porous particles with a narrow particle size distribution and very defined pore sizes. The larger sizes cannot penetrate any of the pores and elute first and smaller sizes, diffuse into pores, are retarded and elute later [112]. Since SL2 at 24h displayed a higher DP_n value than at 3h and the respective value was similar to BC2 at 3h, a comparison of both hydrolysates after HPLC-SEC separation is displayed on Figure 3.22, as well as the MW fraction results (Figure 3.23). Through the analysis of the chromatogram and the MW fraction results, both hydrolysates exhibited a similar profile separation and peak areas as well as MW fractions. Therefore, it was possible to conclude that the DP_n values appeared to be reliable as they are in accordance with the MW fractions and peak areas values, which is also applied to the other hydrolysates. It is also important to refer that this technique did not enable to determine the oligosaccharide compositions of each peak, meaning that the peaks could be β-glucan, MOS or even protein. As all the enzymatic hydrolysates displayed a high oligo-mannan content released and the first come out peak after separation presented the highest area, a fair assumption may be that this peak may corresponds to the oligo-mannan released.

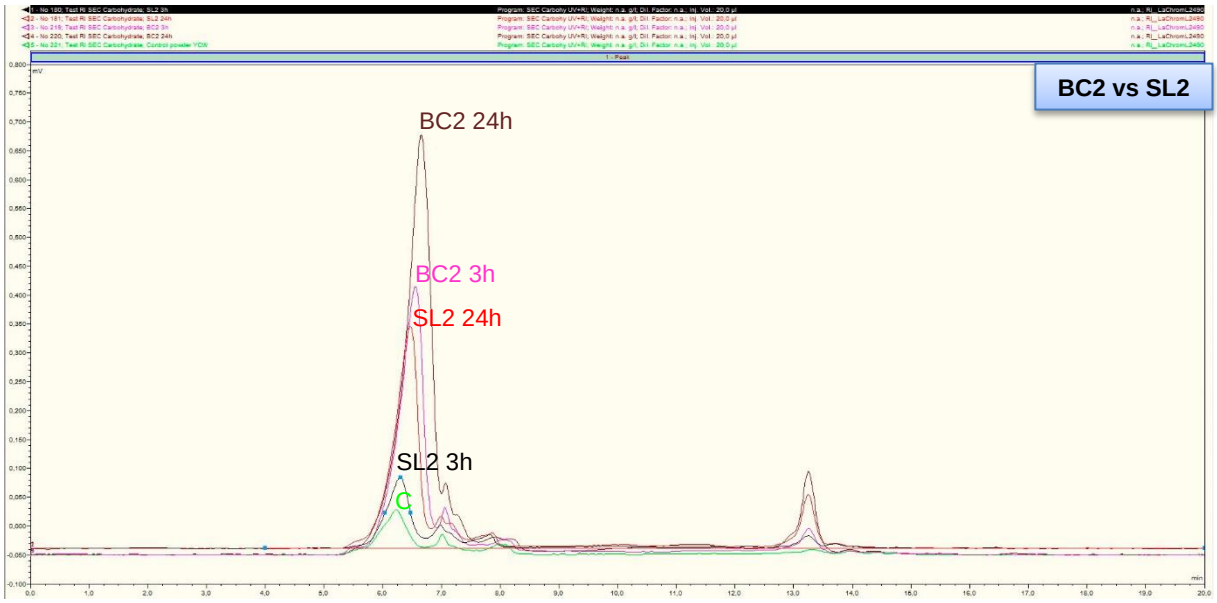

Figure 3.22 Chromatogram of the comparison between BC2 and SL2 hydrolysates over reaction time and with the control sample, C.

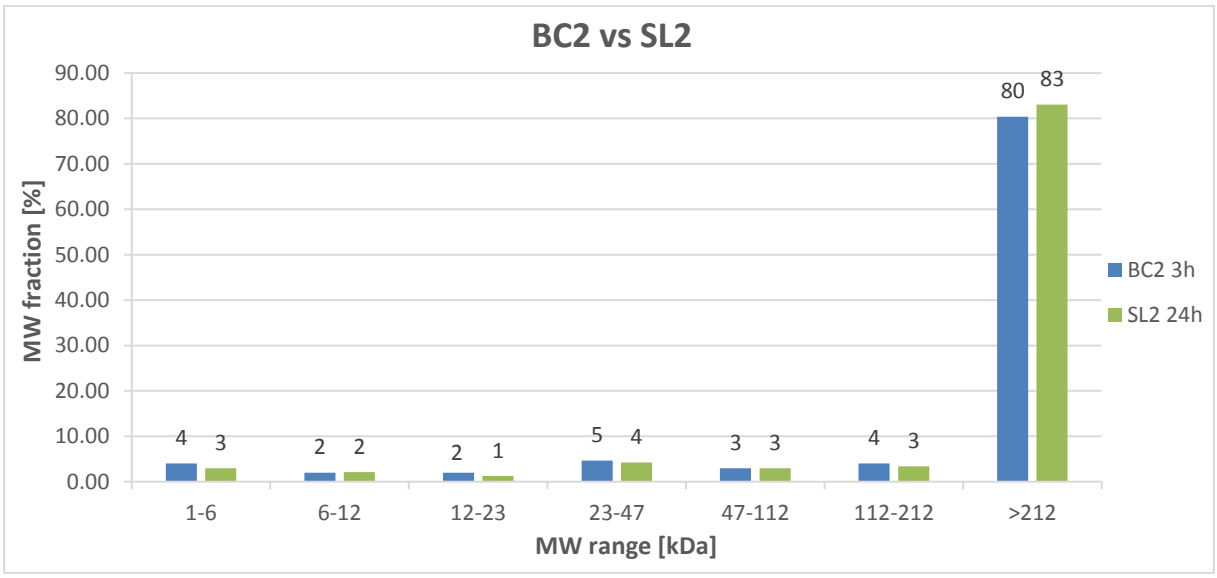

Figure 3.23 Molecular weight (MW) fraction results in the MW range of the standards for BC2 at 3h and SL2 at 24h.

In summary, the enzymatic hydrolysis is shown to be a method which allows the alteration of the molecular size of oligosaccharides, making them more suitable for several applications, such as in food, immune-stimulation or encapsulation [44]. Since, the BC2 hydrolysate exhibited to be a potential candidate for an immune-stimulant application, an additional study was conducted in order to optimise the hydrolysis assay to make it more suitable for an application in animal feed.

As future prospects, it is recommendable to develop a method that is able to distinguish the composition of each peak from the HPLC-SEC chromatograms for a better understanding about the solubilised oligosaccharides content.

CHAPTER 4

4. Optimisation of the BC2 hydrolysates

4.1. Materials and Methods

4.1.1. Materials

The materials, reagents and equipment used for the analytical methods were previously described (see 3.1.1).

4.1.2. Methods

4.1.2.1. Batch to batch trial

To enable the methodology characterization and reproducibility of the results, the enzymatic reactions with BC2 enzyme were done on two different batches of the autolysed YCW in liquid form collected at Ohly's production.

The YCWs batches collected from production presented the code numbers VK16-000529 and VK16-000530 with a 12% and 14% DM, respectively. A pH adjustment to 4.5 was carried out with 2.5M H_2SO_4 by using a pH meter.

The enzymatic reactions with two different batches were performed with the same procedure method and conditions as the enzyme screening (see 3.1.2.1). However, the experiments were executed in triplicates at time intervals of 3h, 6h, 8h and 24h in different baffled conical flasks, in order to construct an enzymatic kinetics in terms of sugar release and to avoid experimental errors. From each time reaction flask, 50g of representative samples were weighed in a precision scale and collected for additional studies. The enzymatic reactions were inactivated by heating at 80°C for 30min in a water bath. Table 4.1 shows all the variables and intervals tested for the batch to batch trials.

Table 4.1 Enzymatic reaction conditions for the batch to batch trials.

Dry matter	VK16-000529 – 12% VK16-000530 – 14%
Weight	200 mL/200 g
Temperature	50°C
pH	4.5
Time	3h, 6h, 8h, 24h
Shaking	120 rpm
Dosage enzyme	1% (w/w) per substrate DM

4.1.2.2. Variation of reaction conditions

Once a reproducibility test was performed and in order to study in detail the BC2 hydrolysates, a variation of reaction conditions of pH and temperature were done on autolysed YCW in liquid form collected at Ohly's production (VK16-000552) with a 13% DM. A pH adjustment to 4.5 was executed with 2.5M H_2SO_4 by using a pH meter.

BC2 enzyme is a β-glucanase, presenting an optimum pH range of 5 to 7 and is stable between 45 to 55°C. Therefore, the pH and temperature variations were selected within these ranges. The experiments were performed at time intervals of 3 and 6h, since from the batch to batch trials were observed a lower glucose and RS amounts release. The enzymatic reactions were performed in duplicates and in different 250mL baffled conical flasks wherein, from each, 50g of representative samples were weighed in a precision scale and collected for additional characterization. The enzymatic reactions were inactivated at 80°C for 30min in a water bath. In Table 4.2 is presented the reaction conditions for the variation of temperature and pH trials.

Table 4.2 Enzymatic reaction conditions for the variation of temperature and pH trials.

Dry matter	13%
Weight	200 mL/200 g
Temperature	45°C, 50°C, 55°C, 60°C
pH	4.5, 5.0, 5.5
Time	3 – 6h
Shaking	120 rpm
Dosage enzyme	1% (w/w) per substrate DM

4.1.2.3. Hydrolysates characterization

Afterwards, a hydrolysates characterization was conducted for the batch to batch and variation of reaction conditions trials. The enzymatic hydrolysates were characterized both analytically and chemically, such as solubilisation and phase separation, reducing and total sugars, free glucose and mannose contents and MW profiles. The procedure methods were previously described in detail in sub-sections 3.1.2.2.2 and 3.1.2.2.4 to 3.1.2.2.8.

4.2. Results

4.2.1. Total reducing sugars, free glucose and mannose contents

Both for the batch to batch and variation of the condition reaction trials, the total reducing sugars (RS), free glucose and mannose contents were measured for the supernatant (SN) of the BC2 hydrolysates. The obtained results are presented in the Figure 4.1 and Figure 4.2, in percentage of amount of total sugar in the starting material (SM). As performed before for the SM in powder form, the total glucose and mannose contents were previously determined by acid hydrolysis for the YCW in liquid form collected at Ohly's production, which presented 17.9% (w/w) and 28.5% (w/w), respectively. A lower total glucose content were observed by the comparison between the YCW in powder form used for the enzyme screening. Appendix E presents the standard deviation results of the RS, free glucose and mannose measurements for both trials.

Batch to batch trials

The sugars content release presented similar results for both production batches. However, from 6h to 24h, the release of RS was slightly lower for the batch VK16-000529. At 24h, a lower free glucose released was observed when compared with the results from 3h to 8h reaction time, indicating to a possible experimental error.

Comparing the results from both production batches with the obtained from the screening, at 3h and 24h, a higher release of sugars was verified for the YCW in powder form used for the screening. Moreover, at 24h, the free glucose released with the YCW in powder form was shown to be two and three times higher when compared with the liquid form of the batch VK16-000530 and VK16-000529, respectively. The RS released was also higher for the YCW in powder, noticing a higher difference at 24h reaction time. All the enzymatic hydrolysates presented a reduced release of free mannose, which was also observed in the enzyme screening.

Variation of reaction conditions

For all the reaction conditions, the sugars content release increased over reaction time and a reduced content of free mannose released was verified, which was also noticed for the enzyme screening and batch to batch trials.

For both 3h and 6h, the obtained hydrolysates at 60 °C shown to be the lowest release of sugars for all the different pHs.

Most of the hydrolysates obtained from different reaction conditions displayed a highest free glucose content in relation of the RS content released. However, for both 3h and 6h, the hydrolysates obtained at pH 4.5; 55 and 60°C, pH 5.0; 45°C and pH 5.5; 60°C, presented the same content released of free glucose and RS. Additionally, at pH 4.5; 45°C and pH 5.5; 50°C, both at 6h reaction time, showed

the same amount released of free glucose and RS, wherein the latter conditions exhibited an increase of two times more of free glucose and RS contents released from 3h to 6h.

