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Fermentation and downstream processing of yeast for production of single-cell proteins and exopolysaccharides

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Master in Chemistry and Biotechnology

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Abstract

Yeasts are known to be sources of highly quality proteins and exopolysaccharides (EPS). The main goal in this study was to produce and process yeasts of biotechnological interest to investigate whether these species can be used as supplements in food and feeds. *Candida utilis, Wickerhamomyces anomalus* and *Arxula adeninivorans* were fermented several times on chicken meat hydrolysate and downstream processed to be able to characterize the cell walls of the yeast, measure the protein content, and to discover the bottlenecks in the different processing steps. The results from the protein analysis showed that *W. anomalus* and *A. adeninivorans* contained a higher amount of proteins than *C. utilis*.

All the yeasts had glucose, mannose and N-acetyl-glucosamine in their cell walls according to the monosaccharide composition analysis. These monosaccharides are building blocks in the exopolysaccharides b-glucan, mannan and chitin. Earlier performed studies have reported that these exopolysaccharides often are percent in the yeast cell wall. The results from this study indicates that b-glucan, mannan and chitin are present in the cell walls of *C. utilis, W. anomalus* and *A. adeninivorans* as well. In addition, galactose was found in the cell wall of *A. adeninivorans*.

In total seven downstream processing steps were performed on *W. anomalus* after optimization of the process. The final processing steps were; 2-phase separation, cell disruption, enzymatic treatment, acid treatment, centrifugation, ultrafiltration and nanofiltration. The obtained fractions after the filtrations contained both proteins and carbohydrates. In depth analysis of these fractions are subjected to ongoing analysis in the lab.

Sammendrag

Gjær er kilder til proteiner med høy kvalitet og eksopolysakkarider (EPS). Hovedmålene med dette studiet var å produsere og prosessere gjær av bioteknologisk interesse for å undersøke om gjæren kan brukes som tilskudd in mat og fôr. *Candida utilis*, *Wickerhamomyces anomalus* og *Arxula adeninivorans* ble fermentert flere ganger med hydrolyserte kylling-innvoller som vekstmedia, og nedstrøms-prosessert for å kunne karakterisere celleveggen til artene, måle proteininnholdet, og detektere eventuelle flaksehalser i prosesserigsstegene. Resultatene fra proteinanalysen viste at *W. Anomalus* og *A. adeninivorans* inneholdt et høyere proteininnhold enn *C. utilis*.

Videre ble celleveggen analysert for monosakkaridsammensetning og karakterisert. Alle artene har cellevegger bestående av glukose, mannose og N-acetyl-glukosamin. Disse monosakkaridene er blant annet byggesteiner i eksopolysakkaridene b-glukan, mannan og kitin. Tidligere studier har rapportert at disse eksopolysakkaridene ofte er tilstede i celleveggen til gjær. Basert på funnene gjort i dette studiet er derfor b-glukan, mannan og kitin mest sannsynlig også tilstede hos *C. utilis*, *W. anomalus* og *A. adeninivorans*. Galaktose ble i tillegg funnet hos *A. adeninivorans*.

Totalt syv nedstrømprosesseringssteg ble utført på *W. anomalus* etter at prosesseringen ble optimalisert. Prosesseringsstegene som ble utført var; 2-fase separasjon, celle ødeleggelse, behandling med enzym, behandling med syre, sentrifugering, ultrafiltrering and nano-filtrering. Fraksjonene generert etter de siste filtreringene inneholdt både proteiner og ulike karbohydrater. Dypere analyser av disse fraksjonene skal blir utført videre.

Abbreviations

BALI	Borregaard advanced lignin™
BSA	Bovine Serum Albumin
CDW	Cell dry weight
Da	Dalton
EPS	Exopolysaccharides
GRAS	Generally regarded-as-safe
HPAEC	High-performance anion-exchange chromatography
HPLC	High-performance liquid chromatography
ICS	Ion chromatography system
kDa	Kilo Dalton
MECO	Nominal molecular weight cut-off
NF	Nano-filtration
o/w	oil-in-water
PSI	Pounds per square inch
RFB	Repeated fed batch
RI	Refractive index
SC Mannan	Mannan (*Saccharomyces cerevisiae*)
SCP	Single-cell proteins
SDDs	Spray-dried dispersions
SEC	Size exclusion chromatography
UF	Ultra-filtration
UV	Ultraviolet
YPD	Peptone from meat, bacteriolocial, yeast extract, D-*(+)-Glucose*

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1 Introduction

1.1 Single-cell proteins and exopolysaccharides

Single-cell proteins (SCP) are proteins derived from single-celled microorganisms such as yeast, bacteria and algae (Ritala et al., 2017) (A T Nassari, 2011). Yeasts are important in industrial processes that is based on fermentation, but also in commercial production of organic acids such as citric acid (Willey et al., 2014). One reason for this industrial use is that yeast cultures have a low risk of being contaminated by other microorganisms, they grow quite fast and they produce high amounts of proteins (Ytrestøyl et al., 2015). This has led to a huge interest in microbial proteins from yeasts as a replacement for several feeds (Sharma et al., 2018) and as a protein source in our diets e.g. as Quorn (Wiebe et al., 1997). Some species of yeasts contain high concentrations of essential amino acids and can therefore act as a high-quality protein source (Ritala et al., 2017). In addition to the world's environmental challenges, we are also facing challenges with a growing world population. To be able to encounter a growing consumption level, and to meet an increasing food demand, new sources for proteins are needed (Sharma et al., 2018). Single-cell proteins may be one of the solutions.

As well as being a source of high-quality protein, yeasts also possess carbohydrates in their cell wall known as exopolysaccharides (EPS) which is of increasing commercial interest. The fungal cell wall consists of mainly three general polysaccharides; glucans, mannans and chitin (Hall & Gow, 2013). These glycans have been shown to be immune stimulating (Gow et al., 2017) (Hall & Gow, 2013) (Lee et al., 2008). In addition, proteins linked to the mannans in the cell wall, so-called mannoproteins, of some yeasts are known to have emulsifying properties (Cameron et al., 1988).Other attractions concern the ability of the polysaccharides to be used in processing of food (Nguyen et al., 1998). Each of the proteins and polysaccharides have interesting properties, and a combination of these molecules can probably provide both health benefits and be a good nutritional source in food and animal feed.

1.2 Aim of study

The aim of this master´s thesis was to produce and process different yeast species to gain single-cell proteins and exopolysaccharides for their potential use in the food and feed industry. The secondary objectives of the study was to a) characterise the cell wall for the three selected yeasts, both on protein content and exopolysaccharide composition, and b) to develop and optimize the downstream processing of yeast that could be implemented for feed ingredient production and provide a knowledge base for future industrial use, and to rank the yeast based on their properties and to see if these characteristics can be used in other applications.

2 Theory

2.1 The fungus kingdom

The fungus kingdom consists of a huge group of organisms, including yeast. Generally, organisms in this kingdom are eukaryotic, spore-bearing, have absorptive nutrition, lack chlorophyll, and can reproduce both sexually and asexually. Fungi live on animals, humans and plants as parasites or symbionts. They are saprophytes which means that they get nutrition from dead organic material with the use of enzymes, making them important for degradation of organic matter in different habitats. Single-celled fungi are called yeast while multicellular fungi are categorised as molds. Yeasts are organisms which reproduce either sexually through spore formation, or asexually by budding and transverse division. In yeast it is commonplace that mitosis may be concurrent with budding to produce a daughter cell during asexual reproduction (Willey et al., 2014). By changing their cell cycle, especially the length of the cycle, yeasts can regulate their growth rate depending on the nutrient availability in the environment. They grow on a wide range of energy sources that serve as substrates and signals for growth. These signals optimize survival under this exact nutritional state. Precursors containing carbon is important for anabolic metabolism and biomass increase, and yeasts prefer any fermentable carbon source over any other energy source. They have a hierarchical pattern of consumption where they often consume glucose and fructose before other sugars (Broach, 2012).

2.2 Ascomycota

Fungi that belong to the Ascomycota phylum are commonly known as sac fungi because they produce spores in a characteristic saclike structure, called ascus. Some of the ascomycetes have a life cycle where they swap between filamentous fungi and yeast. The other ascomycetes are yeasts. In terrestrial, freshwater and marine environments these fungal microorganisms help with the degradation of organic compounds such as cellulose and lignin, which is known to be chemically stable sugar complexes (Willey et al., 2014).

The genus *Candida* is one of many fungal pathogens in the Ascomycota phylum that cause infection in animals and humans (Willey et al., 2014). Despite this pathogenesis, the species *C. utilis* is generally regarded-as-safe (GRAS) and is accepted for human consumption (Bekatorou et al., 2006) (Sharma et al., 2018) (Buerth et al., 2016). *C. utilis* has been used as nutritional supplements for decades (Bekatorou et al., 2006) (Buerth et al., 2016) and is

reported as a protein rich yeast which can be used as a source of essential amino acids, such as lysine, valine and glutamic acid, in e.g. fish diets (Sharma et al., 2018) (Bekatorou et al., 2006).

Wickerhamomyces anomalus (*Pichia anomala*) also belongs to the ascomycetous phylum and is a heterothallic yeast in the *Wickerhamomycetaceae* family. Heterothallic fungi need a mating partner of different mating type. The *Wickerhamomycetaceae* family reproduces asexually by budding and sexually thought ascospores (Ni et al., 2011). *W. anomalus* is not especially tolerant to ethanol and acetate but can grow at both low and high pH (2-12) (Fredlund et al., 2002), under anaerobic conditions and under high osmotic pressure (Passoth et al., 2006). Among *Wickerhamomyces,* there are species producing toxins that can kill other fungi. *W. anomalus* kills other fungi with a glycosylated exo- β-1,3-glucanase (panomycocin), that hydrolyses the β-1,3-glucans, a major polymer in the fungal cell wall (Izgu et al., 2005). This enzyme is a 49 kDa protein and is stable up to 37 ºC and a pH range between 3 and 5,5 (Izgu et al., 2007).

Arxula adeninivorans, also known as *Blastobotrys adeninivorans*, is a yeast of biotechnological interest, due to its flexible properties. It is a relatively recently discovered yeast, the first reported findings of this species dates back to the mid-eighties (Middelhoven et al., 1984). The species has an uncommon metabolic flexibility with a broad substrate spectrum. In addition, the yeast is tolerant to a wide range of temperatures, up to 48 ºC, and can reproduce under conditions with low accessibility of water, it is xerotolerant (Wartmann et al., 1995). Provisionally, the discovery of *Arxula* yeast is limited to asexual reproducing yeast, making the yeast an anamorphic ascomycete (Kunze et al., 2014) (Middelhoven et al., 1991). As mentioned, the species can grow under a wide range of temperatures and depending on this temperature the yeast can have different morphological forms. At temperature above 42 ºC the cells become mycelial, whereas under this temperature the cells reproduce by budding (Figure 1) (Wartmann et al., 1995). This dimorphism occurs in many yeast strains but can be caused by other factors as well, such as pH, carbon source and nitrogen source (Malak et al., 2016). At 42 ºC, an intermediate form, Pseudomycelia is formed, and describes the phenomena when the cells cling together in chains.

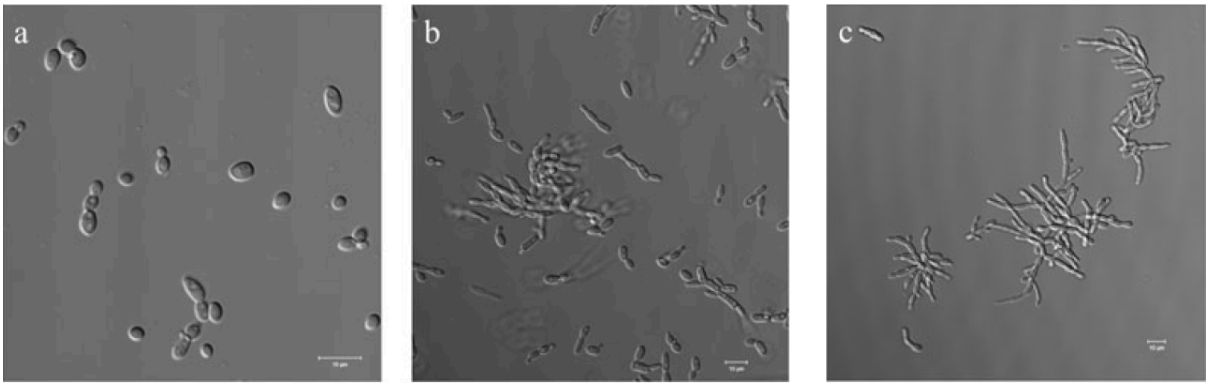

Figure 1: *A. adeninivorans* LS3 having temperature dependent dimorphism, a: budding cells, b: pseudomycelia. c: mycelial cells (Malak et al., 2016).

2.3 The fungal cell walls

The fungal cell wall is a dynamic organelle, with a dynamic structure, built to be robust but at the same time soft and flexible. The composition of the wall is essential for regulation in response to environmental changes, forced stress, pathogenesis, morphogenesis, and cell viability and differentiation (Gow et al., 2017). Although the cell walls are engineered in a comparable way, many of the building blocks are conserved in species (Coronado et al., 2007). Cell walls are often layered, where the outward layers normally are specific for each fungus. The inner layers often consist of branched β -(1,3) glucan interacting with chitin through hydrogen bonds (Figure 2). The exoskeleton that is made from these polysaccharides is the structure that protects the wall from the pressure from the cytoplasm and the membrane (Gow et al., 2017). Fungal cell walls are often made of chitin which is a strong but flexible polysaccharide with N-acetylglucosamine residues. But some cell walls contain other polysaccharides such as mannans and galactans (Willey et al., 2014).

Roughly one-fifth of the yeast genome is dedicated to the creation of the yeast cell wall (Gow et al., 2017). This may be explained by the highly complex cell wall that constitutes 26%-32% of the cell dry weight (Nguyen et al., 1998). Yeast cell walls consist of polysaccharides (85%-90%) and proteins (10%-15%) (Nguyen et al., 1998). The three predominant monosaccharides in the yeasts cell walls are glucose, mannose and glucosamine. The glucosamine is often acetylated (N-acetyl-glucosamine) and is the constituent of the biopolymer chitin in the cells. The monomers of glucose are the building blocks of glucan which is divided into β-1,3-glucan and β-1,6-glucan. The different polymers are present within a cell in different amounts, where approximately 60 % is β-glucan (Klis et al., 2002). Glucan, mannan and chitin are referred to as exopolysaccharides (EPS) that are involved in, among other things,

the protection of the cell from lysis. This EPS layer will therefor most likely increase under stressful conditions. Enzymatic complexes at the plasma membrane are synthesizing the chitin and the glucan polymers, while the mannans are synthesized in the endoplasmic reticulum and Golgi (Ytrestøyl et al., 2015). The outer cell wall of the species *Candida* and *Saccharomyces* have glycoproteins which protects the inner wall layers (Gow et al., 2017). These glycoproteins are proteins attached to mannan (Figure 2). According to (Seviour et al., 2011) the industrial production of EPS have many technical challenges. When producing fungi or bacteria in a fermentation process the temperature, agitation, aeration and power input are reported as crucial factors for the EPS yields in the cells. EPS producing cultures require oxygen, and the EPS yields seems to be affected by pellet size and the morphology of the cells as well.

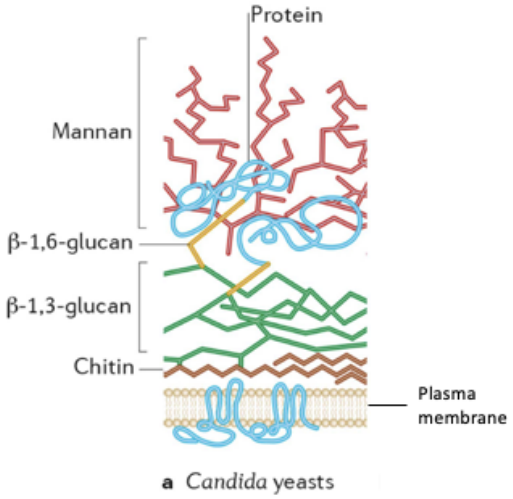

Figure 2: Illustration of the components in the cell wall of *Candida* yeast (Gow et al., 2017).

2.4 Single-cell proteins

Proteins are important in all processes taking place in a cell because they help the cell build a structure, and they are crucial in the transport systems especially across cell membranes. Furthermore, the proteins regulate cell growth, and as enzymes they catalyse biochemical reactions (Wu & Singh, 2012). The production of single-cell proteins is most efficiently produced by fermentation.

2.5 Fermentation

Fermentation has been a key process in food production for several generations with the use of many different microbes (Willey et al., 2014). Industrial fermentation is mainly divided into three methods. In batch fermentation, the level of nutrients decreases as the number of cells expands. This process is carried out in a closed system, where waste gasses are the only components removed, and all the products are collected in the end. A continuous culture allows the products to be harvested continuously and nutrients are added at the same time (not illustrate). This provide the cells with better growth conditions as fresh growth media is added and waste and products are removed. In fed-batch fermentation nutrients are added, but nothing is removed. This makes the process a hybrid between the two other fermentation methods (Watkinson et al., 2016). During a repeated fed batch cultivation cell are harvested after all the media is consumed and new media is added directly after. The batch fermentation is in this way repeated in cycles (Figure 3).