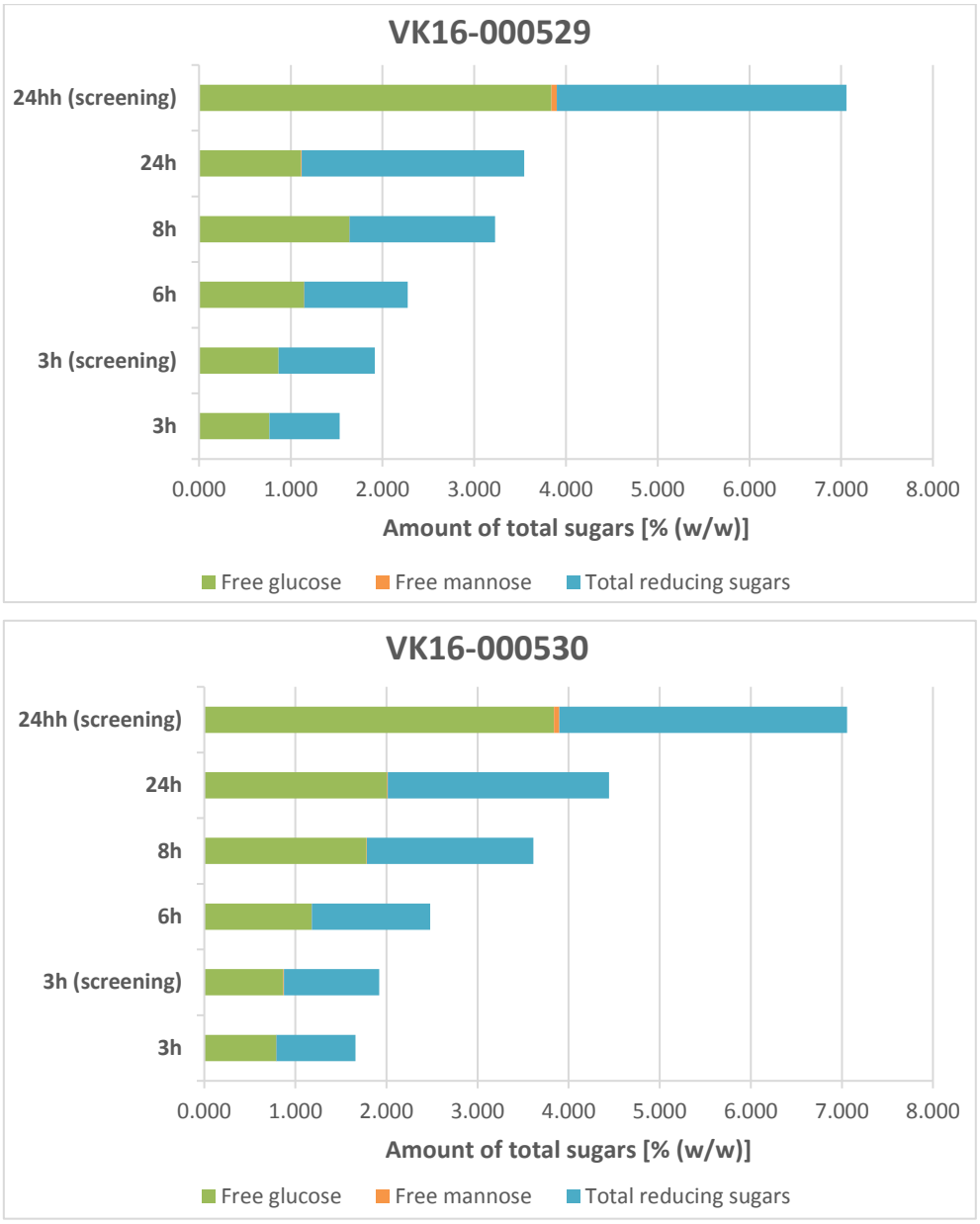

Figure 4.1 Reducing sugars, free glucose and mannose contents in the SN of the BC2 hydrolysates, over reaction time, from different production batches: VK16-000529 (top) and VK16-000530 (bottom).

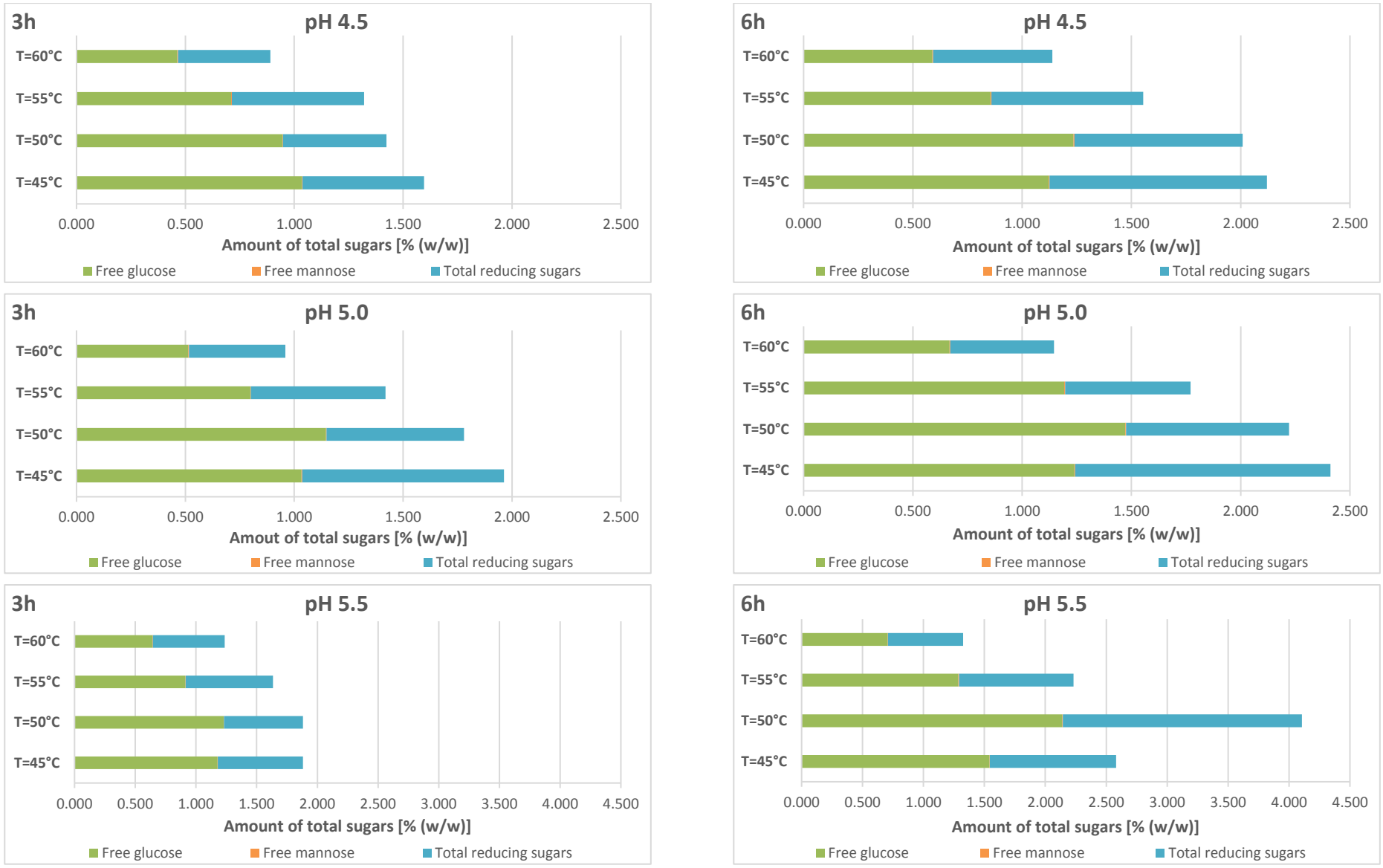

Figure 4.2 Reducing sugars, free glucose and mannose contents in the supernatant of the enzymatic hydrolysates, over reaction time, from different reaction conditions.

4.2.2. Total sugars: oligo-glucan and mannan contents

The total glucose and mannose contents were determined for the SN of the BC2 hydrolysates, at 3h and 6h reaction time, through acid hydrolysis and analysed by HPAE-PAD. The obtained results are presented in Figure 4.3 for the batch to batch trials and Figure 4.4 and Figure 4.5 for the variation of reaction conditions trials, both in percentage of amount per DM. Appendix F contains the standard deviations of the total glucose and mannose results measured by HPAE-PAD.

Batch to batch trials

As the first observation, all the enzymatic hydrolysates, both at 3h and 6h, presented a higher release of oligo-mannan than oligo-glucan content, which was also noticed in the enzyme screening.

Through the comparison of both batches, similar oligo-glucan and mannan contents, over reaction time, were obtained. Additionally, for both production batches, the oligo-mannan released increased over reaction time until 8h and maintained constant from 8h to 24h. In contrast, the oligo-glucan content presented a fluctuation in the results over reaction time, in both batches, varying from around 4 to 6% (w/w), which appear to remain constant over reaction time.

Related to the oligo-glucan and mannan contents released in the enzyme screening, the oligo-mannan contents were similar when compared with the production batches, however, a higher release of oligo-glucan was observed for the enzyme screening.

Variation of reaction conditions

As also observed for the enzyme screening and batch to batch trials, the release of oligo-mannan was higher than oligo-glucan content for both at 3h and 6h reaction time.

The highest release of oligo-glucan, over reaction time, was observed at pH 5.0 and 5.5, both at 45 and 50°C. The obtained hydrolysates at different pHs and 55°C, pH 4.5; 50°C and pH 5.5; 60°C, displayed a constant release of oligo-glucan over reaction time. Additionally, at pH 4.5; 45 and 60°C and pH 5.0; 60°C, the oligo-glucan results showed some fluctuations, which decreased over reaction time.

For most of the hydrolysates, an increase of oligo-mannan released was observed. However, at pH 4.5; 60°C and pH 5.0; 55 and 60°C, the release of oligo-mannan remained constant and at pH 4.5; 45°C a decrease of oligo-mannan over reaction time was verified. The obtained hydrolysates at pH 4.5; 55°C, pH 5.0; 45 and 50°C and pH 5.5; 45, 50 and 55°C, showed the highest release of oligo-mannan.