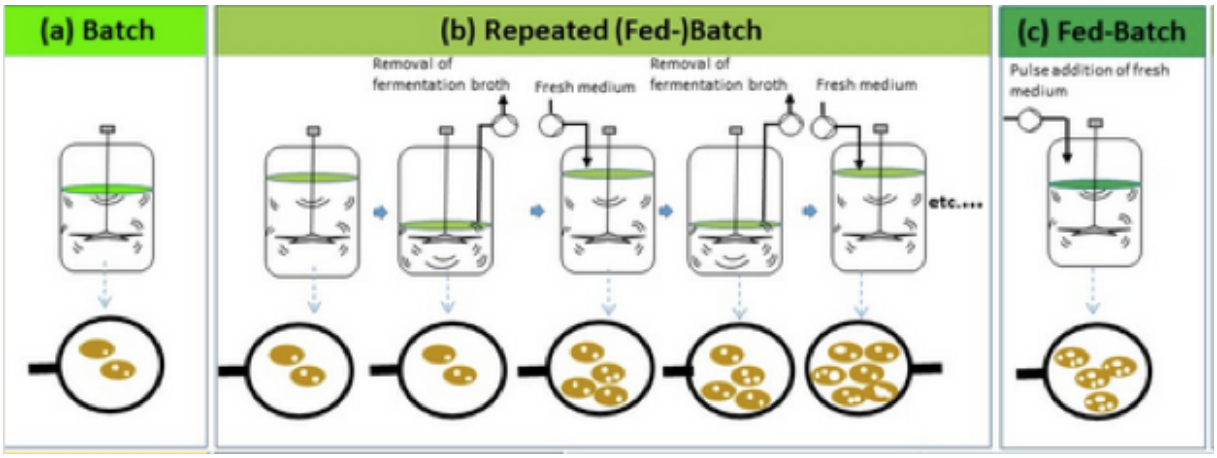

Figure 3: Illustration of batch fermentation (a), repeated fed batch fermentation (b) and fed batch fermentation (c). The modified picture is based on Figure 2 in (Koller, 2018).

2.5.1 Growth parameters

pH, temperature and oxygen are crucial growth parameters (Nagata & Chu, 2003). If the pH is not optimal for the cultivated microorganism the growth can be delayed or stopped due to inhibition or destruction. It is therefore important to measure the pH throughout the fermentation and to have the opportunity to change the pH during the process if necessary. Another key parameter is the temperature. Microorganisms grow differently under different temperatures, e.g. thermophile and hyperthermophile organisms grow best under extremely high temperatures. (Merritt, 1966) reported that under anaerobic conditions yeasts grow best

at 30ºC, and under aerobic conditions yeast grown best at 35 ºC, depending on the yeast species, however exceptions occur. An important factor during the fermentation process is the amount of accessible oxygen for the microorganism to consume. Yeast can grow both with and without oxygen, but the biomass production is at its optimum under aerobic conditions (Bekatorou et al., 2006). In addition, cultures producing EPS require oxygen (Seviour et al., 2011). Therefore, microbial biomass is usually produced under aerobic conditions (Bekatorou et al., 2006).

2.5.2 Growth medium

The growth process of a microorganism requires energy input for the conversion of ingredients from the growth medium into biomass. Carbon, hydrogen, oxygen, nitrogen, sulphur and phosphorus are referred to as macroelements. These elements are required by a microorganism in relatively big amounts. Proteins, lipids and carbohydrates are sources for these macroelements. Elements required in smaller amounts, such as manganese, zinc, nickel and copper, are named microelements or trace elements. Microelements are part of some enzymes, and without these elements, the enzymes will not be able to catalyse reactions. Both macroelements and microelements are needed by most cells for survival and reproduction (Willey et al., 2014). A fermentation media should therefore consist of sources of these elements because the composition can have a significant effect of the growth performance of the microorganism. Some of the chemical elements, such as nitrogen, phosphate, zinc, and iron, are often added to the growth media. Yeast extract (YPD) is also often used as supplements in cultivation media because it contains high amounts of amino acids, nucleotides and vitamins (Bekatorou et al., 2006).

As already mentioned, yeasts have a hierarchical pattern of consumption where they preferably consume the glucose and fructose in a media before other energy sources. This is probably because carbon is one of the main building blocks for the cell. Carbon sources, such as pentoses and hexoses, are known to influence yeast growth performance. The cell wall composition and structure are especially dependent on the carbon source. The proportion of β -glucan and α-mannan have been shown to change when yeasts are grown on different substrates, making the cell wall contribution to the cell dry mass vary a lot (Aguilar-Uscanga & Francois, 2003). (Looijesteijn et al., 1999), showed in a study that the lactic acid bacteria *Lactococcus lactis* is influenced by the carbon source, regarding EPS production. The bacteria produced less EPS when grown on fructose compared to glucose. The cells grown on the two

different sugar substrates showed a big difference in the capacity to produce the EPS precursors. This study may imply that the EPS production in different microorganisms is influenced by the carbon source. This could therefore also have an impact on the later downstream processes necessary to access these polysaccharides. Furthermore, the immune stimulatory properties (Seviour et al., 2011) of some of these EPS could be altered as an effect of which substrates they can grow on.

Nitrogen is often a limiting factor for growth (Sharma et al., 2018). The food industry generates a lot of protein rich (N rich) by-products that can be used as a source of value-added products (Lapena et al., 2018). Enzymatic hydrolysis can provide protein hydrolysates from these by-products which then can be used as high-quality growth medium to increase the protein contents. The protein rich by-products, such as meat, are altered both chemically and functionally during hydrolysis. Enzymes play a central part this hydrolysis to make the nitrogen more available for the organism. Various types of enzymes are used, such as cellulases and proteases (He et al., 2015).

In a study done by (Sharma et al., 2018), *Candida utilis* was produced on a growth medium containing enzymatically hydrolysed sulphite-pulped spruce wood, providing glucose, mixed with enzymatically hydrolysed brown seaweed, supplemented with ammonium sulphate as a nitrogen source. The temperature was set to 30 ºC, and the pH to 5,5. This gave a complete fermentation with good growth condition and cell yields. This study showed that it is important to investigate several available substrates, and maybe consider more complex substrates for an improved growth.

2.5.3 Overflow metabolism

The phenomena of overflow metabolism can be a problem in production of some microorganisms. This happens in the presence of feeding overflow which is induced when glycolysis tops a critical value and as a result from this the organisms produce by-products from pyruvate instead of using the energy for cell growth. Yeast normally produces ethanol and bacteria often produces acetate as the bioproduct (Santos et al., 2012). When this catabolite repression is induced by high glucose or sucrose concentrations the effect is called the Crabtree effect which may lead to incomplete fermentation, loss of biomass and as previously said, production of unfavourable by-products (Bekatorou et al., 2006).

2.6 Emulsions

An emulsion is a mix of two or more liquids that normally cannot blend together and get stable. To form an emulsion, oil, water, an emulsifier and some energy are required. Most emulsions, especially those in food, are oil-in-water (o/w) emulsions. There are mainly five variables that determine properties of emulsions. The first one is the type of emulsion, as said, oil-in-water emulsions is most common. Secondly, the droplet size distribution impacts the physical stability, where small droplets generally give a more stable emulsion (Damodaran & Parkin, 2017). This stability is a physical stability and involves the conservation of small single droplets (Mikkonen et al., 2016). To improve this stability other molecules such as proteins and polysaccharides are used as so-called emulsifiers. Proteins are used as emulsifiers to assist formation, improve stability and offer certain physiological prosperities to the emulsions (McClements, 2004). Some polysaccharides from plants, seaweeds and microbes can also be used as emulsifiers, including some galactomannans (Bhattarai et al., 2019). Thirdly, causing a huge effect on the viscosity of the emulsion, is the volume fraction of the dispersed phase. The fourth variable is the surface layer around the droplets which also has an impact on the physical stability. The last variable is the composition of the continuous phase which among other things have an effect on creaming. Although the droplets in emulsions are spherical and deformable, energy is needed to deform and break up the droplets to make them smaller and to transport the emulsifier to the new interface. Normally, a high-pressure homogenizer is used for this purpose (Damodaran & Parkin, 2017).

2.7 Water removal

Freeze drying, or lyophilization, is a process where water is removed from the material by sublimation. This process is often used to remove water from biological material because the process in general produces the best quality dehydrated material where the inherent biological activity is conserved during the process. The purpose is to eliminate deterioration reactions responsible for degradation in water containing material by decreasing the water activity (Flink & Knudsen, 2002). Freeze drying is used in the pharmaceutical and biotechnological industry, in the food industry and in the theological industry e.g. by concentrating low molecular weighed substances because they often are too small to be removed by filtration membranes (Nail et al., 2002). In a biotechnical approach the method is often used to preserve microorganisms (Miyamoto-Shinohara et al., 2000) as well as foods, which often are freeze dried because the process avoid loss of most volatiles and flavours, and make the food

lightweight, and preserves it. This method prevents biological and chemical reactions to occur, due to the low temperature and the quick conversion of the product from a hydrated structure to a nearly completely dehydrated product (Ratti, 2001). Other products often freeze dried are bacteria, vaccines, cells, chemicals and antibiotics. The vaccines and other pharmaceuticals are often dried this way to increase the shelf life of the products. In addition to minimize decomposition reactions, the method has the advantages that it removes the water without excessive heating, and it enhance stability of the material. Pre-treatment before freezing the material is often favourable. This can be to concentrate the material or to increase the surface area (Nireesha et al., 2013).

The first step in this drying process is the conversion of water from liquid to ice by freezing the product. The second step is called sublimation, where the ice is removed by direct alteration from solid state to vapour state, this step is called the primary drying step (Nireesha et al., 2013). Presumably, not all the water in the material is frozen because some of it is powerfully bound to the solids. This residual moisture content can be up to 20 % of the weight of the dried soils (Nail et al., 2002). Desorption of the strongly bound water is called the secondary drying step and requires warmer temperatures and reduction in the chamber pressure (Nireesha et al., 2013) (Flink & Knudsen, 2002).

Figure 4: The different processing steps in freeze drying from sample preparation to the formation of a final product. Based on Figure 3 in (Nireesha et al., 2013).

One physical event that occur during the freezing process is that ice crystals are formed and grown. As this happens the solutes are concentrated (Flink & Knudsen, 2002), and the ionic strength is increasing. Biological materials are often affected by this high ionic strength which may alter the materials by e.g. denaturation of proteins. Some chemical reactions may also be accelerated by this freeze concentration effect. Other events are supercooling and vitrification (Nail et al., 2002). All these events should be considered when freeze-drying biological materials.

Additional to freeze drying, there are many other methods for drying. Some drying methods are more suitable to some materials than others. Spray draying is for example most suitable for drying of liquids, while others are more appropriate to use when drying solids. Freeze drying on the other hand is suitable for both. In large-scale industrial production the main drying methods are freeze-drying, drum drying, microwave drying, tunnel drying, belt drying, fluidized bed, vacuum evaporation and spray drying. But many of the listed methods can also be used in a laboratory scale. In all the methods, except for freeze drying, water is present as liquid and removed by vaporization. The fact that water is in liquid state, and not in solid form, has a huge impact on the properties of the dried product obtained (Flink & Knudsen, 2002). In addition to the above-mentioned drying techniques, various separation techniques may be used upstream of the process to remove a bulk of water/liquid from the product prior to final drying step.

Spray drying is one of the most common methods used in the food industry e.g. transforming liquid food products into powder, and as an encapsulation method (Gharsallaoui et al., 2007) (Vehring, 2008). The method removes water or other moisture rapid (Bhandari et al., 1997) and the particles are usually dried within one second. The general method for spray-drying is well-known. A spray solution is atomized into droplets in a spray-drying chamber with a hot drying gas. The solvent in the droplets evaporates, and dried droplets exit the chamber to be collected and separated from the gas by a cyclone separator (Figure 5). The dried particles are called spray-dried dispersions (SDDs) (Dobry et al., 2009).

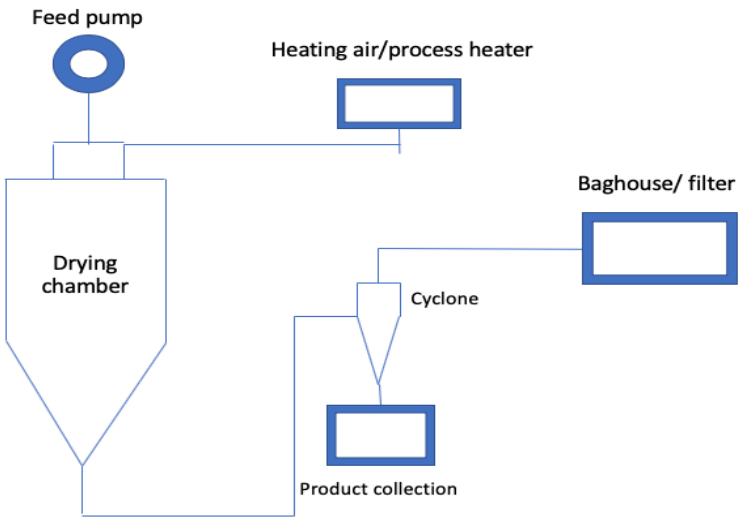

Figure 5: The general equipment used in spray-drying, based on Figure 1 (Dobry et al., 2009).

2.8 Separation techniques

There are numerous of separation techniques available, but the composition of the product in question should be taken into account when choosing the right separation technique. A medium through which a liquid can pass is used to separate solids from the liquid in filtration methods (van Reis & Zydney, 2007). This medium is often filters or membranes categorized by their pore size or nominal molecular weight cut-off (MWCO), normally defined as molecular weight (Mehta & Zydney, 2005). The membranes have three main features. The first one is selectivity, determined by the pore size and surface properties. The others are volumetric flux and capacity. In depth filtration the medium is a depth filter with a specific pore sizes on the filter layers. This provide the ability to remove molecules of different sizes within different layers of the filters (van Reis & Zydney, 2007). 2-phase and 3-phase separators are using centrifugal force to separate suspensions consisting of two or more phases. The phases have different densities and can therefore be used for liquid-liquid separation, liquid-liquid-solid separation or for liquid-solid separation (Brouwers, 1996) (Svarovsky, 2000).

Some membrane separation processes are reverse osmosis, ultrafiltration, microfiltration, vapor separation (Khulbe & Matsuura, 2000) and nanofiltration. In these processes the membranes have different pore sizes and characteristic. Ultrafiltration is one of the most promising method for separation in many industrial processes e.g. food processing and biotechnological industry (Susanto & Ulbricht, 2009). Ultrafiltration membranes concentrate proteins and carbohydrates, while salt, sugars and smaller peptides are passed through the membrane (Mehta & Zydney, 2005). This fractionation is based on chain length. There are different kinds of membranes available for ultrafiltration. Both flat sheet membranes and hollow fibre membranes can be made from a wide range of polymers (van Reis & Zydney, 2007). Nanofiltration membranes have smaller pores than ultrafiltration membranes and only let water, salts and monosaccharides pass through (Bowen & Mukhtar, 1996) (Hong & Elimelech, 1997). They normally have a molecular weight cut off around one nanometer in diameter (Bowen & Mukhtar, 1996). Polymeric membranes are commercially dominant membranes (UF) (Susanto & Ulbricht, 2009). When designing synthetic polymeric membranes, the relationship between the morphology of the polymer, the membrane preparation and the membrane performance need to be considered. The chemical properties of the polymer, the mechanical properties of the polymer, and the pore size, pore size distribution and pore density are important structural features of a membrane (Khulbe &

Matsuura, 2000). The membranes itself is often the key to a significant separation. A high flux and selectivity, low fouling and stability are important characteristics which are based on the previously mentioned properties. Polysulfone and polyethersulfone ultrafiltration membranes are polymeric membranes often used in industry (Susanto & Ulbricht, 2009).

2.9 Kjeldahl method

The Kjeldahl method is the most common method, and the standard process, for determination of organic nitrogen in biological material (Pruden et al., 1985) (Skoog et al., 2014). Most proteins contain nearly the same amount of nitrogen (%). A suitable factor is multiplied with the percentage of nitrogen giving the percentage of protein in the sample. A suitable factor for different samples varies depending on the protein composition of the samples in question; 6,25 for meat and 6,38 for dairy products (Skoog et al., 2014) (Silva et al., 2016). Hot and concentrated sulfuric acid is used to convert the bound nitrogen in the samples to ammonium ions before the solution is cooled down and diluted (Kirk, 1950) (Skoog et al., 2014). This decomposition step is the most critical step in the method where the carbon and the hydrogen are oxidized to carbon dioxide and water. The solution is then made alkaline in order to convert the ammonium ions to ammonia. The last step in the process is a neutralization titration of the ammonia which is separated from the alkaline solution by distillation and collected in the acid solution before titrated (Skoog et al., 2014). Kjeltabs which contains potassium, selenium and sulphate are added together with the sulfuric acid to raise the boiling point of the acid from 180 ºC to 380-400 ºC (Silva et al., 2016). This is to ensure the total degradation of the organic matter and the stabilization of the newly formed ammonium sulphate from the liberated nitrogen. The selenium and sulphate also help with the oxidation process provided by the acid (Kirk, 1950) (Silva et al., 2016).