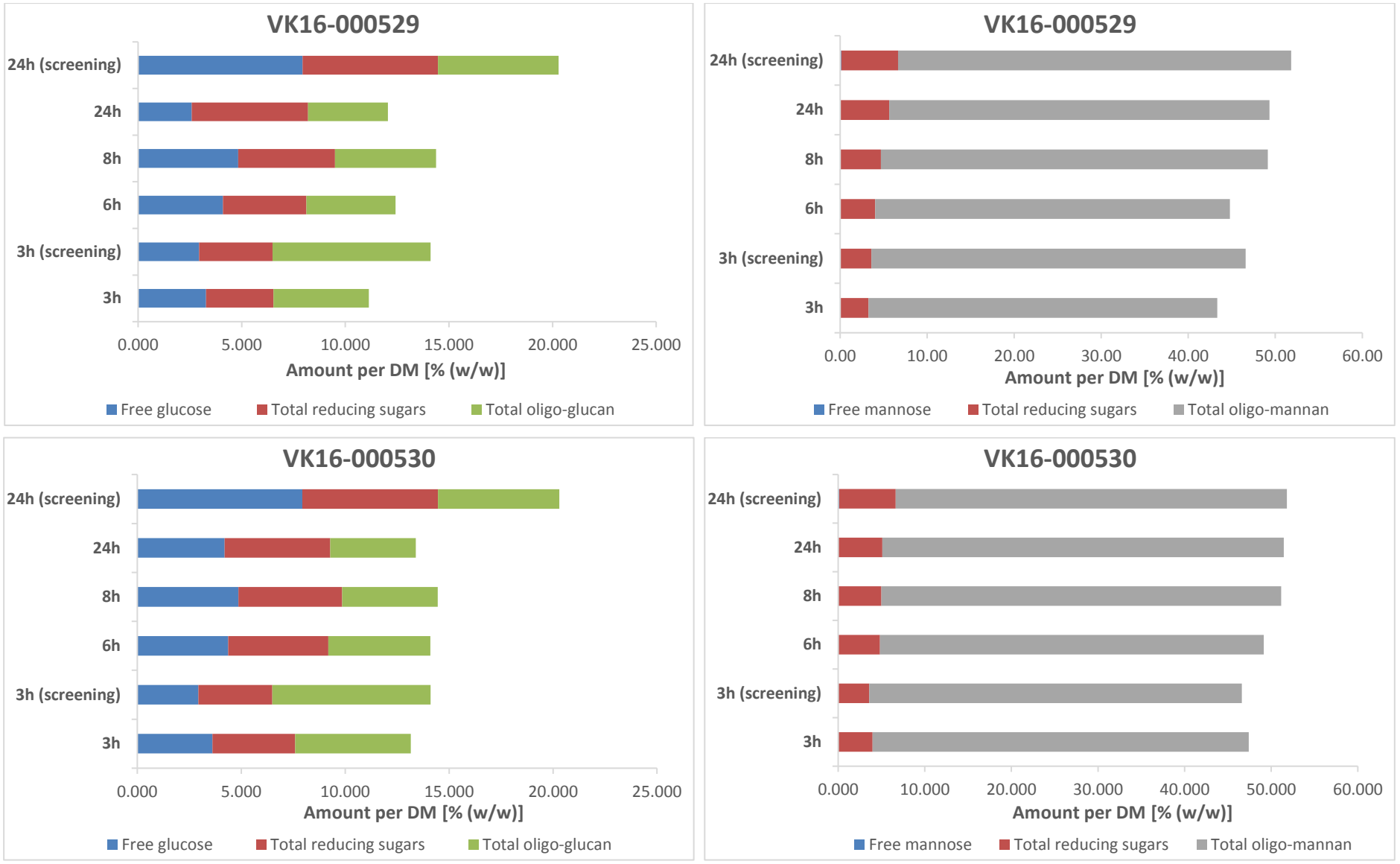

Figure 4.3 Comparison between the total RS, free glucose and mannose, total oligo-glucan and mannan contents, over reaction time, from different production batches.

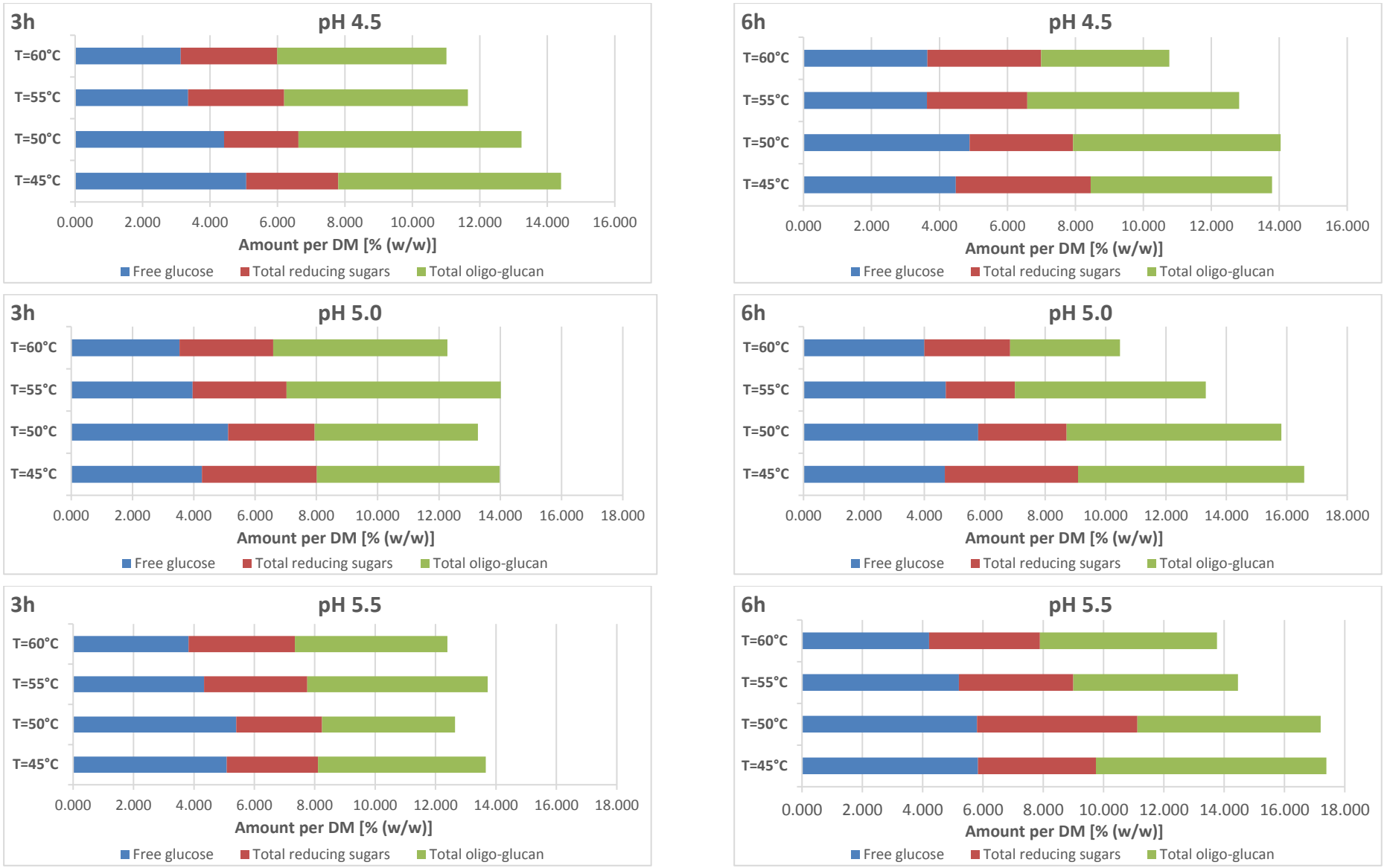

Figure 4.4 Comparison between the total RS, free glucose and total oligo-glucan contents, over reaction time, from different reaction conditions.

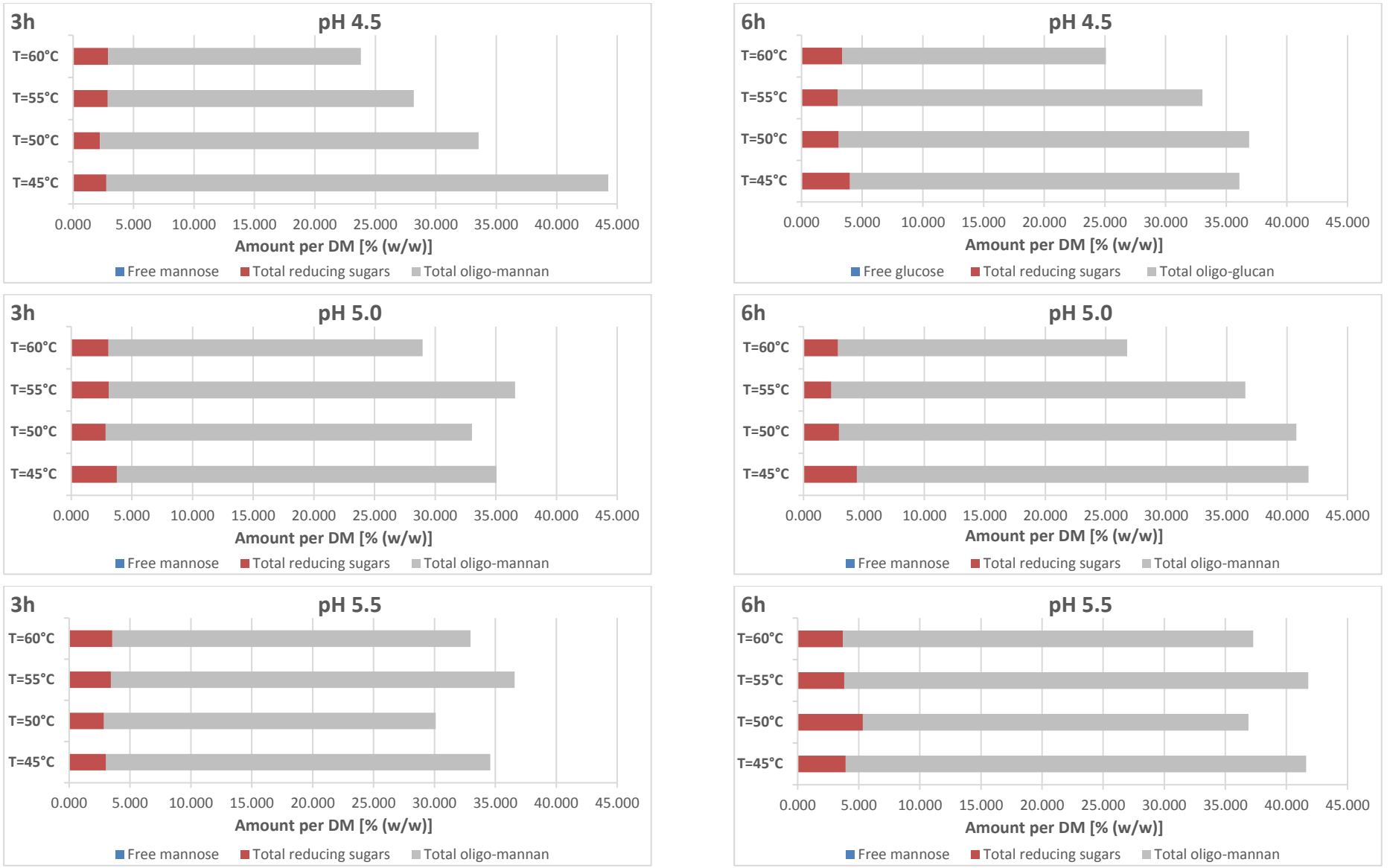

Figure 4.5 Comparison between the total RS, free mannose and total oligo-mannan contents, over reaction time, from different reaction conditions.

4.2.3. Number-average degree of polymerisation (DP_n)

Through the previously presented results, oligo-glucan and mannan contents released in the SN of the BC2 hydrolysates at 3h and 6h reaction time, the DP_n was estimated by using the Equation 4, for both batch to batch and variation of reaction conditions trials (Table 4.3 and Table 4.4).

Batch to batch trials

Comparing the obtained results of both production batches, the DP_n values were similar. Additionally, a decreased of the DP_n values over reaction time was observed.

Table 4.3 DP_n values for the SN of the BC2 hydrolysates, over reaction time, from different production batches.

Time [h]	DP_n	
	VK16-000529	VK16-000530
3	13.70	12.31
6	11.20	10.20
8	10.55	10.17
24	8.47	9.93

DP_n, number-average degree of polymerisation.

Variation of reaction conditions

In most of the enzymatic hydrolysates, the DPn values decreased or remained constant over reaction, what is the case in the reaction conditions at pH 5.0; 45 and 60°C and pH 5.5; 45, 55 and 60°C. Additionally, a fluctuations of the results were observed for some reaction conditions, namely at pH 4.5; 55°C and pH 5.0; 50 and 55°C, wherein the DP_n values increased over reaction time.

Table 4.4 DP_n values for the SN of the BC2 hydrolysates, over reaction time, from different reaction conditions.

Temperature [°C]	pH	DP_n	
		3h	6h
45	**4.5**	17.61	9.43
	5.0	9.93	10.17
	5.5	12.38	11.59
50	**4.5**	17.20	13.11
	5.0	12.58	15.34
	5.5	11.13	7.07
55	**4.5**	10.79	12.31
	5.0	13.21	17.67
	5.5	11.44	11.46
60	**4.5**	9.06	7.62
	5.0	10.34	9.71
	5.5	9.77	10.74

DP_n, number-average degree of polymerisation.