2.10 Chromatography

Chromatography is a tool that is used for separation, identification, and determination of closely related chemical components. All chromatographic methods use a stationary phase and a mobile phase. Components from the applied sample are carried through the stationary phase, which is fixed in place in a column or on a planer surface, by the flow of the mobile phase. The chromatographic methods are mainly divided in two. If the stationary phase is held in a narrow tube while the mobile phase is pushed through the tube by gravity or pressure the method is called column chromatography. The other method is planar chromatography like

e.g. thin layer chromatography. Column chromatography is divided in three categories depending on the nature of the mobile phase that can be either a gas, a liquid or a supercritical fluid (Skoog et al., 2014).

2.11 Hydrolysis with acid

Hydrolysis is used to break down a large molecule to smaller and simpler molecules. In acid hydrolysis a proteolytic acid is used as supplier for H^+ ions in a nucleophilic substitutions reaction to cleave chemical bonds. This is an essential step when studying monosaccharide composition in oligo- and polysaccharides because the acid hydrolysis breaks glycosidic bonds and converts the carbohydrates into monosaccharides. Sugar recovery standards (SRS) are usually hydrolysed together with the samples to monitor the hydrolysis. A conversion factor is calculated based on the amount of sugars hydrolysed in the SRS and taken into account when calculating the abundance of monosaccharides in the samples. Dallies and co-workers concluded in a study that hydrolysis with sulfuric acid coupled with high-performance anion-exchange chromatography (HPAEC) analysis is the best method for determination of carbohydrates in the yeast cell wall (Dallies et al., 1998). They reported that this method is the most complete, accurate and reproducible method in comparison to other techniques.

2.12 Cell disruption

Methods for cell disruption are generally divided in two. The non-mechanical methods explain chemical treatment with base, acid, detergents, enzyme analysis and physical treatment, such as osmotic shock. Examples of mechanical methods are high pressure homogenization, Bead Mill, ultra-sonification, decompression and treatment with grinding particles (Figure 6) (Geciova et al., 2002).

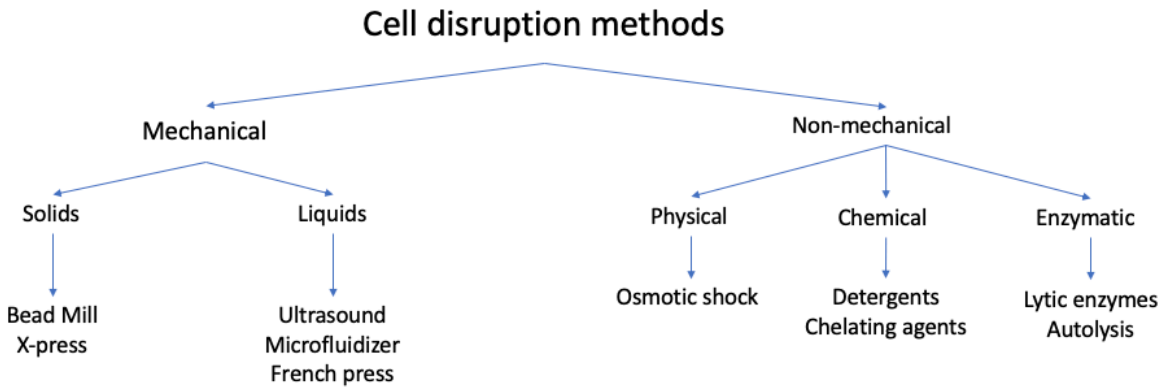

Figure 6: Overview of a selection of cell disruption methods. The figure is based on Figure 1 by (Geciova et al., 2002) adapted from (Middelberg, 1995).

Mechanical methods, such as bead milling, high-pressure-homogenization and microfluidizer, are often favoured for large-scale production (Geciova et al., 2002). Advantages with these methods are that they operate in a continuous matter, and have a short residence time, in addition they have low operating costs and are easy to clean (Middelberg, 1995). However, they are unspecific since the disruption is caused by tearing the structures apart (Geciova et al., 2002). High pressure homogenization is widely used in the industry in a range of different applications like making emulsions in the dairy industry (Bhattarai et al., 2019).

One type of high-pressure homogenization method is the microfluidizer. Other advantages with this method than those already mentioned, are that the cell suspensions that are to be disrupted can have a quite low concentration. The method can actually disrupt cell with a concentration as low as 5g/L (Geciova et al., 2002). Particles of larger sizes than compared to particles made by other homogenizer devises are created after the disruption as well. This can have an impact on the following centrifugation step, if included, with a more efficient separation of the disrupted material (Baldwin & Robinson, 1990). But this separation is also influenced by the number of passes in the process. A declining in the efficiency of the centrifugation is seen when the number of passes increases. This is because of a bigger degradation of the cell debris, and because the viscosity of the liquid decreases (Geciova et al., 2002). Baldwin and co-workers combined the disruption of *Saccharomyces cerevisiae* yeast cells by microfluidizer with an enzymatic pre-treatment step to gain a higher cell disruption. The results showed that this treatment enhanced the disruption by the microfluidizer by soften the cells prior to disruption. A combination of mechanical and non-

mechanical methods and treatments are often considered to gain a higher disruption of the material (Baldwin & Robinson, 1990).

A simple and direct method to measure the disruption is by microscopy where the volume or number of cells disrupted is determined. Another, but indirect method is to measure the release of specific enzymes during the process (Middelberg, 1995).

3 Materials

3.1 Laboratory equipment and materials

Category	Equipment	Supplier
Freezer	Ultra-low temperature freezer	New Brunswick
Freeze dryer	Alpha 2-4 LDplus	Martin Christ
Counting plate	Brand™ Bürker	fisher scientific
Kjeldahl protein determination	Kjeltec TM 8400	FOSS, Tecator
	Autodigestor	Hoganas Sweden
Termomixer	TermoMixer C	Eppendorf
Autoclave	Labor-Autoklav	CertoClav
Incubation shaker	Multitron Standard	INFORS HT
Oven	HERATHERM oven	Thermo Scientific
pH measurements	Doratest pH test strips	VWR Chemicals
	pH probe	Mettler Toledo
Vacuum pump	VCP 80 pump	VWR™
Chromatography machine	Ultimate 3000	Thermo Scientific
	Dionex ICS 3000 (AS-AP)	Thermo Scientific
Water bath	SBB Aqua S plus	Grant
	Julabo 5A ED max 60 ºC	Julabo
Gas analyser	FerMac 368 off-gas analyzer	Electrolab Biotech Tewkesbury, UK
Software	IRIS process control software	Infors
	Chromeleon Console 7,0	Termo Fisher Scientific
Centrifuges	MEGA STAR 1.6R	VWR™
	Avanti J-26S XP Centrifuge	Beckman Coulter™
	Allegra X30R Centrifuge	Beckman Coulter™
	Avanti J-25 XP Centrifuge	Beckman Coulter™
	Centrifuge 5418R	Eppendorf
Balances	Quintix Analytical balance	Sartorius
	Entris Analytical balance	
Chromatography columns	Dionex AminoTrap™ BioLC™ Guard 2x50	Termo Fisher Scientific

	Dionex CarboPac PA1	Termo Fisher Scientific
	Rezex RFQ-Fast Acid H+ (8%) 100 x 7,8 mm	Termo Fisher Scientific
	Yarra 3 um SEC-2000 LC column 300x 46 mm	phenomenex
Microscope	Leitz, Laborlux K	Leitz

3.2 Chemicals

Chemical	Supplier
Sodium hydroxide (NaOH)	Sigma-Aldrich (USA)
Sulfuric acid (H_2SO_4)	VWR Chemicals
Oxalic acid ($C_2H_2O_4$)	Aldrich Chemistry
Sodium acetate (NaOAc)	Sigma-Aldrich
Sicapent (phosphorus pentoxide)	Merck KGaA (Germany)
Anti-foam	Glanapon DB 870antifoam, Busetti, Vienna, Austria
Kjeltabs	Thomson and Capper Ltd (Cheshire UK)

3.3 Enzymes

Enzyme	Supplier
Alcalase 2.4 L FG	Novozymes
Glucanex (β-glucanase, cellulase, protease, chitinase activities)	Sigma Aldrich

3.4 Carbohydrates (mainly monosaccharides) and proteins

Monosaccharide	Supplier
D-(+)-glucose	VWR Chemicals
D-(+)-mannose	Sigma
D-(+)-xylose	Sigma Aldrich
L-(+)-arabinose	Sigma Aldrich
D-(+)-galactose	Sigma Aldrich
D-(+)-glucosamine hydrochloride	Sigma

N-acetyl-D-glucosamine	Sigma
L-(-)-fucose	Sigma
D-(-)-fructose	VWR
Ribose	In house stock
Mannan (*Saccharomyces cerevisiae*)	Sigma
Pullulan $(C_6H_{10}O_5)_n$	PSS-polymer
Proteins	Supplier
Bovine Serum Albumin (BSA)	Sigma-Aldrich

3.5 Fermentation medium

Media	Substances	Supplier
Borregaard Advanced Lignin™ (BALI)		Borregaard
YPD	Peptone from meat, bacteriolocial, Yeast extract, D-*(+)-Glucose*	Sigma-Aldrich
Chicken meat hydrolysate		Nortura Hærland

3.6 Yeast strains

Strains	Supplier
Candida utilis LYCC 7549	Lallemand Yeast Culture Collection, Montreal, Canada
Arxula adeninivorans J121 *Wickerhamomyces anomalus* LS3	Swedish University of Agricultural Sciences, Uppsala, Sweden

3.7 Fermenters

Fermenter	Supplier
Techfase-S 30L	Infors HT
Minifors	Infors

3.8 Biorefinery; machines and equipment

Category	Equipment	Supplier
Cell disruption	Microfluidizer™ Simens SIMATIC HMI LM20	Microfluidizer™
Filtration filters	GEA filtration Danmil depthfilter 05-0,2 MIC Lot no: 2313203497450	GEA DANMIL A/S
Filtration membranes	TS2540 (TRISEP® XN45) 5kDa Alfa Laval UF-PET GR90PE	Lenntech Lenntech
Separation	GEA Westfalia 2-phase separator Pilot Unit Model L	GEA
Hydrolyses	EINAR hydrolyses system	Belach bioteknik

4 Method

4.1 Experimental design

As previously mentioned, the aim of this master thesis was to produce different strains of yeasts grown on different carbon sources and to process this biological material to gain single-cell protein and carbohydrates. Figure 7 is a flowchart showing the process from production of yeast and the downstream processing performed to be able to analyse the produced yeast, laboratory scale.

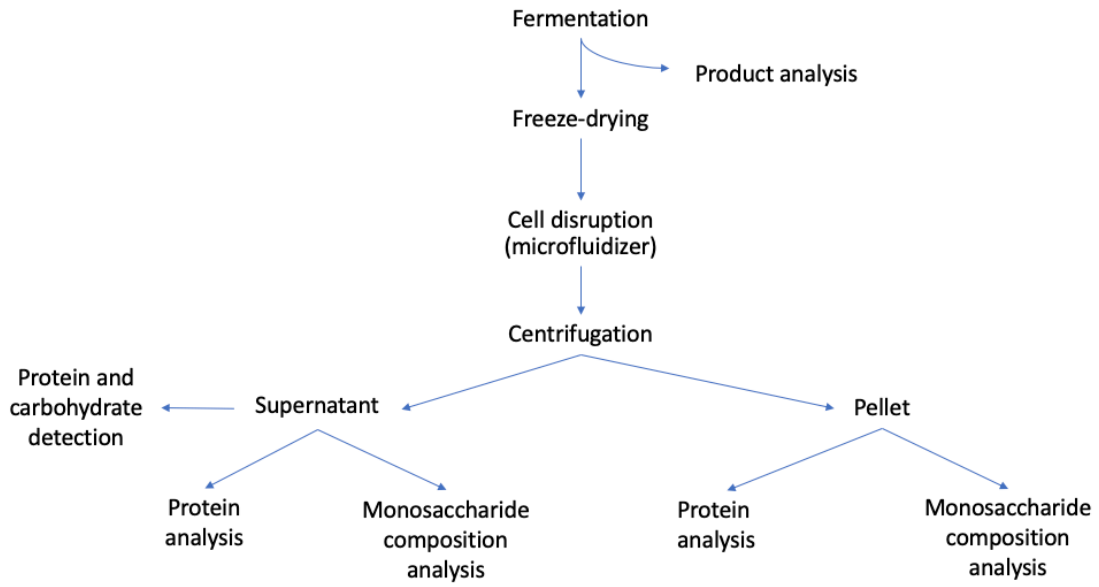

Figure 7: Overview of production, downstream processing and analysis of yeast, laboratory scale.

Bottlenecks, if any, in the downstream processing of yeast needed to be discovered before up-scaling. The flow chart below (Figure 8) shows the final process from production of yeast and all the downstream processing necessary to gain carbohydrate rich and protein rich fractions. The process was first tested on *A. adeninivorans* repeated fed batch fermented in 20 L to find the bottlenecks in every processing step, and then performed on *W. anomalus* repeated fed batch fermented in 25 L.

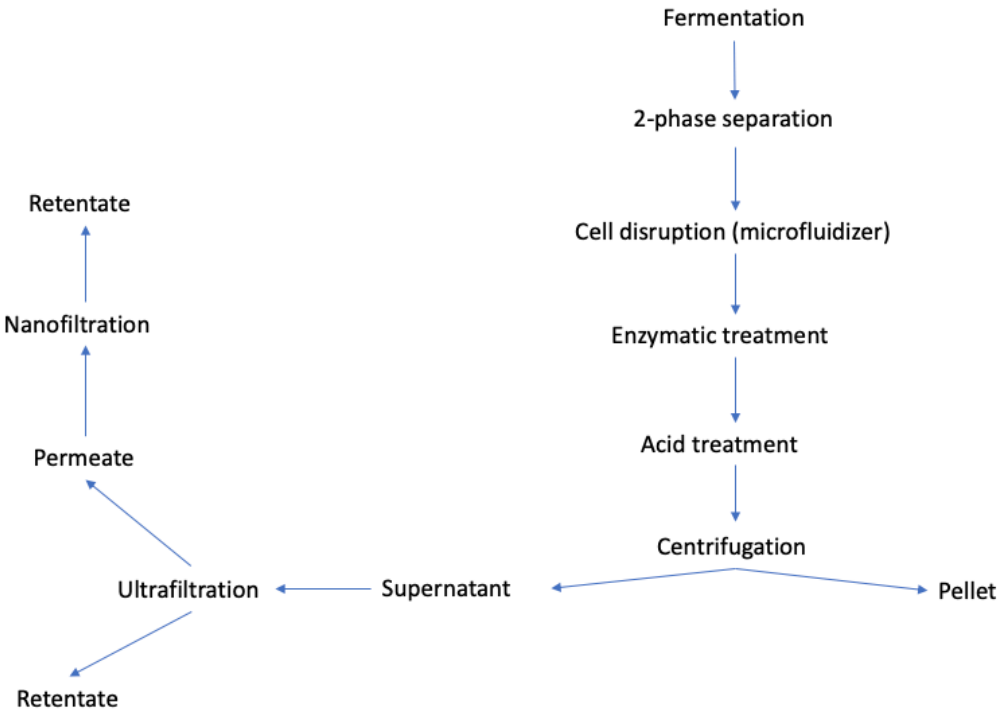

Figure 8: Flow chart showing the different processing step that were performed during the investigation of bottlenecks in the downstream processing of yeast.

4.2 Fermentations

4.2.1 Fermentation processes

The fermentations performed in this master thesis were done together with David Lapena Gomez, Pernille Margrethe Olsen and Gergely Kosa. The company Nortura Hærland (Hærland Norway) has provided the chicken meat used as growth media in the cultivations. The chicken meat is hydrolysed by David Lapena Gomez and co-workers (Lapena et al., 2018). Borregaard, (Sarpsborg Norway) has provided the sugar source, Borregaard Advanced Lignin™ (BALI).

Candida utilis LYCC 7549 (Lallemand Yeast Culture Collection, Montreal, Canada), *Wickerhamomyces anomalus* J121 and *Arxula adeninivorans* LS3 (Swedish University of Agricultural Sciences, Uppsala, Sweden) were batch fermented and repeated fed batch fermented on the complex chicken meat and BALI media in Minifors Infors bioreactors (Minifors, Infors, Bottmingen, Switzerland). The bioreactors had a working volume of 1,5 L. In the repeated fed batch fermentations cells were harvested for the first time after 16 hours, then harvests were conducted every eight hours. Fresh media was added after each harvest.

The repeated fed batch fermentations were run for three days. Furthermore, *Arxula adeninivorans* LS3 and *Wickerhamomyces anomalus* J121 were also repeat fed batch fermented at larger scale with a working volume of 20 L (*A. adeninivorans*) and 25 L (*W. anomalus*), using Techfase-S 30L, total volume 42 L. The production of *A. adeninivorans* was a test where the growth media was changed one time every 24 hours for three days as the same time as cells were harvested. The cultivation of *W. anomalus* was performed by David Lapena Gomez and Pernille Margrethe Olsen. The first harvest was after 16 hours of cultivation, while the next harvests were every eight hours until 72 hours of cultivation.