4.2.4. Molecular weight analysis

In order to relate the estimated DP_n, the molecular weight (MW) for the SN of the BC2 hydrolysates was analysed by HPLC-SEC. The following figures exhibit the resulting chromatograms of the BC2 hydrolysates for both batch to batch and variation of reaction conditions trials, as well as the respective MW fraction results.

All the samples exhibited a similar separation profile from around 5 to 8.5min and between 12 to 14min. Comparing the retention time (RT) of the standards (Figure 3.9), the samples displayed peaks in a range of 212 to 23kDa and soon after the β-laminaripentaose peak (0.99kDa). As also verified for the enzyme screening samples, the peak after β-laminaripentaose may corresponds to free glucose released. Additionally, the peaks in the range of 7 to 8.5min and between 12 to 14 min showed a low height, suggesting a not well separation of the compounds.

Since the MW fraction results were determined based on the area of the peaks, the chromatograms will be analysed and compared in terms of it.

Batch to batch trials

Since it was obtained similar results for both production batches, the MW profile and fractions were only determined and analysed for the batch VK16-000529.

Related to the first peak, in the range of 5 to 7min, the area remained constant over reaction time. For the enzyme screening samples, an increase of height over reaction time was observed in the last peak from around 12 to 14min. However, for this batch, this peak exhibited a higher height at 6h and 8h than 24h. Since this peak may corresponds to free glucose, from the previous analysed results, a decrease of free glucose from 8h to 24h reaction time was also verified for this batch.

In addition, the obtained results from this production batch were compared with the enzyme BC2 hydrolysates obtained at 3h and 24h from the enzyme screening (Figure 4.7 and Figure 4.8). An identical separation profile was observed, however, in relation of the first peak, a higher area was verified BC2 hydrolysates obtained from enzyme screening. Additionally, for the comparison of both control samples from the liquid and powder YCW, C-liq and C-pow, respectively, the first separated peak also presented a higher area for the powder YCW, whereas the liquid YCW does not appear to present any peak.

All the referred analysis are also supported by the evaluation of the graphical representations of the MW fraction results.

Variation of reaction conditions

Through the comparison of the several different reaction conditions, a similar separation profile was observed at pH 5.0 and 5.5 and at different temperatures. However, in the range of 6.5 to 7.5min, two low height peaks were verified only for the reaction conditions at pH 4.5 and different temperatures.

Additionally, at pH 4.5 and 60°C for both reaction times, the first separated peak from around 5 to 7min is not well defined, suggesting a not well separation of the components.

Related to the first separated peak, for all the different pHs, shown to exhibit the highest height at 50°C and 6h reaction time. The area of this peak displayed to remain constant over reaction time for most of the different reaction conditions. Only at pH 4.5; 45 and 50°C a slightly increase of area was observed from 3h to 6h. All the mentioned evaluations are also verified by the analysis of the graphical representations of the MW fraction results.

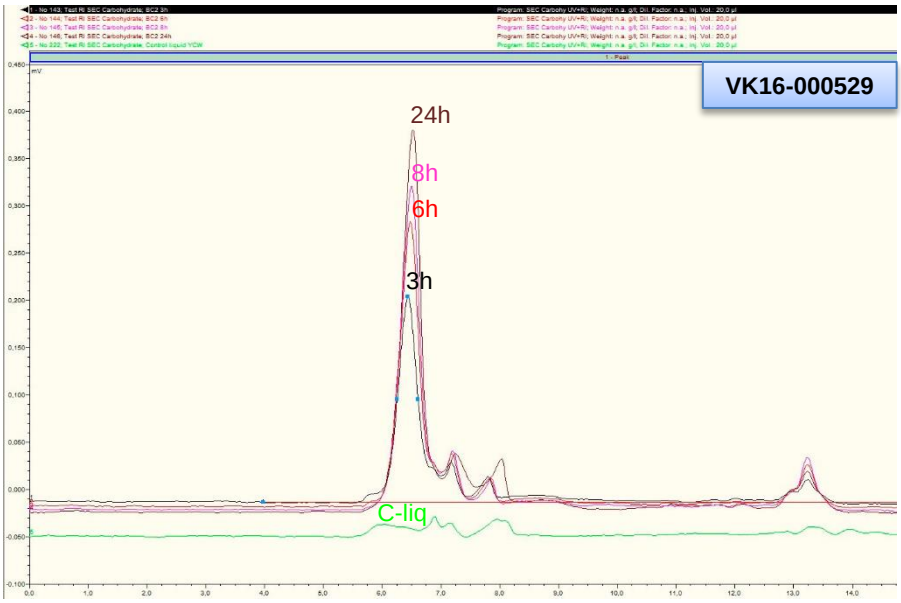

Figure 4.6 Chromatogram of BC2 hydrolysates from the production batch VK16-000529, over reaction time, and comparison with the control sample, C-liq.

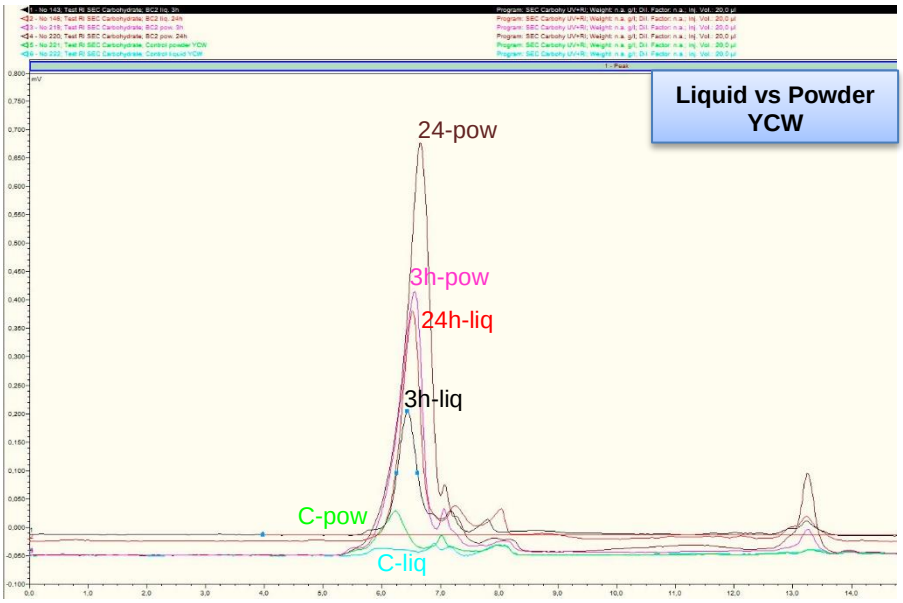

Figure 4.7 Chromatogram of comparison between the BC2 hydrolysates at 3h and 24h from the liquid and powder YCW as well as the respective control samples, C-liq and C-pow.

Figure 4.8 Molecular weight (MW) fraction results in the MW range of the standards. Comparison between the control samples of the liquid and powder YCW, C-liq and C-pow, respectively (top). BC2 hydrolysates from the production batch VK16-000529, over reaction time (middle). Comparison between the BC2 hydrolysates from the liquid and powder YCW, BC2-liq and BC2-pow, respectively (bottom).

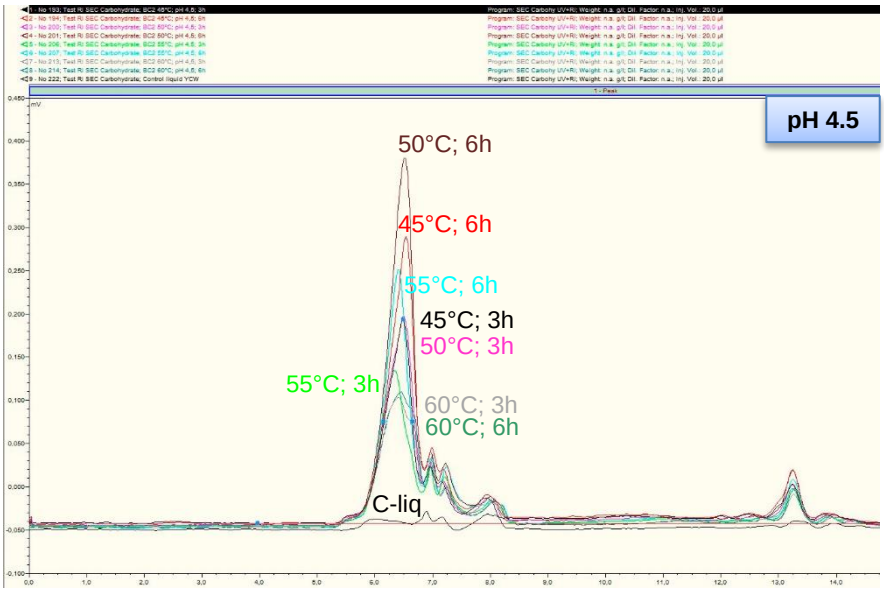

Figure 4.9 Chromatogram of the BC2 hydrolysates obtained at pH 4.5 and different temperatures, over reaction time, and comparison with the control sample, C-liq.

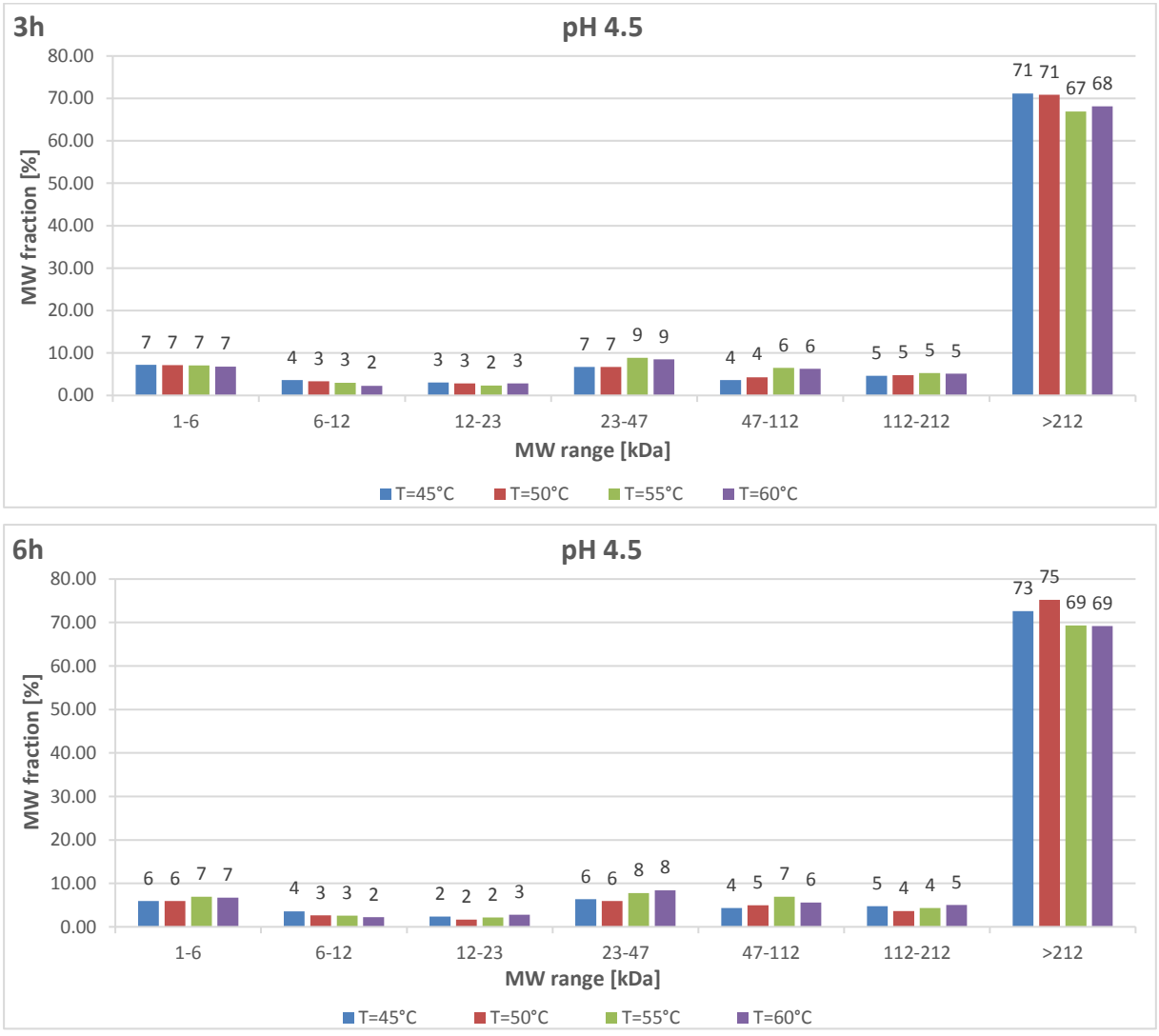

Figure 4.10 Molecular weight (MW) fraction results in the MW range of the standards for the BC2 hydrolysates at pH 4.5 and different temperatures: 3h (top) and 6h (bottom).