The fermentation processes and parameters were documented using IRIS process control software (Infors). 5M sulfuric acid (H_2SO_4) and 5M sodium hydroxide (NaOH) were automatically added to control the pH in the bioreactors. The pH was set to 5,0 and monitored with a pH probe (Mettler Toledo). The temperature was set to 30 ºC. The stirrer speed was set to 300 rpm in the beginning of the cultivation but had to be adjusted manually to regulate the oxygen level, which was analysed with a FerMac 368 off-gas analyser (Electrolab Biotech, Tewkesbury, UK). The CO_2 levels was also evaluated by this off-gas analyser. To prevent foam in the reactors a foam sensor controlled the foam production. A five times diluted antifoam (Glanapon DB 870 antifoam, Busetti, Vienna, Austria) was automatically added when the levels got to high.

4.2.2 Yeast fermented on YPD

Candida utilis LYCC 7549, *Wickerhamomyces anomalus* J121 and *Arxula adeninivorans* LS3 were fermented on peptone from meat, yeast extract, and D-*(+)*-Glucose (YPD) by David Lapena and co-workers (Lapena et al., 2019).

4.2.3 Medium preparation

For cultivation in 2,5 L Infors bioreactors, working volume of 1,5 L, 124 g chicken meat hydrolysate and 863 mL Milli Q water were autoclaved at 121 ºC for 20 min. 138 mL BALI sugar were added right before inoculation in the bioreactors. This gave a concentration of 5,81 g/L nitrogen, and 50 g/L glucose which gave a concentration of 8,33 g/L carbon.

For the cultivation in the 42 L reactor (Techfase-S 30L, Infors), with a working volume of 20 L, the medium was prepared at the same ratio as for the minor fermentations; 1,653 kg chicken meat hydrolysate, 1840 mL BALI sugar and 16,507 L water as the growth media.

4.2.4 Overnight precultures

The yeasts were inoculated in shake flasks approximately 16 hours prior to inoculation in the fermenters. For the fermentations in the minifors bioreactors the precultures were made by adding 200 µl yeast culture to 50 mL media (chicken meat hydrolysate and BALI) in a 250 mL shake flask. In the up-scaling cultivation, 400 mL media was added in a 2 L flask with 1,6 mL yeast culture.

4.3 Analytical analysis after fermentation

25 mL sample was taken every forth hours during the fermentations. Regarding the *A. adeninivorans* production (20 L) 25 mL sample were taken every forth hours for 12 hours, and not during night time. These 25 mL samples were centrifugated at 4700 rpm for 5 min at 4 ºC (MEGA STAR 1.6R). The pellets were washed twice with distillate water. Both the pellets and 1 mL of the supernatants were kept for protein analysis, cell dry weight calculations (CDW) and analysis of biproduct production and sugar consumption. All the biomass pellets from the centrifugation were freeze-dried using an Alpha 2-4 LDplus (Martin Christ, Osterode am Harz, Germany) at -80 ºC at 0,01 mbar vacuum.

4.3.1 Protein analysis

Micro Kjeldahl was used to measure the protein content in the yeast samples. 3 mL 96 % sulfuric acid and one Kjeltablet were added to approximately 0,2 g smashed and freeze-dried yeast. The samples were heated up to 420 ºC for one hour for acid digestion by using the program 3001 Prot Feed AOAC on an autodigestor (FOSS, Tecator, Hoganas, Sweden). The nitrogen content was measured in every sample as % protein with a Foss Kjeltec^{TM} 8400 (FOSS, Tecator, Hoganas, Sweden) by multiplying the total nitrogen by a factor of 6,25.

4.3.2 Biproduct analysis

The supernatants from the fermentations were analysed by high-performance liquid chromatography (HPLC) based on ion exclusion, in order to observe production of biproducts such as ethanol. 5mM sulfuric acid was used as a mobile phase, while the samples were separated on a Rezex ROA-organic acid H+, 300 x 7.8 mm (Phenomenex, Torrance, CA, USA) analytical column fitted with a cation-H cartridge guard column as the stationary phase. The samples were diluted 10 times before 100 µl were vacuum filtrated through a 0,45 µm filter on a 96 well plate before applied on to the system. The flow was 0,6 mL/min, the

temperature on the column was 65 ºC, and the running time was 25 minutes per sample. Chromeleon software 7,0 (Thermo Fischer Scientific) was used in the analysis. 0,5 g/L – 10 g/L ethanol, acetic acid, lactic acid, glucose and xylose were used as standards in the analysis.

4.4 Downstream processing of yeast

The following processing and analysis are done together with Cathrine Nilsen Sebjørnsen (master student at NMBU). Batches of *C. utilis*, *W. anomalus* and *A. Adeninivorans* were chosen for downstream processing to be able to compare properties of the yeast. One batch of repeated fed batch fermented *C. utilis* and *W. anomalus* (last batch) from the 1,5 L fermentations and one batch from the *A. Adeninivorans* 20 L fermentations were also processed further. A schematic overview of the chosen yeasts is shown in Table 1.

Table 1: Yeast samples chosen for downstream processing and analysis.

Strain	Fermentation	Working volume (L)	Growth media
A. Adeninivorans	Repeated fed batch	20	Chicken hydrolysate and BALI
C. Utilis	Repeated fed batch	1,5	Chicken hydrolysate and BALI
W. Anomalus	Repeated fed batch	1,5	Chicken hydrolysate and BALI
A. Adeninivorans	Batch	1,5	Chicken hydrolysate and BALI
C. Utilis	Batch	1,5	Chicken hydrolysate and BALI
W. Anomalus	Batch	1,5	Chicken hydrolysate and BALI
A. Adeninivorans	Batch	1,5	YPD
C. Utilis	Batch	1,5	YPD
W. Anomalus	Batch	1,5	YPD

4.4.1 Cell disruption by high-pressure homogenization

Cell disruption by the use of Microfluidizer™ SIMATIC HMI LM20 was performed on the freeze-dried yeasts from the batch fermentations and the repeated fed batch fermentation. A 12% yeast slurry were made (12% DM) from each sample and disrupted at a pressure of 30 000 PSI in 45 min per sample in loop. 150 µl anti-foam was added before the run to prevent foaming. To be able to measure cell lysis, 100 µl sample was taken out before the lysis and 100 µl after the cell disruption (lysis).

4.4.2 Centrifugation and freeze drying

After 45 minutes of cell disruption, the yeast cells were harvested and centrifugated (Avanti J-25 XP Centrifuge) at 8 000 g in 10 min at 4 ºC. The supernatants were collected and frozen at -80 °C overnight. The pellets were washed two time by centrifugation at the same conditions with Milli Q water before they also were frozen. The frozen supernatants and pellets were then freeze dried (Alpha 2-4 LDplus) at -80 ºC at 0,01 mbar for drying.

4.4.3 Cell lysis, counting

A counting plate, Brand™ Bürker, was used to measure cell lysis. To be able to count the cells the samples were diluted 60 times before 10 µl were added on to the plate. % cell lysis was measured by counting intact cells in the samples taken before cell disruption and after cell disruption in a microscope (Leitiz, Laborlux K).

4.5 Analytical methods

4.5.1 Monosaccharide composition analysis

In order to analyse the monosaccharide composition in the pellets and the supernatants derived from the cell disruption 25 µl 72% H_2SO_4 was added to triplicates of 10 mg/mL (pellets) and 5 mg/mL (supernatants) samples. 700 µl water was added to get a 4% H_2SO_4 solution after incubation at 30 ºC for 1 hour. The samples were then autoclaved (Labor-Autoklav) at 121°C for 1 h and filtrated (VCP 80 pump).

Sugar recovery standards (SRS standards) were made by mixing 400 µl 12 mg/mL glucose, 366,7 µl 11 mg/mL mannose, 100 µl 1 mg/mL N-acetyl-glucosamine (GlcNAc) and 133,3 µl water. 500 µl were transferred to a second eppendorf tube, and 11,4 µl 96% H_2SO_4 were added to both tubes to get a 4% H_2SO_4 solution. The SRS standards were autoclaved together with the samples at 121°C for 1 hour before they were centrifugated (Centrifuge 5418R, Eppendorf) for 5 minutes on maximum speed.

High-performance anion-exchange chromatography (HPAEC) conducted on an ICS 3000 (Dionex Sunnivale, California USA), set up with a pulsed amperometric detector (PAD) with a disposable electrochemical gold electrode, was used to detect monosaccharides in the hydrolysed samples and in the SRS standards. The samples and the SRS were diluted 50 times before applied on to the system. 0,5 mg/mL fucose was used as an internal standard. In

addition to the samples, a mix of glucose, mannose, glucosamine, arabinose, xylose and ribose were made and used as standards for the ICS run (Table 2). 1mM KOH (electrolytically generated) was used as the mobile phase, and an anion-exchanger, Dionex CarboPac PA1 250 mm x 2mm kept at 30 ºC, was used as the stationary phase during the analysis. A Dionex AminoTrap™ BioLC™ Guard 2x50 mm was used to prevent amino acids to interrupt the detection. The solutes were eluted using a flow of 0,250 mL/min. The running time was 35 minutes per samples. Chromeleon software 7,0 (Thermo Fischer Scientific) was used for the analysis.

Table 2: Concentrations of glucose, mannose, glucosamine, arabinose, xylose and ribose used as standards in the monosaccharide composition analysis.

	Concentration (mg/mL)					
	Glucose	Mannose	Glucosamine	Arabinose	Xylose	Ribose
Level 1	0,1	0,1	0,05	0,05	0,05	0,05
Level 2	0,01	0,01	0,005	0,005	0,005	0,005
Level 3	0,005	0,005	0,0025	0,0025	0,0025	0,0025
Level 4	0,001	0,001	0,0005	0,0005	0,0005	0,0005

4.5.2 Dry weight analysis

A dry weight analysis was done to be able to calculate the exact amounts of sugars in the yeast samples. Because of limited amount of some samples the analysis is performed on three pellets and three supernatants, see Table 3. 300 mg samples, triplicates, were weighted and dried in an oven at 105 ºC. After 20 hours the samples were transferred to a vacuum exicator (with Sicapent) for evaporation for 1 hour and then weighted again.

Table 3: Dry weight analysis was performed on a selection of yeast samples, both pellets and supernatants, cultivated on different growth media.

Strain	Sample	Growth medium
W. Anomalus	Supernatant	YPD
W. Anomalus	Supernatant	Chicken hydrolysate and BALI
C. Utilis	Supernatant	Chicken hydrolysate and BALI
A. Adeninivorans	Pellet	Chicken hydrolysate and BALI
C. Utilis	Pellet	Chicken hydrolysate and BALI
W. Anomalus	Pellet	Chicken hydrolysate and BALI

4.5.3 Detection of ribose

Ion exclusion chromatography was used to confirm that the ribose detected in the monosaccharide composition analysis was correct. All samples containing ribose were run on a Rezex RFQ-Fast Acid H+ (8%) 100 x 7,8 mm with 5mM H_2SO_4 as the mobile phase for 6 minutes at 85 ºC with a flow of 1,0 mL/min. 50 mM and 100 mM N-acetyl-glucosamine was used as a standard. Chromeleon software 7,0 (Thermo Fischer Scientific) was used to monitor the analysis (results not shown).

4.5.4 Size exclusion chromatography (SEC)

Size exclusion chromatography was used to detect the protein fraction and the carbohydrate fraction in the repeated fed batch supernatants. 10 mg sample was dissolved in 1 mL Milli Q water (total volume). Seven replicates from each sample were weighted and treated differently with enzymes according to Table 4. Because of small volumes the highly concentrated Alcalase 2,4 L FG was diluted (1:2000) and 1µl was added to the samples. In addition, a protein standard of 10 mg/mL Bovine Serum Albumin (BSA), a carbohydrate standard of 10 mg/mL *Saccharomyces cerevisiae* (SC) Mannan, and a positive control of 10 mg/mL SC Mannan treated with 50 µl glucanex were made. 250 µl of 200 mM NaOAc (pH 5) was used as a buffer in some samples. 2 mg/mL Pullulan with molecular sizes of 21,1 kDa, 0,342 kDa, 6,2 kDa, 48,8 kDa and 133 kDa were used as molecular weight standards.

Table 4: Enzymatic treatment with glucanex and alcalase on supernatants from *C. utilis, W. anomalus* and *A. adeninivorans* repeated fed batch fermented for analysis by size exclusion chromatography.

			Amount added			
Strain	Sample	Treatment	Alcalase (µl)	Glucanex (µl)	NaOAc (µl)	Water (mL)
C. Utilis	1	Water	0	0	0	1
	2	Alcalase	1		0	0
	3	Glucanex	0	50	0	0
	4	Alcalase and glucanex	1	50	0	0
	5	Alcalase and NaOAc	1	0	250	0
	6	Glucanex and NaOAc	0	50	250	0
	7	Alcalase, Glucanex and NaOAc	1	50	250	0
W. Anomalus	1	Water	0	0	0	1
	2	Alcalase	1		0	0
	3	Glucanex	0	50	0	0
	4	Alcalase and glucanex	1	50	0	0
	5	Alcalase and NaOAc	1	0	250	0
	6	Glucanex and NaOAc	0	50	250	0
	7	Alcalase, Glucanex and NaOAc	1	50	250	0
A. Adeninivorans	1	Water	0	0	0	1
	2	Alcalase	1		0	0
	3	Glucanex	0	50	0	0
	4	Alcalase and glucanex	1	50	0	0
	5	Alcalase and NaOAc	1	0	250	0
	6	Glucanex and NaOAc	0	50	250	0
	7	Alcalase, Glucanex and NaOAc	1	50	250	0

After starting the enzymatic degradation by adding the enzymes, the samples were incubated in a thermomixer (Termo Mixer C) over-night (1000 rpm at 37 ºC). To stop the enzymatic reactions the samples were boiled for 15 minutes (SBB Aqua S plus, Grant). 100 µl sample were transferred to new tubes and run on the SEC system with a Yarra 3 um SEC-2000 LC column 300x 46 mm, and water as the mobile phase. The samples were analysed for 35 minutes with a temperature of 40 ºC, 0,200 mL/min flow, with a refractive index (RI) and an ultraviolet (UV) detector at 280 nm. Chromeleon software 7,0 (Thermo Fischer Scientific) was used to document the analysis.

4.5.5 Protein analysis

Micro Kjeldahl was used to measure the protein content in the disrupted yeast samples. See previously described procedure (section 4.3.1).

4.6 Discover of bottlenecks in an up-scaling process

4.6.1 Cell disruption

1,5 L *A. adeninivorans* from 20 L fermentation were run through a high-pressure homogenizer (Microfluidizer™ SIMATIC HMI LM20) at 30 000 PSI three times. Before stored in a cold room over-night. A total of 4,5 L was disrupted.

4.6.2 Enzymatic treatment and centrifugation

2,5 mL alcalase 2,4 L FG was added to the 4,5 L disrupted yeast and incubated at 60 ºC for three hours (Multitron Standard, Infors HT). The enzymatic treated yeast was then centrifugated for 5 min at 10 000g (Avanti-J265 Centrifuge). The supernatant was kept for further processing.

4.6.3 Filtration

Depth filtration (Danmil depthfilter, DANMIL A/S) was used to filtrate the supernatant to get a clear suspension. The size of the pores in the filter were 0,05-0,2 µm. Further, the solution was filtrated through a cross-flow membrane filtration system (GEA filtration) with a 5 kDa cut off ultra-filtration membrane made of polyethersulphone with polyester as the support material at 10 bar and 45 ºC. The collected permeate was then nano-filtrated through a Lenntech TS2540 nano-filtration membrane with a molecular weight cut off between 300-500 Da at a pressure of 40 bar and a temperature of 40 ºC.

4.6.4 Testing the effect of oxalic acid and enzymatic treatment

0,4 L *A. adeninivorans* from 20 L fermentation were run through a high-pressure homogenizer for three passes at 30 000 PSI. The 0,4 L disrupted cells were then divided into two 0,2 L bottles. One bottle was then treated with 100 µl Alcalase 2.4 L FG for three hours. Both bottles were transferred to 15 mL falcon tubes, 7,5 mL in each. These samples were then treated with different concentrations of oxalic acid at different temperatures and time periods according to the table below (Table 5). The pH after adding oxalic acid is measured with pH paper (Doratest pH test strips).

25 mL Alcalase treated yeast and untreated yeast were also centrifugated at 3000 rpm for 5 min at 20 ºC (Allegra X30R Centrifuge). The supernatants were transferred to 15 mL falcon tubes, treated with 100 mM oxalic acid, and incubated at 60 ºC over-night. To test for how long the treatment with oxalic acid should be, 7,5 mL disrupted and Alcalase treated yeast was treated with oxalic acid and incubated at 60 ºC for 1 hour, 2 hours and 4,5 hours. All samples were centrifugated at 4000 g for 5 min at 20 ºC (Allegra X30R Centrifuge) right after incubation.

Table 5: Concentration, incubation time and temperature of treatment with oxalic acid and alcalase on cell disrupted *A. adeninivorans*. Light blue: samples treated with alcalase and oxalic acid without centrifugation. White: samples treated with oxalic acid without centrifugation. Orange: Samples centrifugated before treatment with oxalic acid. Green: Samples treated with oxalic acid for different time periods.

Treatment	Concentration (mM)	Temperature	Time	pH
Alcalase + oxalic acid	100	60	over night	3
Alcalase + oxalic acid	100	40	over night	3
Alcalase + oxalic acid	100	25	over night	3
Alcalase + oxalic acid	50	60	over night	3
Alcalase + oxalic acid	50	40	over night	3
Alcalase + oxalic acid	50	25	over night	3
Oxalic acid	100	60	over night	3
oxalic acid	100	40	over night	3
oxalic acid	100	25	over night	3
oxalic acid	50	60	over night	3
oxalic acid	50	40	over night	3
oxalic acid	50	25	over night	3
Alcalase + Oxalic acid	100	60	over night	3
Oxalic acid	100	60	over night	3
Alcalase + Oxalic acid	100	60	1 hour	3
Alcalase + Oxalic acid	100	60	2 hours	3
Alcalase + Oxalic acid	50	60	2 hours	3
Alcalase + Oxalic acid	100	60	4,5 hours	3

4.7 Production and downstream processing of *W. anomalus*

W. anomalous were produced by repeated fed batch fermentation by David Lapena Gomez and Pernille Margrethe Olsen in a 25 L fermentation. The growth media was a mix of chicken meat hydrolysate (80%), urea (20%), and 2,33 L BALI sugar (50 g/L).

18,5 L *W. anomalus* was run through a 2-pahase separator for washing (120 sec per shot). Cell disruption of 4,5 L solid from this separation was performed with a high-pressure homogenizer, Microfluidizer, for three passes at 30 000 PSI. After this cell lysis 2,5 mL alcalase (2.4 L FG) was added and incubated in a Belac hydrolysis system for 3,5 hours at 30 ºC. 500 mL 1M Oxalic acid was added after Alcalase treatment (end concentration 100mM), and slowly cold down to room temperature. The suspension from this acid treatment was centrifugated at 10 000 g for 10 min twice. The transparent supernatant was collected while the pellet was dissolved and washed in 15 L water before running through the 2-phase separator (240 sec per shot) again. The liquid phase was combined with the supernatant from the centrifugation before flown through on a UF 5kDa membrane (10 bar at 45 ºC). Both the permeate and the retentate were collected after the ultra-filtration. Further, the permeate was filtrated again through a Lenntech TS2540 membrane (40 bar at 40 ºC), and the retentate from this nano-filtration was collected as well. Samples were taken at every processing step and analysed by Fanny Buffetto.

5 Results

5.1 Analytical results from the fermentations

Five different fermentations were performed during the work with this master thesis. One batch fermentation, and four repeated fed batch fermentations. 25 mL sample were taken at different time points during the fermentations and analysed for cell dry weight, protein content and bioproduct production. The cell dry weight calculations and the protein analysis from all these cultivations are presented in Table 6-9. The results from the biproduct analysis are shown in Figure 9-11.

5.1.1 Cell dry weight and protein content

The calculated cell dry weight (CDW g/L) and the protein content (g/L) from the different fermentations are shown below. The 25 mL samples are taken in duplicates in the batch fermentation. Table 6 shows that after 24 hours of yeast production on chicken meat hydrolysate and BALI *C. utilis* had the lowest protein concentration, while *A. adeninivorans* had the highest concentration of proteins.

Table 6: Overview of the cell dry weight (g/L) and the protein content (g/L) from batch fermentation with *C. utilis, W. Anomalus, A. Adeninivorans*. Sampling points are after 12 hours and 24 hours of cultivation.

Strain	Time	CDW (g/L)	% Protein	Protein (g/L)
C. utilis	12	14,0 ± 1,18	47,1 ± 0,66	6,6
	24	17,9 ± 1,66	45,1 ± 0,85	8,1
W. anomalus	12	17,7 ± 0,44	50,8 ± 0,39	9,0
	24	29,7 ± 0,23	45,9 ± 0,29	13,6
A. Adeninivorans	12	16,4 ± 1,59	47,2 ± 0.35	7,8
	24	44,0 ± 0,03	41,9 ± 2.61	18,4

The analysed samples from the repeated fed batch fermentation of *A. Adeninivorans* were taken every four hours until 12 hours of production, and then one time just before starting the next cultivation cycle. CDW (g/L) and protein concentration (g/L) from the 24 hours samples are presented in Table 7. The protein content and cell density are higher for the repeated fed batch fermentation (Table 7), compared with the batch fermentation of *A. adeninivorans* (Table 6).

Table 7: Cell dry weight (CDW g/L) and protein content (g/L) of *A. adeninivorans* after a 20 L repeated fed batch fermentation. The samples are taken after 24 hours of cultivation from each cycle.

Strain	Cycle	Sampling time (h)	CDW (g/L)	% Protein	Protein (g/L)
A. Adeninivorans	1	24	48,7	41,6	20,3
	2	24	58,0	41,7	24,2
	3	24	56,9	41,3	23,5

C. utilis and *W. anomalus* were repeated fed batch fermented with a working volume of 1,5 L. 25 mL samples were taken every four hours. This cultivation was run with eight cycles where new media was added for the first time after 16 hours, and second time after 28 hours. The rest of the harvests were conducted every eight hours. The last harvest was after 76 hours of cultivation. Table 8 presents the calculated CDW (g/L) and the protein content (g/L) from a selection of sampling points.

Table 8: In a selection of samples from different sampling points the cell dry weight (CDW g/L) and protein content (g/L) in repeated fed batch fermented *C. utilis* and *W. anomalus* (1,5L working volume) were calculated.

Strain	Time (h)	CDW (g/L)	% Protein	Protein (g/L)
C. utilis	16	12,2	46,7	5,7
	28	28,4	43,9	12,5
	36	22,7	39,3	8,9
	44	29,6	41,6	12,3
	52	25,9	41,6	10,8
	60	26,5	43,5	11,5
	68	26,1	38,1	9,9
	76	26,9	43,0	11,6
W. anomalus	16	20,4	49,5	10,1
	28	32,0	48,6	15,6
	36	29,1	50,7	14,7
	44	29,9	50,8	15,2
	52	28,8	50,9	14,7
	60	25,0	52,8	14,4
	68	27,4	49,4	16,6
	76	33,7	48,3	16,3

A selection of the CDW (g/L) calculations and the measured protein content (g/L) from the repeated fed batch fermentation with *C. utilis*, *W. anomalus* and *A. adeninivorans* (1,5 L working volume) are presented below in Table 9. The harvests were conducted every eight hours until 72 hours of cultivation.

Table 9: Overview of protein content (g/L) and cell dry weight (CDW g/L) from repeated fed batch fermentations of *C. utilis, W. anomalus* and *A. adeninivorans*. Growth medium were chicken meat hydrolysate and BALI sugar.

Strain	Sampling time (h)	CDW (g/L)	% Protein	Protein (g/L)
C. Utilis	16	16,4	42,3	6,9
	24	21,0	40,2	8,4
	32	17,8	43,2	7,7
	40	20,5	41,1	8,4
	48	22,6	40,1	9,1
	56	17,7	40,1	7,1
	64	18,6	42,7	7,9
	72	22,1	38,9	8,6
W. Anomalus	16	7,3	48,4	3,6
	24	23,0	47,4	10,9
	32	19,9	47,4	9,4
	40	21,2	48,4	10,3
	48	21,5	47,8	10,3
	56	24,4	48,5	11,8
	64	17,2	49,6	8,5
	72	18,8	49,5	9,3
A. Adeninivorans	16	13,7	47,0	6,5
	24	1,6	46,5	0,7
	32	10,8	49,7	5,3
	40	13,4	48,6	6,5
	48	16,0	41,9	6,7
	56	12,2	48,4	5,9
	64	14,3	46,9	6,7
	72	16,1	50,1	8,0

5.1.2 Biproduct analysis

High performance liquid chromatography (HPLC) was used to analyse the production of bioproducts during the cultivation processes. The amount of glucose, xylose and ethanol in the supernatants from the batch fermentation and repeated fed batch fermentation (*C. utilis* and *W. anomalus*) on chicken meat hydrolysate and BALI are presented in Figure 9,10 and 11. In addition, Figure 9 and 10 are presenting the chromatograms of one standard and one typical sample from the analysis.

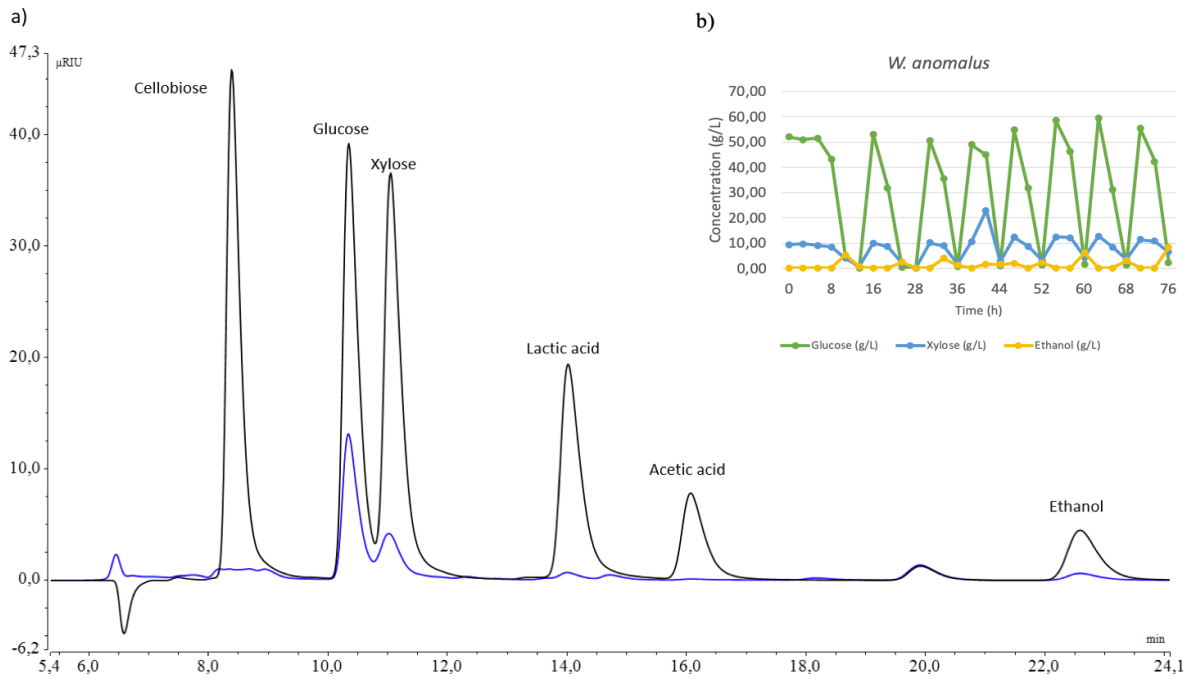

Figure 9: Analysis of biproducts in the supernatants of *W. anomalus* repeated fed batch fermented on chicken meat hydrolysate and BALI sugar analysed by high performance liquid chromatography. A chromatogram of a standard of cellobiose, glucose, xylose, lactic acid, acetic acid and ethanol (black) together with one sample analysed (blue) are shown in a). Concentration of glucose (green), xylose (blue) and ethanol (yellow) after different time points are presented in b).

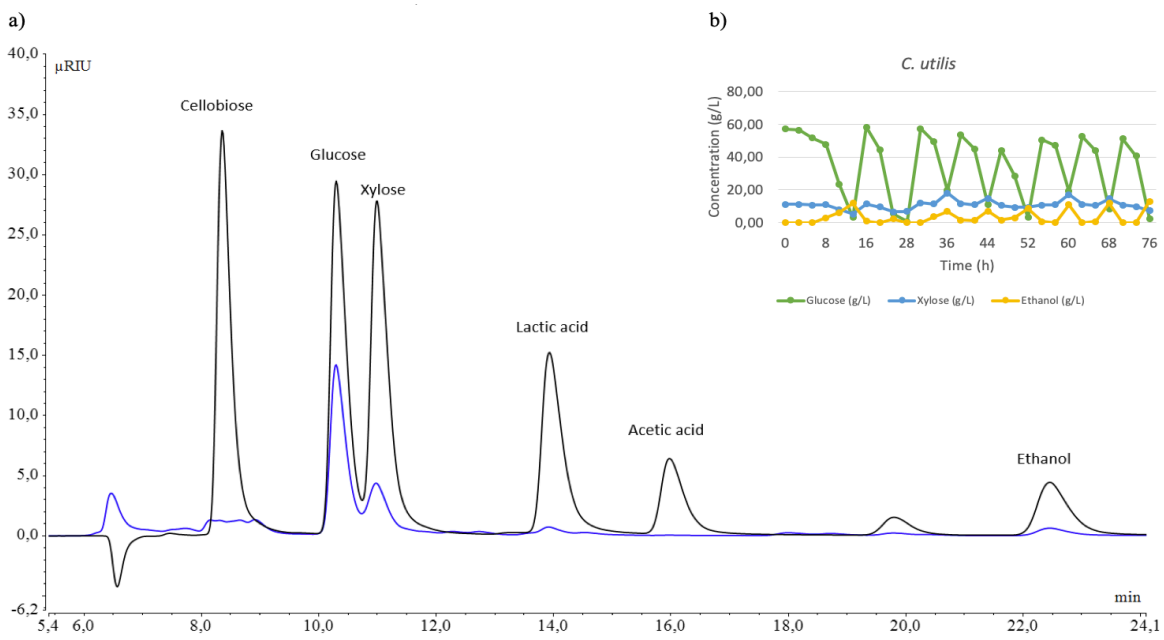

Figure 10: Analysis of biproducts in the supernatants of *C. utilis* repeated fed batch fermented on chicken meat hydrolysate and BALI sugar analysed by high performance liquid chromatography. A chromatogram of a standard of cellobiose, glucose, xylose, lactic acid, acetic acid and ethanol (black) together with one sample analysed (blue) are shown in a). Concentration of glucose (green), xylose (blue) and ethanol (yellow) after different time points are presented in b).

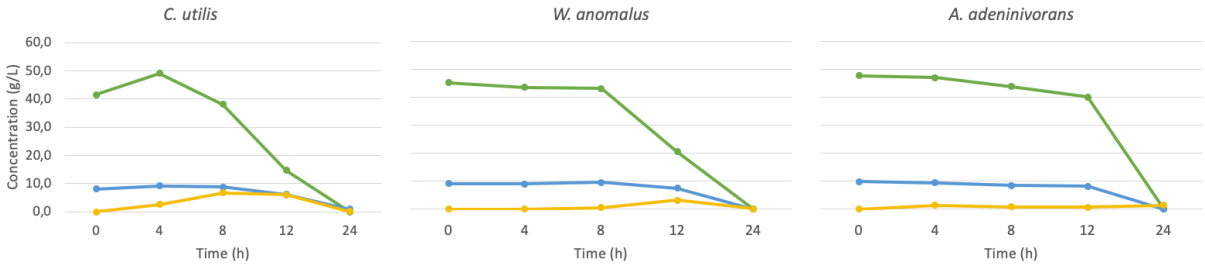

Figure 11: Concentration of glucose (green), xylose (blue) and ethanol (yellow) after different time points in the supernatants from *C. utilis, W. anomalus* and *A. adeninivorans* batch fermented on chicken meat hydrolysate and BALI sugar analysed by high performance liquid chromatography.

5.2 Downstream processing; analytical results

C. utilis, W. anomalus and *A. adeninivorans* from three of the fermentation processes were chosen for further analysis, in addition to *C. utilis, W. anomalus* and *A. adeninivorans* batch fermented on yeast peptone and glucose (YPD). Yeast from these four fermentations were disrupted and analysed for protein content and monosaccharide composition. Chromatograms from the size exclusion chromatography were processed with Chromeleon software 7,0. The results from the monosaccharide composition analysis and from the protein analysis are presented in Table 12-14 and Figure 13-16. The monosaccharides are calculated into polymers of glucose, polymers of mannose, polymers of N-acetyl-glucosamine, polymers of galactose, polymers of xylose and polymers of ribose. Intact cells were counted using a counting plate before and after cell disruption to check if all the cells were lysed. All samples had >95% cell lysis.

5.2.1 Cell wall composition analysis

Results from the proteins analysis, monosaccharide composition analysis and dry weight analysis performed on the pellets and supernatants after cell disruption of *C. utilis, W. anomalus* and *A. adeninivorans* are presented below in Table 10-13 and Figure 12-19. Table 10 shows the results from the dry weight analysis performed on a selection of the yeast samples.

Table 10: Dry weight analysis of a selection of samples after cell disruption and freeze drying. *W. anomalus (1)* and *W. anomalus (3)* are from the same fermentation but the cells are disrupted as independent samples.

Strain	Growth media	Sample	Water content (%)
W. anomalus (1)	Chicken meat and BALI	Pellet	2,8
C. utilis	Chicken meat and BALI	Pellet	3,6
A. adeninivorans	Chicken meat and BALI	Pellet	1,9
W. anomalus (3)	Chicken meat and BALI	Supernatant	8,1
W. anomalus	YPD	Supernatant	4,8
C. utilis	Chicken meat and BALI	Supernatant	2,8

Table 11, 12 and 13 are overviews of the analysis done on the pellets and the supernatants categorised based on fermentation process and growth media. All the presented numbers are relative to the weight of the dried pellets and supernatants. The presented results are protein content and monosaccharide composition within each yeast strains.