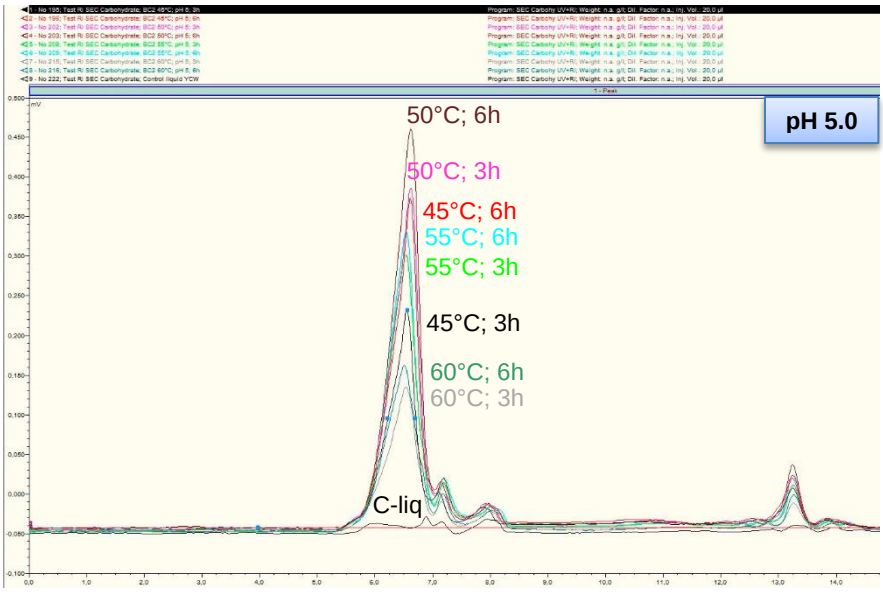

Figure 4.11 Chromatogram of the BC2 hydrolysates obtained at pH 5.0 and different temperatures, over reaction time, and comparison with the control sample, C-liq.

Figure 4.12 Molecular weight (MW) fraction results in the MW range of the standards for the BC2 hydrolysates at pH 5.0 and different temperatures: 3h (top) and 6h (bottom).

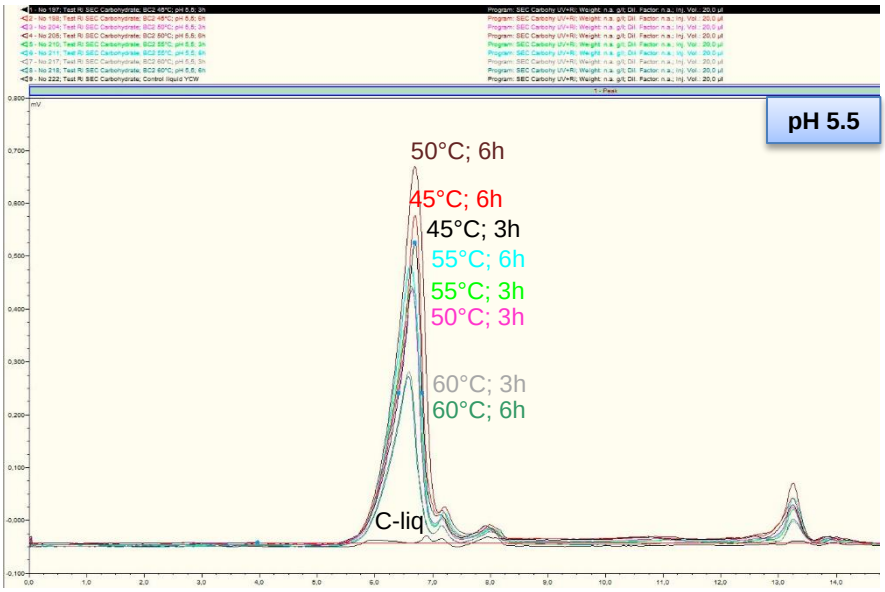

Figure 4.13 Chromatogram of the BC2 hydrolysates obtained at pH 5.5 and different temperatures, over reaction time, and comparison with the control sample, C-liq.

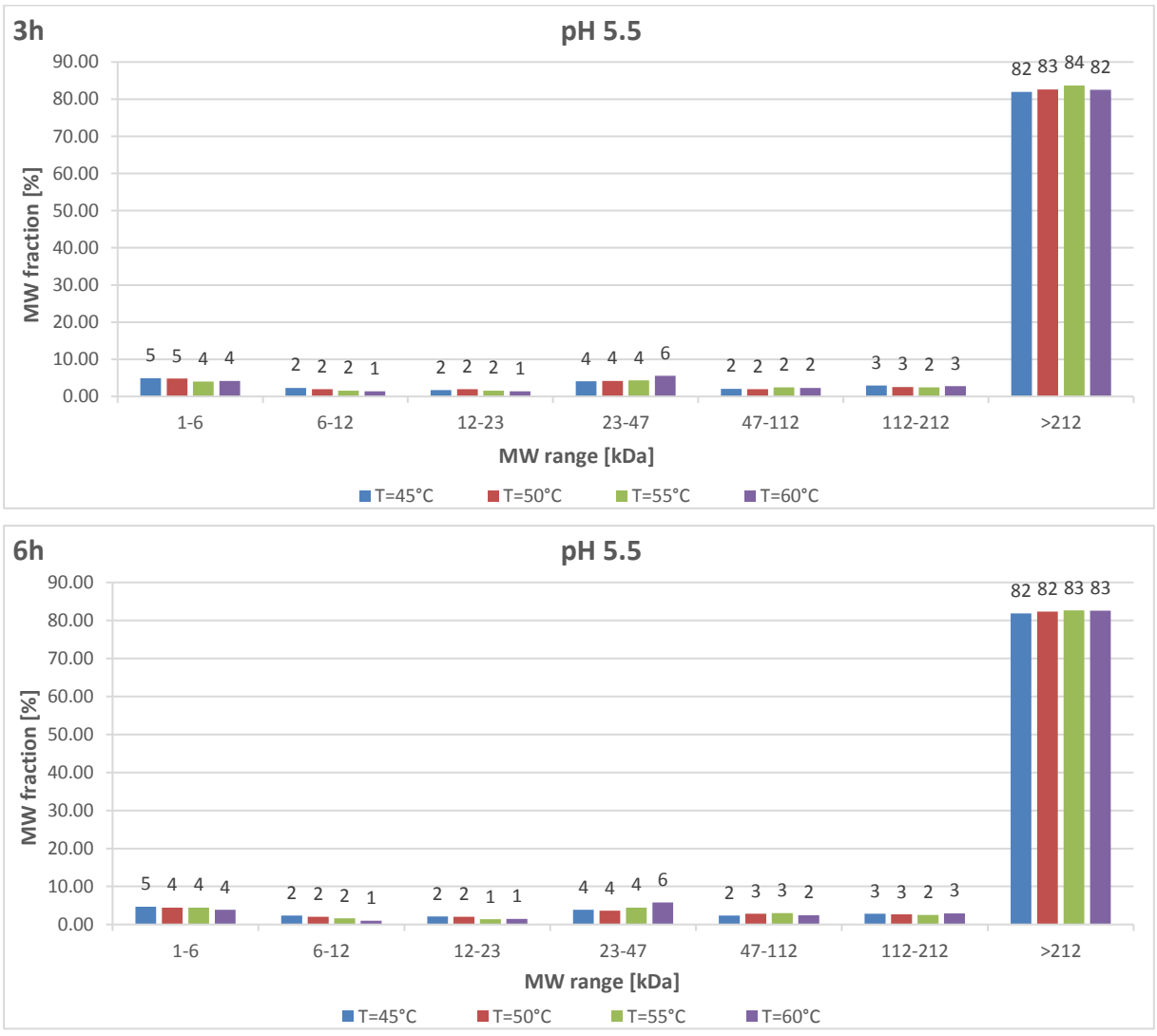

Figure 4.14 Molecular weight (MW) fraction results in the MW range of the standards for the BC2 hydrolysates at pH 5.5 and different temperatures: 3h (top) to 6h (bottom).

4.3. Discussion and Conclusion

Through the analysis of the batch to batch trials results regarding the number-average degree of polymerisation (DP_n) (Table 4.3), molecular weight (MW) fractions (Figure 4.6 and Figure 4.8) as well as the reducing and free sugars (Figure 4.1), oligo-glucan and mannan released (Figure 4.3), both studied batches (VK16-00529 and VK16-000530) exhibited similar results, confirming the reproducibility of the results. However, the batch VK16-000529 at 24h presented a lower release of free glucose when compared at 3h, 6h and 8h reaction time, which may indicate to an experimental error, for instance on the applied dilution to measure the free glucose or the enzyme dosage added before the enzymatic reaction.

Since the batch to batch trials were performed in same reaction conditions as the enzyme screening experiments, the reducing and free sugars and oligo-glucan and mannan contents released, obtained in both trials, were compared (Figure 4.1 and Figure 4.3). Related to the reducing and free sugars, a higher amount was released at 3h and 24h in the enzyme screening experiments. Additionally, the same observation was verified for the oligo-glucan content released, however the oligo-mannan content displayed similar results in both experiments. Through the analysis of the obtained chromatograms by HPLC-SEC and the molecular weight (MW) fraction results (Figure 4.7 and Figure 4.8), a higher peak areas and MW fractions were observed for the BC2 hydrolysates of the enzyme screening experiments. The higher content of reducing and free sugars and oligo-glucan in the enzyme screening experiments may be due to the use of different starting material (SM) forms, wherein the enzyme screening experiments were performed with YCW in powder form and the BC2 optimisation experiments were done with YCW in liquid form collected at Ohly's production. According to Oliveira et al. (2012), they studied the influence of different drying temperatures on quality of β-glucans and concluded that high temperatures used in drying may have caused a degradation and depolymerisation of β-glucans into fragments of low molecular weight (MW) [87]. As in the enzyme screening experiments, the SM was used in powder form, the latter statement may be a fair explanation for the higher amounts of free and reducing sugars and oligo-glucan released, which may promote a slight open to the cell wall (CW), providing a more accessible digestion through the enzymes [87]. This assumption can also be supported by the different obtained amounts of total glucose determined for the SM in powder and liquid form, wherein the former presented a higher total glucose content, corresponding to 23.9% (w/w) than the latter (17.9% (w/w)). Additionally, both different forms of the SM exhibited similar contents of total mannose, in the range of 29 to 30% (w/w), supporting the latter observations regarding the similar results of oligo-mannan by the enzyme screening and BC2 optimisation experiments.

The BC2 enzyme presents a β-glucanase activity, exhibiting an optimum pH range of 5 to 7 and is stable between 45 to 55°C. From the latter batch to batch trials, the BC2 hydrolysates displayed DP_n values higher than 10 at 3h and 6h, thus the optimisation of reaction conditions were performed at these reaction times.

Comparing the temperature and pH conditions applied, at 60°C and for the different pHs, a lower reducing and free sugars were observed. This observation is evident since at 60°C the enzyme is declared as unstable.

At pH 4.5, 5.0 and 5.5; 45 and 50°C at 6h, a high enzyme activity was observed by the release of free glucose and RS equal or more than 2% (w/w) in total sugars. However, for an immune-stimulant activity, low content of RS and free glucose is required, as high free glucose may disrupts the oligosaccharide recognition function [108] and low RS may be associated to high amount of oligosaccharides. Therefore, at pH 4.5; 45 and 50°C, pH 5.0; 50 and 55°C and pH 5.5; 45 and 55°C at 3h, a lower release of free glucose and RS, less than 2% (w/w) in total sugars, was observed which may be considered as the reaction conditions to obtain DP_n values higher than 10.

Regarding the oligo-glucan content, the highest released amounts were verified at pH 4.5; 45 and 50°C and pH 5.0; 45, 50 and 55°C, followed by pH 5.5; 45 and 55°C, over reaction time. In contrast, the oligo-mannan content released for all the reaction conditions remained almost constant over reaction time. As also observed in the enzyme screening results, some discrepancies on the oligo-glucan and mannan results were verified over reaction time, whereby the same assumptions which were done before, are also applied in this case [64,101,102].

Related to the obtained DP_n values, the highest were at pH 4.5; 45 and 50°C, pH 5.0; 50 and 55°C and pH 5.5; 45 and 55°C, in the range of 18 to 11, over reaction time. As also mentioned before, a polymer chain length of 7 to 15 glucose units is required for binding in an immune recognition, as it was reported by Hanashima et al. (2013) and Adams et al. (2008) [43,106,107]. Therefore, it was possible to conclude that for a high immune-stimulant activity, the optimal reaction conditions may be at pH 4.5; 45 and 50°C at 3h reaction time. Through the analysis of the obtained chromatograms and the MW fraction results from these reaction conditions (Figure 4.9 and Figure 4.10), both presented similar areas for all the peaks. Consequently, the estimated DP_n values are reliable as they are in accordance with the peak areas and MW fractions results.

In summary, the batch to batch trials allowed to test the reproducibility of the results as well as to conclude that different amounts of sugars are released from YCW in powder and liquid form. This may be due to the drying temperature that promoted the degradation of the oligosaccharides content and thus, making the YCW more accessible digestion by the enzymes. Reaction conditions pH 4.5; 45 and 50°C at 3h reaction time, were shown to be the optimal conditions to obtain BC2 hydrolysates suitable to be used as immune-stimulant in animal feed, presenting a DP_n value of 17.

As future prospects, in order to complete this study, a reduction and variation of the enzyme dosage should be also done, to reduce costs associated with a switch to production scale. Moreover *in vitro* tests should also be performed to study the affinity of this hydrolysate to bind and recognize the immune cells.

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Appendix A Auxoferm HCT specification

Inactive Yeast

Auxoferm HCT

Ohly®
An ABF Ingredients Company

01/14

Auxoferm HCT is the cell wall fraction gained from pure culture yeast of the species S. cerevisiae. The soluble parts of the yeast cells are removed by a gentle digestion, the remaining cell wall fraction is then purified and finally dried.
Auxoferm HCT stands out for its content of high molecular polysaccharides, the glucan and mannan. The fine powder is of light beige to beige appearance and characterized by its typical yeast flavour.

Average analysis

• Dry matter		96,0 %
• Protein (Nx6,25)	<	30,0 %
• Polysaccharides (total)		52,0 %
• Sodium		0,5 %
• Lipids		11,0 %
• Ash		4,0 %
• PH (in 2% solution)		5,8 – 6,8

Composition of polysaccharides

• Glucan		20,0 %
• Mannan		25,0 %
• Chitin	<	1,0 %
• Glycogen	<	2,0 %

Selected minerals

• Sodium	275 mg/100 g
• Potassium	300 mg/100 g
• Magnesium	20 mg/100 g
• Calcium	50 mg/100 g
• Phosphorus	1200 mg/100 g

M4 FB102 -01

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Inactive Yeast

Auxoferm HCT

01/14

Applications: Our high quality and pure yeast *beta*-glucans can be used in feed applications such as aquaculture. Material is listed as straight feeding stuff within EU – 12.1.5 Yeasts and like products [Brewer´s yeast] [Yeast product]. Material is certified according to the QS (Qualität und Sicherheit) requirements on feeding stuff.

Benefits: This *beta*-(1,3)/(1,6) glucan is suitable for feed applications and has shown positive effects on the farming.

Storage: Store under cool and dry conditions. Keep containers closed when not in use.

Shelf life: 24 months, if unopened and stored under 30 °C (85 °F) and less than 80 % humidity.

Ingredients: Yeast cell wall fraction

Packaging: 25 kg (55.1 lbs) multi wall paper bag with PE-inliner

Kosher PARVE Certification

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Inactive Yeast

Auxoferm HCT

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PRODUCT SPECIFICATION

Chemical and physical data

Parameter	Unit	Min.		Max.	Method
Dry matter	%	93,0			105° C, 4 h
Protein in product	%			30,0	Kjeldahl
NaCl	%	-		1,0	Potentiometric titration with $AgNO_3$
PH (in 2% solution)		5,8		6,8	Electrochemical method
Ash	%	-		8,0	550° C, 4 h

Microbiological data

Parameter	Unit		Method
Total plate count	cfu/g	max. 10.000	Standard I Agar, 48 h, 30° C
Yeasts and moulds	cfu/g	max. 100	YGC-Agar, 96 h, 25° C
E. coli	cfu/g	neg.	Fluorocult-BRILA Broth, 24 h, 37° C. Followed by test on Indol formation
Coliformes	cfu/g	max. 10	Fluorocult-BRILA Broth, 48 h, 37° C.
Sulphite reducing clostridia	cfu/g	max. 200	Meat liver agar, anaerobic, 48 h, 37° C
B. cereus	cfu/g	max. 1000	Cereus selective agar base acc. to Mossel, 48 h, 30° C
Salmonella	neg./25 g	neg.	ELISA-Test, AOAC-OMA May 1996, No996.08

Typical Nutritional Information (average value per 100g product)

Energy (kcal)	337		Thiamin (mg)	~
Energy (kJ)	1415		Riboflavin (mg)	~
Protein (g)	30		Niacin (mg)	~
Carbohydrates, by difference (g)	52		Vitamin B6 (mg)	~
Sugars (g)	~		Folic acid (mg)	~
Carbohydrates, digestible (g)	~		Ca-pantothenate (mg)	~
Fat, total (g)	11			
Fat, saturated (g)	~			
Fat, mono-unsaturated (g)	~			
Fat, polyunsaturated (g)	~			
Trans fatty acids (g)	~			
Cholesterol (mg)	~			
Dietary fibre (g)	45			
Sodium (g)	≤ 0,5			

~ data not available

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Appendix B Screened Enzymes

Table B.1 List of screened enzymes, including activity profile, GMO, temperature and pH ranges and activity.

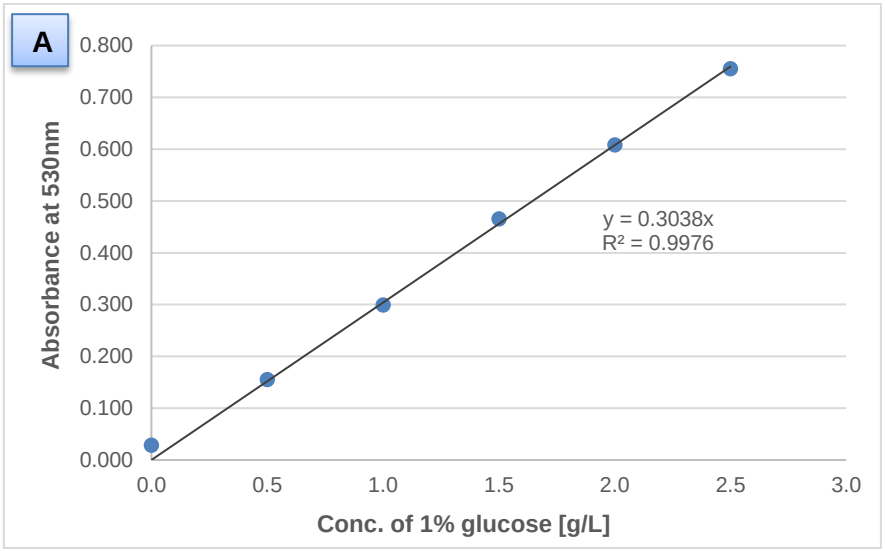
Enzymes	Activity profile	Organism	Activity	T range [°C]	pH range	GMO
AE1	Pectinase with hemicellulolytic side activities	*Aspergillus* sp. *Trichoderma* sp.	9,600 PGU/mg	50-65	4-7	Yes
AE2	Cellulase with many side activities	*Trichoderma reesei*	15,000 ECU/mg	65 (opt. 50-60)	3-6 (opt. 4-5)	Yes
AE3	β-(1,3/1,6)-Glucanase with high protease side activities	*Trichoderma* sp.	>2,500 LAM/g	60 (opt. 50)	4-7	Yes
AE4	Mannanase as main activity with β-glucanase, xylanase and cellulase side activities	*Trichoderma reesei*	>1,000,000 MNU/g	40-75 (opt. 70-75)	2-7 (opt. 3-5.5)	
AE5	β-(1,3)-Glucanase	*Trichoderma citrinoviride*	50 LAMU/g	50-70 (opt. 60)	3.5-6 (opt. 5)	
AO1	Protease and peptidase with many side activities	*Aspergillus oryzae*	>40,000 U/g	30-50 (opt. 50)	3-6 (opt. 6)	
AO2	Protease, lipase, β-mannosidase and α-galactosidase as side activities	*Aspergillus niger*	>10,000 U/g	20-70 (opt. 80)	2-7.5 (opt. 4)	
AO3	Xylanase	*Aspergillus niger*	>90,000 U/g	30-55 (opt. 50)	4-9 (opt. 4.5)	
BC1	α-(1,4)-Amylase	*Aspergillus oryzae*	25,000 U/g	35-55	opt. 3-6	No
BC2	β-Glucanase	*Trichoderma* sp.	12,000 U/g	45-55	opt. 5-7	No
BC3	β-(1,4)-Glucanase with cellulase and xylanase side activities	*Trichoderma longibrachiatum*	12,500 U/g	50-65	opt. 3-6.5	No
BC4	Cellulase with cellobiase, β-glucosidase, β-glucanase side activities	*Trichoderma* sp.	1,500 U/g	50-70	opt. 3.5-6	No
DA1	Cellulase and β-(1,3/1,6)-glucanase	*Trichoderma reesei*	>6,200 IU/g	Opt. 60	3.5-5.5 (opt. 4)	No
DA2	Cellulase and β-(1,4)-glucanase	*Trichoderma reesei*	> 2,250 BGLU/mL		4.9-5.3	No
DA3	β-(1,4)-Glucosidase		>3,000 U/g		5-6	Yes
DA4	Glucoamylase	*Aspergillus niger*	>350 GAU/g	Opt. 68	3.5-5.5 (opt. 4.5)	No
DS1	Endo-β-(1,3/1,4)-Glucanase	*Talaromyces emersonii Trichoderma longibrachiatum*	>100,000 BGF/g		4-5	No
DS2	Endo-β-(1,3/1,4)-Glucanase	*Talaromyces emersonii*	>40,000 BGF/g		4-4.5	No
DS3	Glucoamylase	*Aspergillus niger*	80,000 AGI			No
DS4	Cellulase	*Aspergillus niger*	7,900 CXU/g			No
DC1	Xylanase, β-(1,3/1,4)-glucanase and cellulase with pectinase, mannanase, xyloglucanase, laminarase, β-glucosidase, β-xylosidase and α-L-arabinofuranosidase side activities	*Trichoderma longibrachiatum* (formerly *Trichoderma reesei*)	Xylanase 90,000 to 110,000 U/g; β-glucanase 30,000 to 35,000 U/g; Cellulase 115,000 to 140,000 U/g	40-57	4.2-6.5	