Table 11: Protein content and monosaccharide composition in *C. utilis, W. anomalus* and *A. adeninivorans* after batch fermented on yeast meat peptone and glucose.

Batch fermentation YPD														
	C.utilis													
	Sample	Protein content (%)	Protein (g/g sample)	Galactose (% of DM)	Galactose (g/L)	Glucose (% of DM)	Glucose (g/L)	Mannose (% of DM)	Mannose (g/L)	GlcNAc (% of DM)	GlcNAc (g/L)	Xylose (% of DM)	Xylose (g/L)	Ribsoe (% of DM)	Ribose (g/L)
	Pellet	63,407	0,709	0	0	76,647 (±5,310)	0,857	40,821 (±1,827)	0,457	1,514 (±0,126)	0,0169	0	0	0	0
	Supernatant	8,741	0,008	0	0	26,557 (±3,887)	0,026	11,830 (±1,973)	0,011	0	0	0	0	0,872 (±0,197)	0,001
	A. Adeninivorans														
	Sample	Protein content (%)	Protein (g/g sample)	Galactose (% of DM)	Galactose (g/L)	Glucose (% of DM)	Glucose (g/L)	Mannose (% of DM)	Mannose (g/L)	GlcNAc (% of DM)	GlcNAc (g/L)	Xylose (% of DM)	Xylose (g/L)	Ribsoe (% of DM)	Ribose (g/L)
	Pellet	58,13	0,571	3,078 (±0,484)	3,023	24,110 (±2,104)	0,237	14,390 (±2,200)	0,141	6,866 (±1,226)	0,067	0	0	1,148 (±0,520)	0,011
	Supernatant	35,696	0,306	1,697 (±0,185)	1,456	17,465 (±2,422)	0,150	10,878 (±1,628)	0,093	0	0	0	0	0,723 (±0,211)	0,006
	W. Anomalus														
	Sample	Protein content (%)	Protein (g/g sample)	Galactose (% of DM)	Galactose (g/L)	Glucose (% of DM)	Glucose (g/L)	Mannose (% of DM)	Mannose (g/L)	GlcNAc (% of DM)	GlcNAc (g/L)	Xylose (% of DM)	Xylose (g/L)	Ribsoe (% of DM)	Ribose (g/L)
	Pellet	64,827	2,110	0	0	56,833 (±0,902)	1,850	35,901 (±1,362)	1,168	2,277 (±0,161)	0,074	0	0	0,021 (±0,030)	0,001
	Supernatant	18,617	0,206	0	0	29,080 (±2,275)	0,321	12,944 (±1,338)	0,143	0	0	0	0	0,659 (±0,197)	0,007

Table 12: Protein content and monosaccharide composition in *C. utilis, W. anomalus* and *A. adeninivorans* after repeated fed batch fermented on chicken meat hydrolysate and BALI sugar.

Repeated fed batch fermentation														
	C.utilis													
	Sample	Protein content (%)	Protein (g/g sample)	Galactose (% of DM)	Galactose (g/L)	Glucose (% of DM)	Glucose (g/L)	Mannose (% of DM)	Mannose (g/L)	GlcNAc (% of DM)	GlcNAc (g/L)	Xylose (% of DM)	Xylose (g/L)	Ribsoe (% of DM)	Ribose (g/L)
	Pellet	42,926	1,319	0	0	38,030 (±6,976)	1,169	14,681 (±3,324)	0,451	0,353 (±0,259)	0,011	0	0	0	0
	Supernatant	41,417	0,703	0	0	21,685 (±1,000)	0,368	11,671 (±0,984)	0,198	0	0	0,595 (±0,120)	0,010	0,0553 (±0,120)	0,001
	A. Adeninivorans														
	Sample	Protein content (%)	Protein (g/g sample)	Galactose (% of DM)	Galactose (g/L)	Glucose (% of DM)	Glucose (g/L)	Mannose (% of DM)	Mannose (g/L)	GlcNAc (% of DM)	GlcNAc (g/L)	Xylose (% of DM)	Xylose (g/L)	Ribsoe (% of DM)	Ribose (g/L)
	Pellet	50,843	1,308	5,612 (±0,697)	0,144357476	25,752 (±3,887)	0,662	18,010 (±2,120)	0,46327123	7,185 (±1,188)	0,185	0	0	0,936 (±0,246)	0,024
	Supernatant	29,650	0,687	1,218 (±0,171)	0,028216188	1,436 (±0,554)	0,033	8,780 (±1,820)	0,20339748	0	0	0	0	0,298 (±0,030)	0,007
	W. Anomalus														
	Sample	Protein content (%)	Protein (g/g sample)	Galactose (% of DM)	Galactose (g/L)	Glucose (% of DM)	Gucose (g/L)	Mannose (% of DM)	Mannose (g/L)	GlcNAc (% of DM)	GlcNAc (g/L)	Xylose (% of DM)	Xylose (g/L)	Ribsoe (% of DM)	Ribose (g/L)
	Pellet	46,791	0,713	0	0	26,774 (±2,742)	0,408	15,862 (±1,848)	0,242	0,330 (±0,098)	0,005	0	0	0	0
	Supernatant	42,559	0,791	0	0	1,392 (±0,447)	0,026	8,965 (±0,369)	0,167	0	0	0	0	0,0404 (±0,183)	0,001

Table 13: Protein content and monosaccharide composition in *C. utilis, W. anomalus* and *A. adeninivorans* after batch fermented on chicken meat hydrolysate and BALI sugar.

Batch fermentation Chicken meat hydrolysate and BALI														
	C.utilis													
	Sample	Protein content (%)	Protein (g/g sample)	Galactose (% of DM)	Galactose(g/L)	Glucose (% of DM)	Glucose (g/L)	Mannose (% of DM)	Mannose (g/L)	GlcNAc (% of DM)	GlcNAc (g/L)	Xylose (% of DM)	Xylose (g/L)	Ribsoe (% of DM)	Ribose (g/L)
	Pellet	52,731	1,581	0	0	31,342 (± 1,891)	0,940	12,148 (± 0,863)	0,364	0,330 (± 0,016)	0,010	0	0	0	0
	Supernatant	45,994	1,398	0	0	35,888 (± 2,678)	1,091	11,856 (± 1,009)	0,360	0	0	0	0	0,808 (± 1,67)	0,025
	A. Adeninivorans														
	Sample	Protein content (%)	Protein (g/g sample)	Galactose (% of DM)	Galactose (g/L)	Glucose (% of DM)	Glucose (g/L)	Mannose (% of DM)	Mannose (g/L)	GlcNAc (% of DM)	GlcNAc (g/L)	Xylose (% of DM)	Xylose (g/L)	Ribsoe (% of DM)	Ribose (g/L)
	Pellet	43,353	1,005	3,088 ± 0,223	0,072	21,467 (±0,340)	0,498	13,939 (± 0,325)	0,323	5,602 (± 0,218)	0,130	0	0	0,968 (± 0,030)	0,022
	Supernatant	34,421	0,691	1,392 (± 0,134)	0,028	16,922 (± 1,361)	0,340	8,037 (± 0,595)	0,161	0	0	0	0	0,319 (± 0,052)	0,006
	W. Anomalus 1														
	Sample	Protein content (%)	Protein (g/g sample)	Galactose (% of DM)	Galactose (g/L)	Glucose (% of DM)	Glucose (g/L)	Mannose (% of DM)	Mannose (g/L)	GlcNAc (% of DM)	GlcNAc (g/L)	Xylose (% of DM)	Xylose (g/L)	Ribsoe (% of DM)	Ribose (g/L)
	Pellet	45,11	0,941	0	0	5,307 (± 1,864)	0,111	4,257 (± 1,149)	0,089	0	0	0	0	0	0
	Supernatant	55,81	1,424	0	0	38,171 (±3,139)	0,974	13,421 (± 0,812)	0,342	0	0	0	0	0,553 (± 0,060)	0,014
	W. Anomalus 2														
	Sample	Protein content (%)	Protein (g/g sample)	Galactose (% of DM)	Galactose (g/L)	Glucose (% of DM)	Glucose (g/L)	Mannose (% of DM)	Mannose (g/L)	GlcNAc (% of DM)	GlcNAc (g/L)	Xylose (% of DM)	Xylose (g/L)	Ribsoe (% of DM)	Ribose (g/L)
	Pellet	51,252	1,889	0	0	54,723± 2,834	2,017	35,609 (± 1,678)	1,312	1,150 (± 0,132)	0,042	0	0	0	0
	Supernatant	20,791	0,229	0	0	39,107 (± 2,1367)	0,430	14,854 (± 1,290)	0,163	0	0	0	0	0,553 (± 0,030)	0,608
	W. Anomalus 3														
	Sample	Protein content (%)	Protein (g/g sample)	Galactose (% of DM)	Galactose (g/L)	Glucose (% of DM)	Glucose (g/L)	Mannose (% of DM)	Mannose (g/L)	GlcNAc (% of DM)	GlcNAc (g/L)	Xylose (% of DM)	Xylose (g/L)	Ribsoe (% of DM)	Ribose (g/L)
	Pellet	49,261	0,931	0	0	45,751 (± 4,259)	0,864	26,750 (± 5,335)	0,505	0,911 (± 0,298)	0,017	0	0	0	0
	Supernatant	27,811	0,273	0	0	38,541 (± 5,785)	0,379	15,862 (± 2,633)	0,156	0	0	0	0	0,532 (± 0,080)	0,005

The distribution of glucose, mannose and N-acetyl-glucosamine from the batch fermented *C. utilis, W. anomalus* and *A. adeninivorans* with chicken meat hydrolysate and BALI sugar as growth media is presented in Figure 12 based on Table 13.

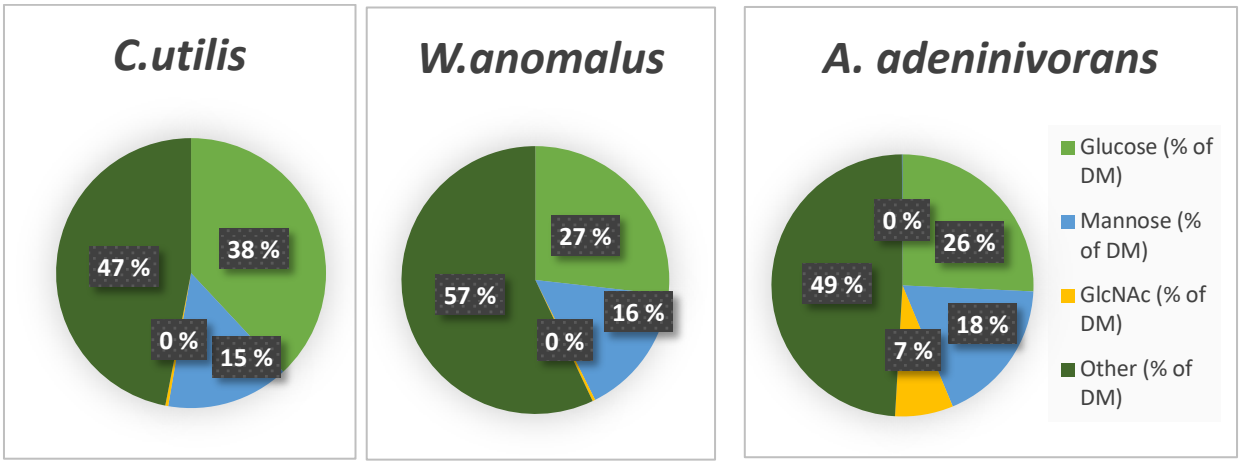

Figure 12: The distribution of glucose, mannose and N-acetyl-glucosamine (GlcNAc) in the pellets from batch fermented *C. utilis, W. anomalus* and *A. adeninivorans* on chicken meat hydrolysate and BALI sugar.

The % of dry matter of glucose, mannose, N-acetyl-glucosamine and galactose in *C. utilis* (Figure 13), *W. anomalus* (Figure 14) and *A. adeninivorans* (Figure 15) categorised based on growth medium are presented below.

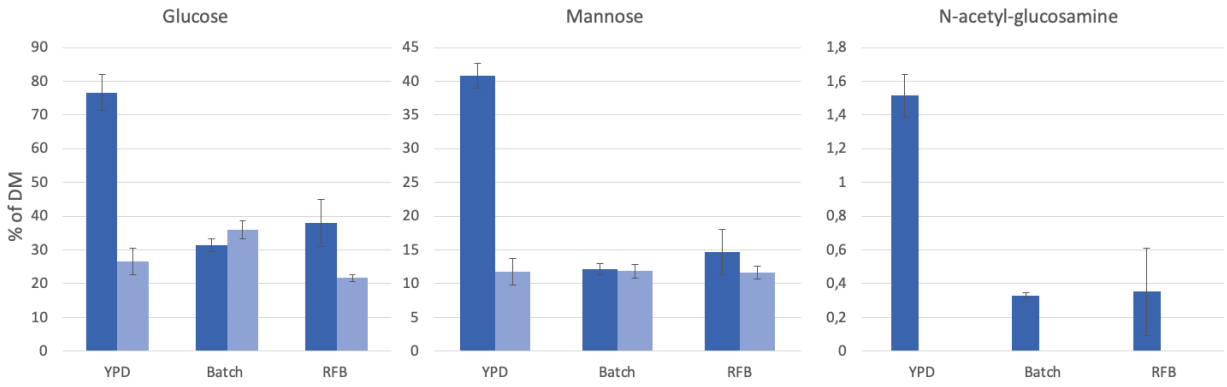

Figure 13: Distribution of glucose, mannose and N-acetyl-glucosamine in the pellets (dark blue) and supernatants (light blue) in *C. utilis* from three different fermentation processes. YPD: batch fermented on YPD. Batch: batch fermented on chicken meat hydrolysate and BALI. RFB: repeated fed batch fermented on chicken meat hydrolysate and BALI.

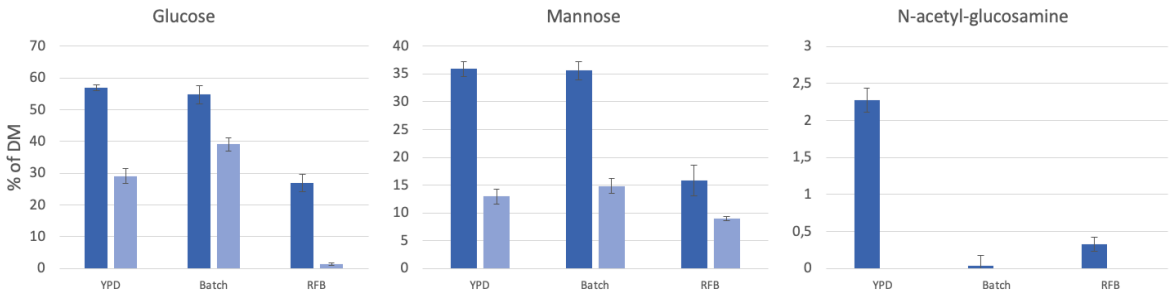

Figure 14: Distribution of glucose, mannose and N-acetyl-glucosamine in the pellets (dark blue) and supernatants (light blue) from *W. anomalus* from three different fermentation processes. YPD: batch fermented on YPD. Batch: batch fermented on chicken meat hydrolysate and BALI sugar. RFB: repeated fed batch fermented on chicken meat and BALI sugar.

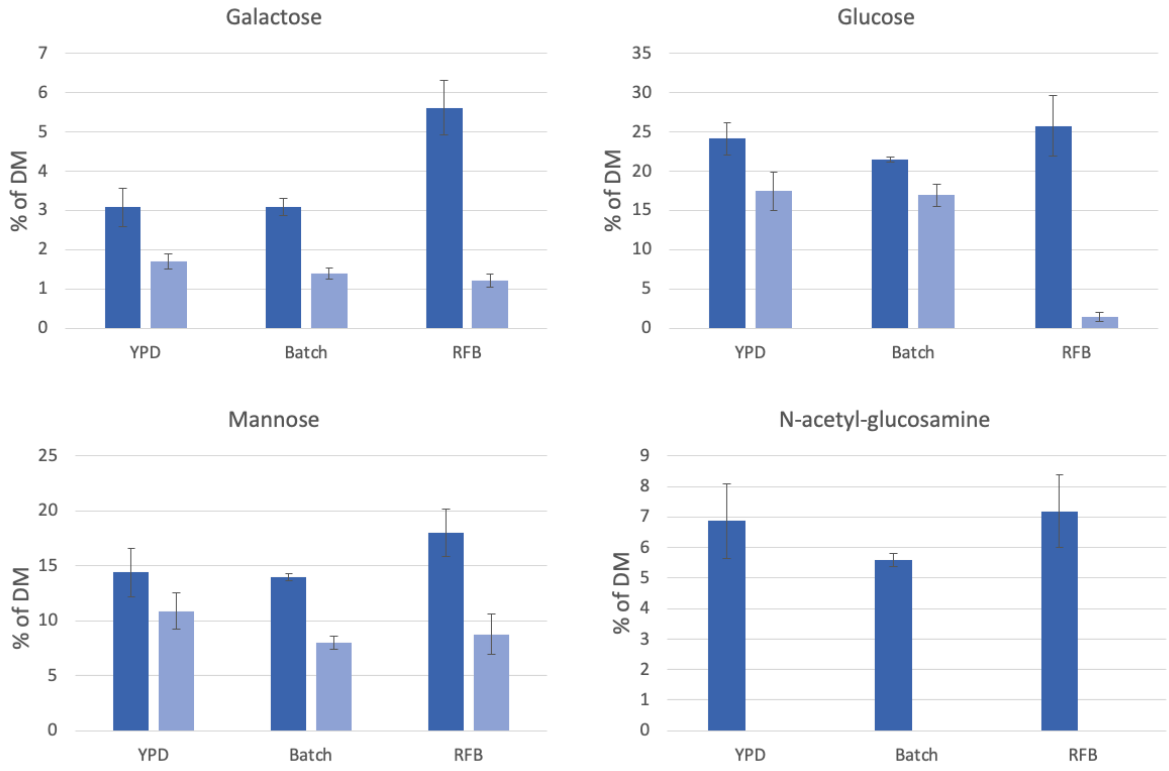

Figure 15: Distribution of glucose, mannose, galactose and N-acetyl-glucosamine in the pellets (dark blue) and supernatants (light blue) in *A. adeninivorans* from three different fermentation processes. YPD: batch fermented on YPD. Batch: batch fermented on chicken meat hydrolysate and BALI. RFB: repeated fed batch on chicken meat hydrolysate and BALI.