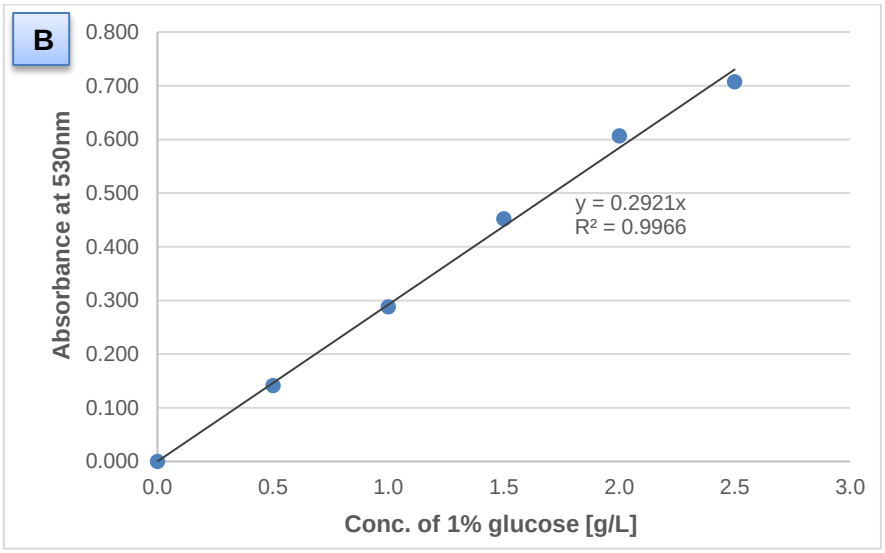
Enzymes	Activity profile	Organism	Activity	T range [°C]	pH range	GMO
ET1	Pectinlyase	*Aspergillus* sp.	22,500 PECTU/mL	Up to 65		Yes
ET2	Pectinase and polygalacturonase	*Aspergillus* sp.	46,000 PGU/mL	10-50		Yes
ET3	Pectinase with exo-β-(1,3)-glucanase side activity		2,500 PGNU/g; 100 BGXU/g			
ET4	Purified β-glucanase	*Streptomyces* sp.				Yes
ES1	Endo- and exo-β-(1,4)-glucanase, cellobiohydrolase and β-(1,4)-glucosidase	*Trichoderma reesei*	3,000 U/g	25-75 (opt. 40-65)	4-7 (opt. 4.5-6)	Yes
ES2	Exo- and endo-peptidase with cellulase, hemicellulase and amylase side activities		>500 PAC/g	30-60 (opt. 45-55)	2.3-6 (opt. 2.5-3.5)	
HE1	Galactomannanase and cellulase	*Aspergillus niger*	10,000 GMA/g	60 (opt. 75)	3-9 (opt. 4)	
NC1	Purified β-glucanase	*Streptomyces* sp.		up to 60 (opt. 50-60)	5-6.5	Yes
NC2	Endo-*N*-acetylglucosaminidase	*Streptomyces violaceroruber*				
NC3	Pectate-lyase	*Bacillus* sp.		40-60 (opt. 60)	5-7 (opt. 8)	
NC4	Chitinase	*Streptomyces* sp.	16 U/g	20-60 (opt. 50-60)	3.5-8 (opt. 4-12)	
NS1	Polygalacturonase	*Aspergillus aculeatus*	3,300 PGNU/g	45-60	Opt. 4-5	No
NS2	Endo-(1,3/1,4)-β-glucanase with xylanase and hemicellulase side activities	*Aspergillus aculeatus*	100 FBG/g	40-60 (opt. 55)	3.3-5.5 (opt. 4.5)	No
NS3	Glucoamylase	*Aspergillus niger*	300 AGU/mL	Opt. 60	Opt. 4.5	No
NS4	Cellulase	*Trichoderma reesei*	700 EGU/mL	55-65	Opt. 4-6	No
NS5	Mannan endo-(1,4)-β-mannosidase		4 MIUM/g		5.5-7.5	Yes
NS6	Exo-(1,3)-β-Glucanase	*Trichoderma hazianum*	46 BGXU/mL	Opt. 40	3-5 (opt. 4)	No
NS7	Endo-(1,3/1,4)-β-glucanase with cellulase side activity	*Trichoderma reesei*	250 FBG/g			No
NS8	Endo-α-(1,4)-amylase	*Bacillus amyloliquefaciens*	480 KNU/g	45-80 (opt. 60-75)	6-10 (opt. 6)	
SL1	Pectin lyase, polygalacturonase, pectinesterase and polygalacturonate lyase	*Aspergillus niger*		25-65 (opt. 50-60)	2.5-4.5 (opt. 3.3-4)	
SL2	β-(1,3/1,4)-Glucanase, β-(1,3)-glucanase, β-(1,2/1,4)-glucanase, β-(1,4)-glucanase and β-(1,3/1,6)-glucanase	*Trichoderma reesei*		30-70 (opt. 50-60)	3.5-5.5 (opt. 4.8-5.5)	
SN1	α-Galactosidase	*Aspergillus niger*	30,000 U/g	Up to 60 (opt. 60)	4-8 (opt. 5)	
SN2	Cellulase and hemicellulase	*Trichoderma longibrachiatum* (formerly *Trichoderma reesei*)	4,000 U/g	Up to 30 (opt. 50)	3-6 (opt. 4.5)	
SN3	β-mannanase, cellulase and hemicellulase	*Aspergillus niger*	15,000 U/g	50-60	3-6 (opt. 4)	

Enzymes	Activity profile	Organism	Activity	T range (°C)	pH range	GMO
SN4	Protease, endo-(1,3/1,6)-β-glucanase and little exo-β-glucanase	*Rhizomucor miehei*		Opt. 30-40	Opt. 6-7	
SN5	Glucoamylase	*Aspergillus niger*	1,500 U/g	20-60 (opt. 65)	3-9 (opt. 5)	
SN6	Pectinase and endo- and exo-arabinase	*Aspergillus niger*	5,000 U/g	40-65 (opt. 55)	3.5-5.5 (opt. 4.5)	
SE1	Endo-β-(1,3/1,4)-glucanase	*Trichoderma reesei*		30-60	4-6.5	No
ST1	β-Glucanase, galactomannanase, cellulase, β-glucosidase	*Aspergillus niger*		Opt. 50-75	3-9 (opt. 3-5)	No
ST2	Glucoamylase	*Aspergillus niger*	10,000 U/g	50-60 (opt. 50)	3.5-6 (opt. 4.5)	No
ST3	Purified β-glucanase	*Streptomyces* sp.		Up to 60 (opt. 50-60)	Opt. 5-6.5	Yes
P-MC1	Phospholipase A1	*Aspergillus oryzae*	10,000-13,000 U/g	30-60 (opt. 50)	4-10 (opt. 4.5)	
P-NE1	Purified phospholipase	*Streptomyces* sp.	10,000–13,000 PLA2UN/mL	40-55 (opt. 50)	7-10 (opt. 9)	
P-N1	Lipase		10 KLU/g			No
P-SN1	Phospholipase A1	*Aspergillus oryzae*	3,000 U/g	Up to 55 (opt. 55)	3.5-6 (opt. 4.5)	

T, Temperature; GMO, Genetically modified organisms; Opt., Optimum

Appendix C Calibration curves for the RS calculations





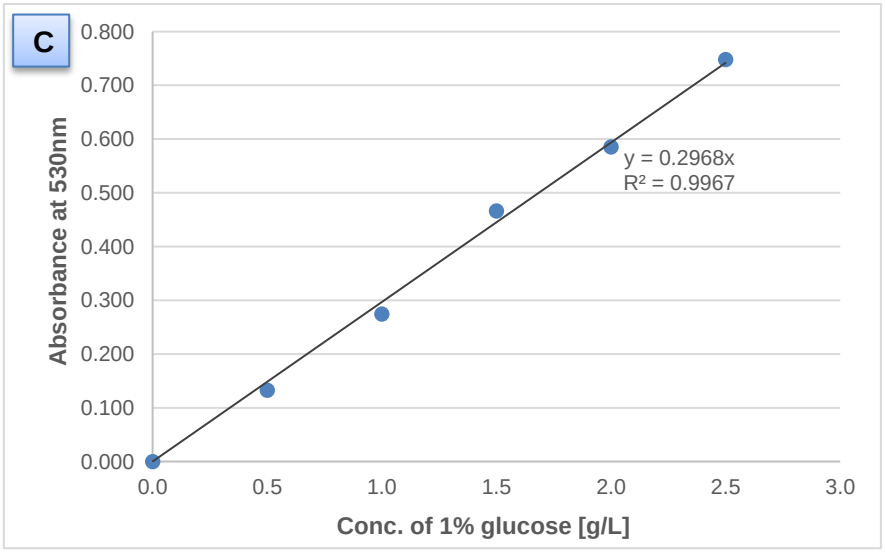


Figure C.1 Calibration curves A, B and C for 1% of glucose stock solution used to determine the RS concentration.

Appendix D Solubilisation and phase separation results for the screened enzymes

Table D.1 Volumes of the pellet and supernant after centrifugation as well as the pellet form and dry matter of the supernatant of the hydrolysate products.

Enzymatic hydrolysate	3h				24h			
	Pellet form	Vol. Pellet [%]	Vol. SN [%]	SN DM [%]	Pellet form	Vol. Pellet [%]	Vol. SN [%]	SN DM [%]
AE1	S	60	40	1.7	S	62.5	37.5	2.5
AE2		65	35	1.9		62.5	37.5	3.2
AE4		50	50	3.8		50	50	5.5
AE5		55	45	2.5		50	50	4.6
AO1		50	50	6.1		45	55	7.5
AO2		62.5	37.5	1.8		55	45	2
BC1		50	50	1.7		70	30	2.2
BC2		55	45	4.2		50	50	6.2
BC3		62.5	37.5	1.8		60	40	2.6
BC4		50	50	1.8		62.5	37.5	2.5
DA1		50	50	1.9		55	45	3.5
DA2		62.5	37.5	1.7		62.5	37.5	2.4
DA3		60	40	1.9		62.5	37.5	3.7
DA4		60	40	1.7		62.5	37.5	2.3
DS1		62.5	37.5	2.2		52.5	47.5	4.6
DS2		50	50	4.2		45	55	6.8
DS3		50	50	2.2		62.5	37.5	3.3
DS4		62.5	37.5	1.8		60	40	2.9
DC1		45	55	3.2		47.5	52.5	4.8
ET1		60	40	1.7		62.5	37.5	1.8
ET2		50	50	1.7		55	45	2
ES1		52.5	47.5	1.7		57.5	42.5	3
ES2		52.5	47.5	2.1		62.5	37.5	3.5
NC2		50	50	2.9		50	50	3
NC3		50	50	1.7		55	45	1.8
NC4		50	50	1.7		60	40	2.1
NS1		50	50	2.8		50	50	4.6
NS2		50	50	3.8		42.5	57.5	6
NS3		62.5	37.5	1.8		62.5	37.5	2.4
NS4		55	45	1.7		62.5	37.5	2.4
NS5		50	50	1.7		55	45	1.7
NS7		62.5	37.5	1.6		62.5	37.5	2.4
NS8		50	50	1.7		62.5	37.5	2
SL2		60	40	2.2		50	50	4.9

Enzymatic hydrolysate	3h				24h			
	Pellet form	Vol. Pellet [%]	Vol. SN [%]	SN DM [%]	Pellet form	Vol. Pellet [%]	Vol. SN [%]	SN DM [%]
SN1	S	60	40	2.2	S	60	40	3.7
SN3		55	45	1.9		55	45	3
SN4		62.5	37.5	5.3		55	45	7.5
SN5		60	40	1.8		62.5	37.5	2.7
SN6		65.5	37.5	2.1		55	45	3.3
SE1		55	45	1.9		62.5	37.5	3.5
ST1		47.5	52.5	4.4		42.5	57.5	6.6
ST2		55	45	2.1		62.5	37.5	3.2
P-MC1		55	45	2.4		55	45	3.6
P-NE1		60	40	1.7		60	40	1.8
P-N1		60	40	1.7		60	40	2.6
P-SN1		55	45	2.7		60	40	4.4

DM, dry matter. S, solid. SN, supernatant. Vol., volume.