5.2.2 Size exclusion chromatography

The freeze-dried and enzymatic treated supernatants from the repeated fed batch fermentation (1,5 L) were analysed by size exclusion chromatography. RI and UV analysis of *W. anomalus* are shown in Figure 16, 17, 18 and 19.

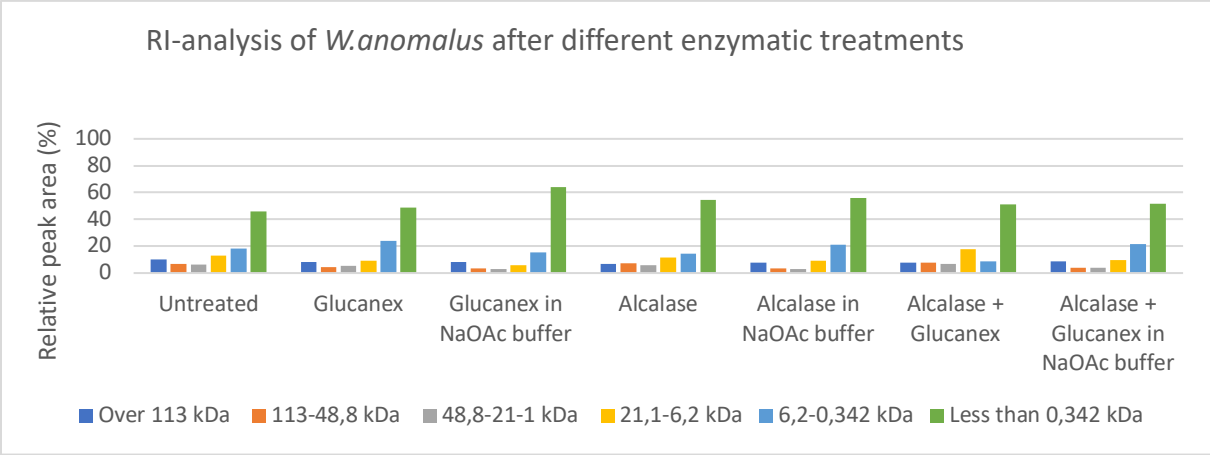

Figure 16: RI analysis by size exclusion chromatography of repeated fed batch supernatant from *W. anomalus.*

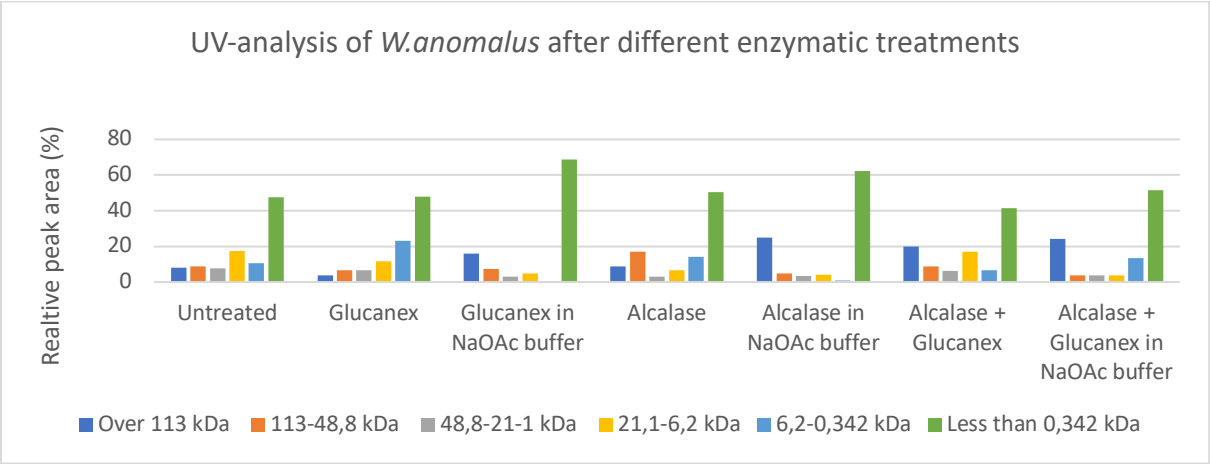

Figure 17: UV analysis by size exclusion chromatography of repeated fed batch supernatant from *W. anomalus*.

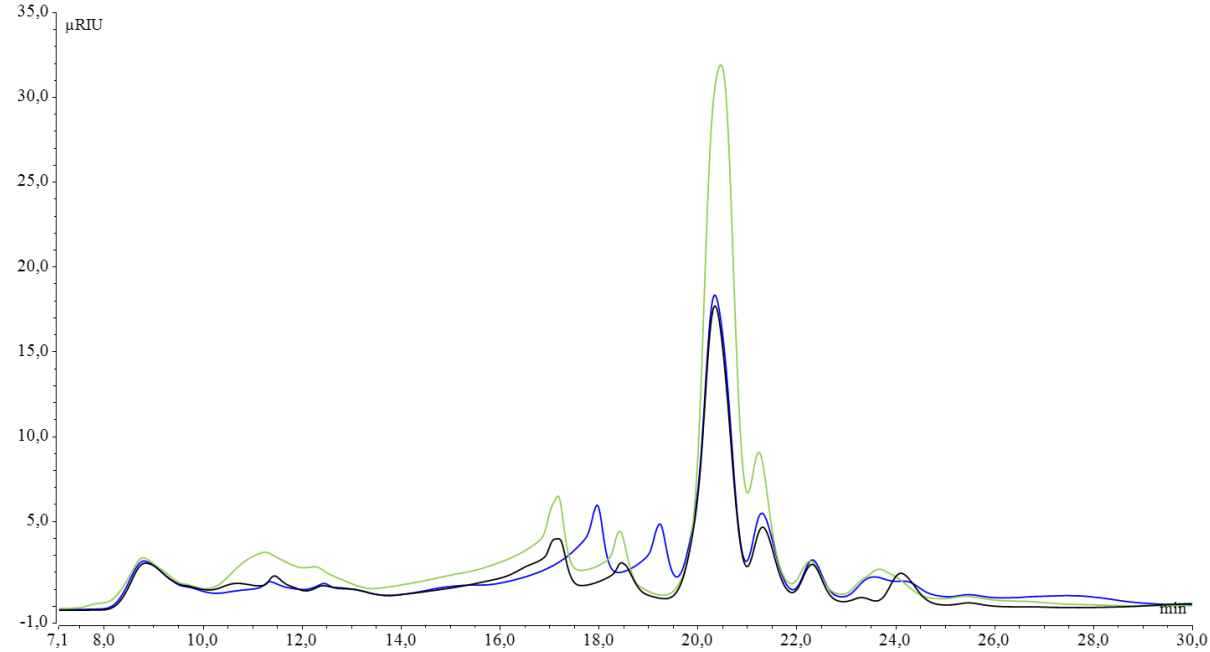

Figure 18: RI chromatogram from size exclusion chromatography of *W. anomalus.* Black line: untreated sample. Blue line: glucanex treated sample. Green line: alcalase treated sample.

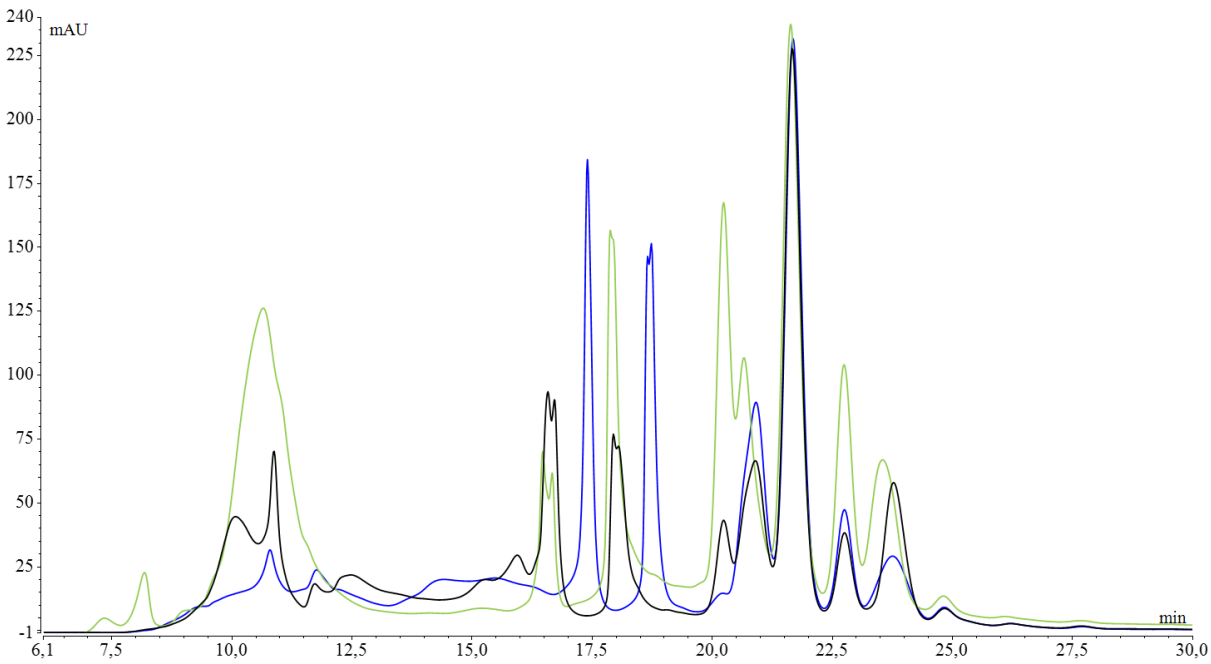

Figure 19: UV chromatogram from size exclusion chromatography of *W. anomalus*. Black line: untreated sample. Blue line: glucanex treated sample. Green line: alcalase treated sample.

5.3 Downstream processing; discovering of processing bottlenecks

5.3.1 Treatment with Alcalase and oxalic acid

When adding oxalic acid to the samples a visual colour change to a lighter colour could be seen right away. After two hours the 1M oxalic acid samples had already started to separate into a supernatant and a solid fraction. The samples incubated at 60 ºC had the biggest and clearest supernatants. The samples treated with 0,5M oxalic acid started to separate at the same time as the 1M acid samples, but the separation was not as evident as for treatment with 1M acid.

5.4 Downstream processing of *W. anomalus*

W. anomalus was repeated fed batch cultivated, washed, cell disrupted, enzymatically and acidic treated, and in the end ultra-, and nano-filtered. An overview of the sampling steps is sketched in Figure 20. 25 mL samples were taken at every processing step and analysed for monosaccharide composition and protein content. The abundance of mannose, glucose and N-acetyl-glucosamine in every sample are shown in Figure 21. The protein content was measured in all the samples by the Dumas method (Table 14).

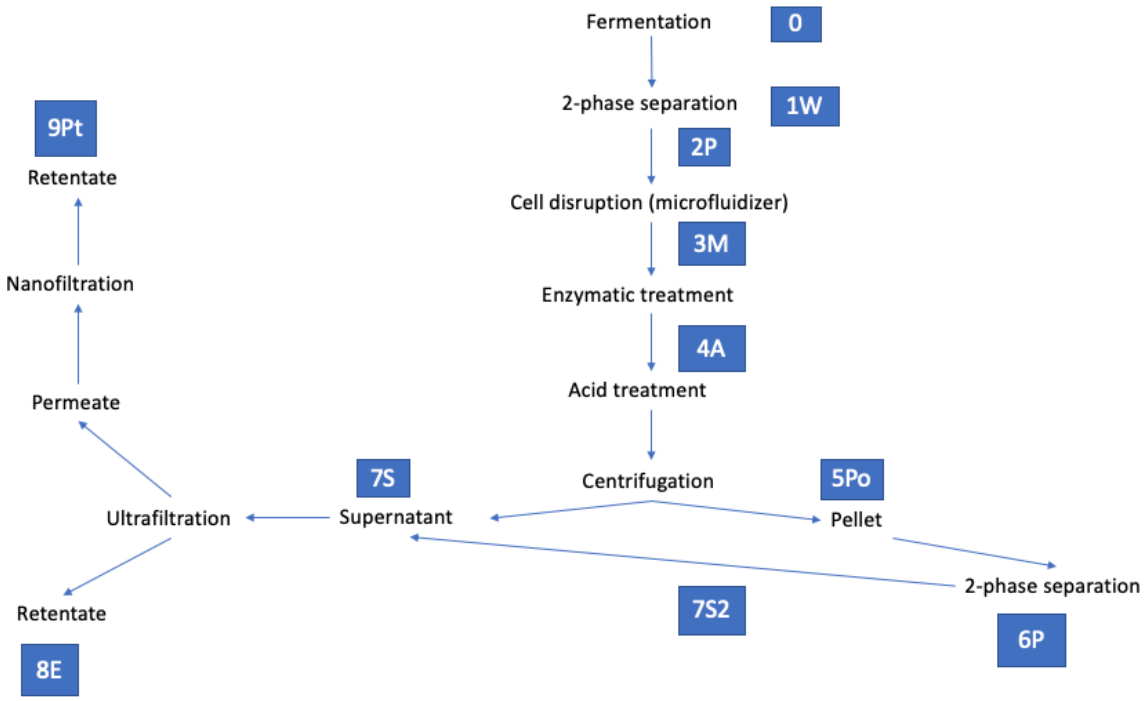

Figure 20: Flow chart showing the sampling steps in the downstream processing of *W. anomalus*.

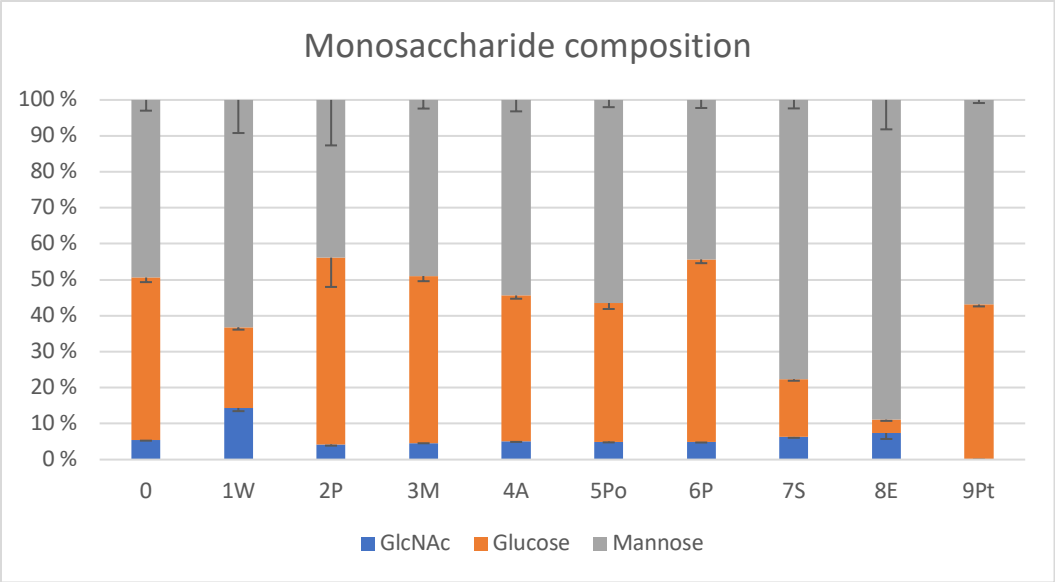

Figure 21: The abundance of mannose, glucose and N-acetyl-glucosamine (GlcNAc) in each processing step in the downstream processing of *W. anomalus* (see Figure 20 for sampling points).

Table 14: The protein content in every sample from the downstream processing of *W. anomalus* (se Figure 20 for sampling points) are measured by the Dumas method with a factor of 5,88 as % protein.