Appendix E Standard deviation results of the RS, free glucose and mannose measurements

- **Enzyme screening**

Table E.1 Standard deviation values of the RS, free glucose and mannose measurements for the enzyme screening.

Enzymatic hydrolysate	RS	Free glucose	Free mannose	RS	Free glucose	Free mannose
SM	0,003	0,000	0,001	--	--	--
		3h			**24h**	
C	0.027	0.000	0.033	0.010	0.000	0.000
AE1	0.002	0.001	0.001	0.057	0.028	0.001
AE2	0.024	0.008	0.000	0.008	0.003	0.000
AE3	0.548	0.291	0.007	0.479	0.475	0.007
AE4	0.034	0.062	0.000	0.257	0.043	0.001
AE5	0.195	0.050	0.000	0.137	0.076	0.000
AO1	0.017	0.042	0.003	0.000	0.099	0.004
AO2	0.315	0.205	0.001	0.495	0.707	0.000
AO3	0.034	0.010	0.000	0.034	0.139	0.035
BC1	0.013	0.002	0.001	0.022	0.006	0.001
BC2	0.227	0.022	0.000	0.202	0.276	0.002
BC3	0.002	0.000	0.001	0.224	0.122	0.000
BC4	0.030	0.003	0.000	0.083	0.050	0.001
DA1	0.005	0.095	0.000	0.093	0.073	0.000
DA2	0.007	0.012	0.000	0.066	0.023	0.001
DA3	0.027	0.004	0.001	0.842	0.631	0.003
DA4	0.034	0.012	0.001	0.040	0.342	0.001
DS1	0.347	0.274	0.008	0.240	0.108	0.010
DS2	0.093	0.006	0.003	0.303	0.086	0.002
DS3	0.071	0.065	0.000	0.040	0.061	0.000
DS4	0.094	0.023	0.001	0.209	0.009	0.000
DC1	0.120	0.070	0.001	0.103	0.094	0.001
ET1	0.008	0.000	0.060	0.027	0.002	0.087
ET2	0.000	0.000	0.072	0.030	0.004	0.083
ET3	0.086	0.146	0.002	0.068	0.054	0.010
ET4	0.094	0.000	0.000	0.017	0.000	0.000
ES1	0.002	0.065	0.002	0.259	0.095	0.001
ES2	0.015	0.001	0.000	0.091	0.080	0.000
HE1	0.103	0.214	0.002	0.462	0.270	0.012
NC1	0.000	0.000	0.000	0.000	0.011	0.002
NC2	0.056	0.000	0.012	0.079	0.000	0.003
NC3	0.024	0.003	0.007	0.042	0.003	0.005
NC4	0.305	0.000	0.006	0.015	0.001	0.007
NS1	0.088	0.026	0.001	0.171	0.112	0.005
NS2	0.205	0.030	0.004	0.188	0.043	0.000
NS3	0.008	0.002	0.001	0.044	0.011	0.002
NS4	0.010	0.006	0.047	0.059	0.079	0.005
NS5	0.000	0.005	0.000	0.019	0.001	0.001

Enzymatic hydrolysate	3h			24h		
	RS	Free glucose	Free mannose	RS	Free glucose	Free mannose
NS6	0.548	0.281	0.002	0.411	0.281	0.004
NS7	0.007	0.000	0.003	0.027	0.014	0.000
NS8	0.002	0.001	0.001	0.008	0.020	0.004
SL1	0.428	0.231	0.006	0.616	0.099	0.004
SL2	0.051	0.037	0.001	0.154	0.142	0.000
SN1	0.027	0.287	0.000	0.025	0.017	0.001
SN2	0.043	0.032	0.001	0.377	0.227	0.011
SN3	0.088	0.043	0.002	0.172	0.043	0.001
SN4	0.034	0.022	0.000	0.753	0.328	0.001
SN5	0.056	0.020	0.001	0.111	0.032	0.001
SN6	0.010	0.006	0.000	0.024	0.026	0.001
SE1	0.008	0.009	0.001	0.192	0.060	0.004
ST1	0.231	0.013	0.000	0.017	0.052	0.009
ST2	0.037	0.007	0.000	0.059	0.066	0.001
ST3	0.051	0.000	0.006	0.137	0.000	0.002
P-MC1	0.163	0.090	0.000	0.325	0.018	0.000
P-N1	0.000	0.006	0.081	0.040	0.013	0.078
P-NE1	0.013	0.000	0.081	0.008	0.006	0.079
P-SN1	0.291	0.032	0.001	0.120	0.036	0.002

C, control. SM, starting material. RS, reducing sugars.

- **Optimization of BC2 hydrolysates**

Batch to batch trials

Table E.2 Standard deviation values of the RS, free glucose and mannose measurements for the batch to batch trials.

Enzymatic hydrolysate	VK16-000529			VK16-000530		
	RS	Free glucose	Free mannose	RS	Free glucose	Free mannose
3h	0.260	0.120	0.001	0.048	0.019	0.001
6h	0.239	0.144	0.001	0.107	0.059	0.002
8h	0.263	0.116	0.002	0.503	0.291	0.003
24h	0.021	0.060	0.001	0.304	0.169	0.001

C, control. RS, reducing sugars.

Variation of the reaction conditions

Table E.3 Standard deviation values of the RS, free glucose and mannose measurements for the variation of reaction conditions.

T [°C]	pH	3h			6h		
		RS	Free glucose	Free mannose	RS	Free glucose	Free mannose
45	4.5	0.227	0.107	0.001	0.227	0.104	0.000
	5.0	0.489	0.085	0.000	0.590	0.288	0.000
	5.5	0.093	0.034	0.000	0.034	0.029	0.000
50	4.5	0.261	0.116	0.000	0.067	0.049	0.000
	5.0	0.025	0.084	0.000	0.059	0.050	0.000
	5.5	0.278	0.110	0.001	0.362	0.197	0.001
55	4.5	0.202	0.066	0.000	0.017	0.020	0.000
	5.0	0.042	0.036	0.000	0.261	0.029	0.000
	5.5	0.042	0.072	0.000	0.379	0.149	0.000
60	4.5	0.078	0.030	0.001	0.135	0.015	0.000
	5.0	0.071	0.075	0.000	0.084	0.001	0.000
	5.5	0.007	0.007	0.000	0.098	0.043	0.000

C, control. RS, reducing sugars.

Appendix F Standard deviation results of the total glucose and mannose measurements by HPAE-PAD

- **Enzyme screening**

Table F.1 Standard deviation values of the total glucose and mannose measurements by HPAE-PAD for the enzyme screening.

Enzymatic hydrolysate	Total glucose	Total mannose	Total glucose	Total mannose
SM	0.033	0.190	--	--
	3h		**24h**	
C	--	--	0.106	2.015
AE1	0.135	0.000	0.078	0.826
AE2	0.068	2.561	0.087	1.400
AE3	0.275	0.706	1.150	1.266
AE4	0.001	0.105	1.227	0.504
AE5	0.467	1.423	0.215	1.166
AO1	0.111	0.130	0.517	1.571
AO3	0.048	0.097	2.563	3.588
BC1	0.083	1.844	0.066	1.130
BC2	0.942	3.509	1.230	3.283
BC3	0.324	0.073	0.504	1.974
BC4	0.288	3.304	0.216	0.367
DA1	0.468	2.892	0.001	0.725
DA2	0.026	0.427	0.294	0.802
DA3	0.145	1.577	0.212	0.718
DA4	0.116	0.116	0.515	3.743
DS1	1.009	4.835	1.009	2.659
DS2	0.079	0.668	0.625	1.966
DS3	0.216	0.990	1.514	2.840
DS4	0.391	0.360	0.072	2.343
DC1	0.935	2.408	1.079	2.862
ET3	1.098	3.552	2.026	3.392
ET4	0.359	0.314	0.477	0.765
ES1	0.525	2.119	1.488	2.225
ES2	0.165	0.215	0.172	0.139
HE1	0.504	1.716	0.178	0.574
NC1	1.066	1.297	0.174	0.903
NC2	0.032	2.324	0.946	0.463
NC3	0.036	2.452	0.648	2.742
NS1	0.540	3.330	0.219	1.623
NS2	0.476	1.550	0.054	0.096
NS3	0.079	1.414	0.370	2.606
NS4	0.072	1.794	0.504	1.022
NS6	0.347	0.440	1.741	1.939
NS7	0.235	3.288	1.297	3.328
NS8	0.011	0.041	0.244	0.392
SL1	0.234	2.590	0.272	1.038

Enzymatic hydrolysate	3h		24h	
	Total glucose	Total mannose	Total glucose	Total mannose
SL2	0.432	1.501	2.511	2.769
SN1	1.421	1.160	0.214	0.209
SN2	0.100	0.847	0.224	0.478
SN3	0.505	1.941	0.427	3.561
SN4	0.778	2.546	0.045	0.907
SN5	0.279	1.370	0.055	0.832
SN6	0.036	0.768	0.216	0.219
SE1	0.325	1.468	0.576	3.658
ST1	0.540	3.020	1.781	3.773
ST2	0.239	1.803	0.716	3.217
ST3	2.240	2.408	0.051	0.125
P-MC1	1.873	2.580	0.002	0.139
P-N1	0.396	2.429	0.198	1.934
P-SN1	0.432	2.339	2.385	1.108

C, control. SM, starting material.

- **Optimization of BC2 hydrolysates**

Batch to batch trials

Table F.2 Standard deviation values of the total glucose and mannose measurements by HPAE-PAD for the batch to batch trials.

Enzymatic hydrolysate	VK16-000529		VK16-000530	
	Total glucose	Total mannose	Total glucose	Total mannose
SM	0.091	0.226	--	--
3h	1.354	2.577	0.679	0.727
6h	1.161	3.095	0.340	1.019
8h	0.378	0.246	0.958	3.272
24h	0.784	2.386	0.670	0.915

SM, starting material.

Variation of the reaction conditions

Table F.3 Standard deviation values of the total glucose and mannose measurements by HPAE-PAD for the variation of reaction conditions.

T [°C]	pH	3h		6h	
		Total glucose	**Total mannose**	**Total glucose**	**Total mannose**
45	**4.5**	0.614	2.592	1.698	5.837
	5.0	0.663	2.028	0.298	0.315
	5.5	0.686	2.343	0.032	1.757
50	**4.5**	0.632	3.507	0.820	1.742
	5.0	1.316	3.850	0.288	0.920
	5.5	0.190	0.824	2.860	6.399
55	**4.5**	0.433	2.782	0.793	2.563
	5.0	0.865	4.247	1.225	3.954
	5.5	1.513	5.343	0.576	2.123
60	**4.5**	0.648	2.341	0.649	0.953
	5.0	1.153	3.514	0.144	0.804
	5.5	0.072	0.585	0.288	2.122

T, temperature.

Appendix G Number-average degree of polymerisation (DP_n) results

Table G.1 DP_n values for the SN of the enzymatic hydrolysates at 3h and 24h reaction times.

Enzymatic hydrolysate	DP_n	
	3h	24h
AE1	7.76	7.53
AE2	8.41	8.24
AE4	8.38	5.00
AO3	6.64	2.45
BC1	7.91	6.32
BC3	6.41	5.71
BC4	6.07	7.18
DA1	9.76	10.06
DA2	7.11	6.65
DA3	6.33	7.28
DA4	9.73	7.13
DS1	8.37	6.23
DS3	5.45	7.77
DS4	6.52	6.12
DS3	5.45	7.77
DC1	6.81	7.34
ES1	9.04	9.70
ES2	7.86	6.86
HE1	7.88	15.06
NC2	9.31	7.40
NC3	5.62	3.79
NS3	8.41	5.22
NS4	9.07	11.52
NS7	8.70	7.92
NS8	7.73	7.68
SL1	5.44	3.28
SN1	7.41	8.90
SN3	9.61	7.99
SN5	7.90	8.91
SN6	7.90	6.32
SE1	10.55	7.38
ST1	6.05	4.19
ST2	6.76	10.65
P-MC1	5.59	9.37
P-N1	8.74	9.63
P-SN1	4.76	7.34

DP_n, number-average degree of polymerisation.