Sample	% Protein
0	50,6
1W	47,2
2P	47,3
3M	47,8
4A	47,6
5Po	36,6
6P	31,7
7S	52,3
8E	36,5
9Pt	67.7

6 Discussion

6.1 Choice of methods

In this master thesis there have been tested many different processing techniques in order to develop a rational way to conduct downstream processing of yeast. The equipment used, especially those in the biorefinery, are typical processing equipment that are also used in industry and are easy to scale up. Lyophilization has been the main method throughout the study to remove water from the biological material. This is because the process is the most gentle method for keeping the material intact and preventing chemical degradation reactions that may alter the yeast. Furthermore, we were operating in scales that did not require testing of more efficient techniques, such as vacuum evaporation and spray drying.

There are numerous of methods for cell disruption. One way to disrupt the cells is with the use of glass beads and mechanical shaking. This was done in a study by (Nguyen et al., 1998) where they also used 0,1M sodium phosphate buffer with pH 8,5 in the cleaning of the cells and during the cell disruption. They reported higher cell wall fractions than previously reported for yeast. They concluded that these values were higher because of their choice of method with the specific condition of pH 8,5. This high pH inhibit the activity of endogenous glucanases which degrade the cell wall EPS into smaller carbohydrate fractions.

The amount of sample required for analysis in this study had to be taken into account when choosing a method for cell disruption. A quite low concentration of the material was required for the analysis to be performed. But at the same time, a lot of product needed to be disrupted later for the up-scaling. In an industrial, and even bigger up-scaling, which is a final goal, a lot of product will be processed as well. The microfluidizer, a method that was able to disrupt low concentrations, but at the same time was able to process a lot of material was chosen. The learning outcome from using this microfluidizer has also been huge regarding yeast material behaviour and how to handle this performance. One example is the change of time from 45 min run in loop to run the material through three sequential times. It was much more efficient to disrupt the cells by running it through several times than in loop for 45 min. Challenges regarding separation of solids and liquids after the cell disruption occurred. This was solved by addition of oxalic acid to the material (discussed further in section 6.3.4). The big particle sizes produced by the microfluidizer should in theory enhance the separation, but as previously mentioned, the viscosity of the material is decreasing with the number of passes,

having a huge impact on the separation. Other methods that could have been used is a twin-screw press method which is used for cell disruption of seeds in oil production (Evon et al., 2009). A screw press has been used in cell disruption of bacteria and other microorganism as well (Hughes, 1951).

It would be interesting to try a pH adjustment when disrupting the cells in the microfluidizer to try to maximize the cell wall yields and to get one fraction with all the EPS present by inhibit the glucanases present. My fellow master student Cathrine Nilsen Sebjørnsen has tried to get access to the EPS with enzymes, but without success. This would make the process less time consuming, at least if the cell disruption step and also the hydrolysis step could be removed. Other enzymes than α-mannanase, endo- and exo-1,3-ß-d-glucanase, mannosidase, chitinase and chitobiase should be tested on the yeast material, as well as other conditions (dosage, temperatures, pH) of the tested enzymes should be tested further.

In the filtrations the membrane sizes and prosperities of the membranes had to be considered. The depth filtration step was omitted in the downstream processing of *W. anomalus* because the supernatant was transparent enough after the centrifugation to be ultra-filtered. We wanted to test the membranes already available on the yeast material, and from experience with other material the 5 kDa ultrafiltration membrane is a good filtration membrane when working with these types of carbohydrates regarding the size of the polymers. Membranes with other pore sizes and properties could have been tested e.g. with disc stack housing.

6.2 Fermentation

The main focus in this master thesis has been on downstream processing. Therefore, not all the results from the fermentations are discussed in detail. In all the fermentations the pH was set to 5. This was primary to avoid contamination. Yeast can grow at lower pH than most other bacteria. As mentioned earlier most yeast grow best around 30-35 ºC, therefore the standard temperature used in cultivation processes is 30 ºC.

6.2.1 Protein analysis

A. adeninivorans and *W. anomalus* had a much higher protein concentration after 24 hours of cultivation than *C. utilis* in the batch fermentation. But after 12 hours the concentrations were approximately the same (Table 6). The protein contents from the *A. adeninivorans* 20 L

repeated fed batch fermentations taken after 24 hours of cultivation is approximately 20 g/L protein in each cycle (Table 7). This is a little higher than for the 24-hour sample from the batch fermentation. Cycle one shows a little lower concentration than in the other two cycles which probably is because the yeast needs some time to adjust to the cultivation environment and growth media. The last harvests of *W. anomalus,* repeated fed batch fermented twice with a working volume of 1,5 L, had protein contents around 50% in both cultivations (Table 8 and 9). But the protein concentrations are on the other hand dissimilar when comparing the two cultivations. This is due to low CDW values in one of the cultivations (Table 9) because of biomass loss during media change.

6.2.2 Biproduct analysis to validate the performance of fermentations

According to the results in Figure 9, 10 and 11 ethanol is produced in small amounts as biproducts from all three species which can indicate that the glucose concentration in the growth media should be adjusted. This biproduct production should be avoided when fermenting yeast for production of single-cell proteins and exopolysaccharides because this overflow metabolism induces cell growth. Analysis of bioproduct production in the fermentations was therefore of interest for further optimization of growth media in regard to the sugar source.

6.3 Downstream processing

6.3.1 Monosaccharide composition analysis

The sugar recover standards (SRS) were autoclaved and analysed together with the samples to monitor the acid hydrolyzation process. The SRS standards should have contained xylose, ribose, arabinose and galactose and not just glucose, mannose and N-acetyl-glucosamine. Xylose is detected in one of the supernatants of *C. utilis* repeated fed batch fermented (Table 12), but is most likely remains from the growth media, and an additional cleaning step should therefore be included. Some of the samples should have been diluted even more because the measurements are higher than the highest standard used, 0,09 mg/mL. The dry weight analysis is meant as a correction that takes into account the water left in the samples after the freeze drying. Since it was not possible to use every yeast sample in the analysis and since the analysed samples differed so much, from 1%-8%, no adjustments are made in the compositional analysis. All presented values are therefore underestimated. The monosaccharide standards used in the analysis were not dried in an exicator before weighted

in either. The concentrations of the monosaccharides are therefore lower than reported du to interactions between water molecules and the sugar. Exact quantification of the different monosaccharides is therefore not possible based on the measured amounts.

The main difference between the yeast species, regarding cell wall composition, is galactose found in *A. adeninivorans*. In addition, *A. adeninivorans* possess an overall higher amount of N-acetyl-glucosamine. On the other hand, all of the species contain glucose, mannose, N-acetyl-glucosamine and ribose (Figure 5-8). These sugars are present independent of cultivation process and growth media. The results from the ion exclusion chromatography showed no peaks corresponding to N-acetyl-glucosamine, indicating ribose as the true sugar present (section 4.5.3). Signals from ribose and N-acetyl-glucosamine overlap in HPAEC analysis. Therefore, the ribose detected needed to be analysed by another chromatographic method.

How the monosaccharides detected are interacting is so far unknown. But the monosaccharides analysed is calculated into polymers of mannose, polymers of glucose, polymers of N-acetyl-glucosamine, polymers of xylose, polymers of ribose and polymers of galactose (Table 11,12 and 13). Based on literature, b-glucan, mannan and chitin are the most abundant sugar polymers in yeast cell. Therefore, we assume that the glucose, mannose and N-acetyl-glucosamine present are polymers of these. This should be analysed further to find out which EPS that are present in the cell walls of the yeast species.

When producing yeast or other microorganisms for commercial use the amounts of EPS in the feed ingredient need to be monitored because too high amounts are known to overstimulate the immune system. To investigate if the yeast material could be separated into one protein rich fraction and one carbohydrate rich fraction was of significant interest because of the demand of a high protein content, but at the same time a low fraction of immune stimulating EPS. In the centrifugation step after cell disruption the crushed yeast cells were separated into two fractions, one pellet and one supernatant. What Table 11-13 are showing is that it is not possible to conclude with a complete separation of the proteins and the carbohydrates. The proteins are present in high amounts in both the pellets and the supernatants based on % protein. The monosaccharides are also present in both fractions, except for the N-acetyl-glucosamine, assumed to be chitin, which is present just in the pellets. According to Figure 2, this chitin layer is the innermost layer of the cell wall and will therefore not dissolve into the

supernatant as easy as the polysaccharides in the outer layers. The uncomplete separation of the fractions can also be seen in the results from the size exclusion chromatography on the selected supernatants (Figure 16-19). The UV signals is based on absorption and is therefore presenting the proteins in the samples (UV 280 nm). The Refractive index (RI) is a universal detector reporting everything in the samples. When comparing the UV and RI signals for each sample and substitute the UV signal from the RI signal there is still a lot left, indicating that sugars or other molecular components are present in the supernatants together with the proteins.

The three *W. anomalus* samples presented in Table 12 have approximately the same protein contents, but the amounts of the monosaccharides are lower in one of the samples (1). In addition, N-acetyl-glucosamine is not present in this sample, but present in the other two. One explanation is that the polysaccharides might not be properly broken down to monosaccharides in the hydrolysis. But on the other hand, all the samples are an average of triplicates measured and analysed the same way, and there is not likely that the hydrolysis did not work properly in all the replicates from the same sample. Whether the yeast samples are homogeneous or not also needs to be discussed. The samples were not smashed and mixed to obtain a homogeneous material prior to weighting samples for cell disruption. This can explain why just two of the three *W. anomalus* samples have the same amount of carbohydrates. *W. anomalus* was cell disrupted as three individual samples because the reproducibility of the microfluidizer as a cell disruption method of yeast needed to be tested. But since other factors seems to have an impact on the analysis in this case, no conclusion can me made about the reproducibility.

6.3.2 Protein analysis

As mentioned earlier proteins are present in both the supernatants and the pellets after cell disruption. But the proteins are present in a bigger fraction in the pellets except for in one *W. anomalus* sample (1) from the batch fermentation on chicken meat and BALI sugar. The biggest difference in protein content between pellets and supernatants is in the YPD batch fermented samples. No conclusion can be drawn on which growth media that produces the highest amount of protein based on the g/g sample values in Table 11-13.

6.3.3 Emulsifying properties

The viscosity of *A. adeninivorans* and *W. anomalus* after the first round of cell disruption was higher than after the second and the third rounds. One possible explanation could be that because of the high protein content together with the carbohydrate composition, the yeasts make emulsions that are destroyed again during the second and third passes. Previously it has been reported by (Cameron et al., 1988) that the mannoproteins of the yeast *S. cerevisiae* has emulsifying properties. This should of course be investigated further in other yeast species that possess mannoproteins in their cell wall because this property could be of commercial significance.

6.3.4 Downstream processing of *W. anomalus*

In the downstream processing of *W. anomalus* the highly effective oxalic acid was added to the disrupted material because the material that are to be ultra-filtered needed to be a transparent non turbid solution. As mentioned in the results (section 5.4.1) the oxalic acid made a separation of the material almost right away when added to the disrupted cells. And after just a couple of hours of treatment a clear liquid could be seen on the top layer. Alcalase was added together with the oxalic acid and incubated at 60 ºC (in theory) and centrifugated which provided a liquid solution that could be filtered. Alcalase is a protease and was added to degrade proteins to increase access to the cell wall. Because of troubles with the Belac hydrolysis system both the enzymatic treatment and the acid treatment were at 30 ºC. The enzyme has a temperature optimum at 60 ºC, this is why this temperature originally was chosen for the both the enzymatic treatment and acid treatment. When testing the time for acid treatment no big differences could be seen between the samples treated for one, two, four and 24 hours, but in the up-scaling process with *W. anomalus* the oxalic acid treatment lasted for over 12 hours because of practical reasons. The concentration of the acid was set to 100 mM because a lower concentration seemed to be less efficient.

According to the visual observations of the separation of the yeast material after addition of oxalic acid, the most reasonable conclusion is that the acid is protonating the charged chemical groups present and neutralising them. This interrupts the chemical interactions between the molecules. Oxalic acid is also reported to have chelating properties (Stevens & Selvendran, 1984) but whether it is this or simply the pH adjustment that is affecting the separation of the material is difficult to tell.

In the processing of *W. anomalus* low amounts of N-acetyl-glucosamine was quantified, while the main sugars collected were mannose and glucose (Figure 18). These three sugars make up 35% of the total mass after the fermentation while the protein makes up approximately 50% (Table 14). After 2-phase separation the sugar content increased to 59% of the dry matter (2P). The monosaccharide mainly quantified in 7S is mannose. No N-acetyl-glucosamine was detected in 9Pt. The retentate after UF (8E) is highly concentrated of mannose, and it would be interesting to investigate if most of the proteins present in this fraction are linked to this monosaccharide. Fraction 8E fraction has to be polymeric because a 5kDa membrane was used in the filtration. It would also be interesting to analyse 8E by size exclusion chromatography because it is a pure fraction that most likely will give clear UV and RI signals for comparison. In the retentate after NF (9Pt) both glucose and mannose were present in approximately equal amounts but represents only 18% of the total weight while the protein is the main component with 67 %. The distribution of mannose and glucose in fraction 8E and 9Pt may be explained by the activity of β-glucanases which is degrading β-glucan to oligosaccharides. These oligosaccharides are collected in the permeate after the ultrafiltration and concentrated in the retentate after the nanofiltration (9Pt). Further, they are hydrolysed to monosaccharides, and detected as glucose.

As mentioned earlier, pH seems to have a huge impact on how the polysaccharides and protein fractions are portioned. If you want to prevent indigenous enzymes, such as β-glucanases, from degrading the cell wall during processing, pH may be used. High pH has been shown to inhibit the activity of the exo- β-1,3-glucanase (killer protein) as well as other glucanases in *W. anomalus* and other yeasts (Nguyen et al., 1998). This causes less degradation of the cell wall. Based on the observations in this study, and the knowledge about the effects of pH adjustments, regulation of the different fractions is assumed to be possible.

7 Conclusion and future perspectives

7.1 Conclusion

In this study *C. utilis, W. anomalus* and *A. adeninivorans* were successfully fermented and analysed for protein content. A selection of yeast samples from the strains were further processed in order to characterise the cell walls. Furthermore, the downstream processing of yeast was optimized and generated an EPS fraction of high purity. The potential immune stimulatory effects of this fraction will be tested.

Prominently including but not limited to is the protein content measured in the yeast *C. utilis* which possess an overall lower content of proteins than *W. anomalus* and *A. adeninivorans*. This makes *W. anomalus* and *A. adeninivorans* yeast more attractive due to production of a higher proportion of single-cell proteins for use in foods and feed. Another important factor when choosing a microorganism for industrial use is the ability of the organisms to be used in other applications as well. Based on the characterization of the yeast cell wall, *A. adeninivorans* is the most appealing considering the composition of the cell wall.

An interesting observation is the change in the viscosity when disrupting the yeasts cells in the microfluidizer. One idea is that an emulsion is made and causes the increase in the viscosity. Proteins and carbohydrates, especially mannan, are known to be good emulsifiers which stabilize emulsions. If this is the case, yeast could be used directly or indirectly in foods such as sour cream or mayonnaise, providing stability to the product, giving high amounts of proteins and offering immune stimulating polysaccharides.

7.2 Future perspectives

Production of single-cell proteins from microorganisms holds significant promise for overcoming the world population´s protein quantity and quality requirements. One example that are currently being utilized commercially is Quorn. Further research is important to provide more examples of sustainable, digestible and economical favourable products. Several yeast species can provide high amounts of proteins and in addition, they can add immune stimulating polysaccharides to the product. But a high level of immune stimulatory EPS can cause to adverse negative effects as an overstimulation of the immune system. Because of this it is necessary to control the production of EPS and proteins together. Both can be adjusted either by growing conditions or during the downstream processing where

protein and EPS may be efficiently separated. When choosing a promising yeast strain the fermentation process needs to be adjusted based on the yeast growth rate, growth media, temperature optimum and pH. Both the upstream processing steps and the downstream processing steps of yeast production can be altered to become as economically viable, sustainable and efficient as possible. Both *A. adeninivorans* and *W. anomalus* are promising yeasts for biotechnology use due to their high protein content, cell wall composition, interesting morphology and, and after this study, believed to have emulsifying properties.

I suggest looking further into the use of microfluidizer as the method for cell disruption to conclude whether it is reproducible or not. Adjustment of pH during and after cell disruption with other chemicals than oxalic acid should be investigated further. Then it might be possible to conclude which properties that is altering the yeast material, and if it is possible to regulate the amounts of EPS in the different fractions. Whether addition of ions to the material when increasing the pH would have a significant effect or not on the generation of the separation could also be interesting to test. Furthermore, since some studies have shown promising emulsifying effects, and based on our initial observations, it would be worthwhile testing whether the yeast material can be used for stabilization in emulsions. The fractions obtained during the downstream processing of *W. anomalus*, especially 8E and 9Pt, should be analysed in more detail, e.g. with MALDI-ToF, size exclusion chromatography, Kjeldahl method and amino acid composition to find out which proteins that are present. The high purity 8E would be a great candidate for testing potential immune stimulatory effects of the EPS from the not so well studied *W. anomalus*. These tasks are subjected to ongoing analysis in the lab.

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Appendix

Appendix A

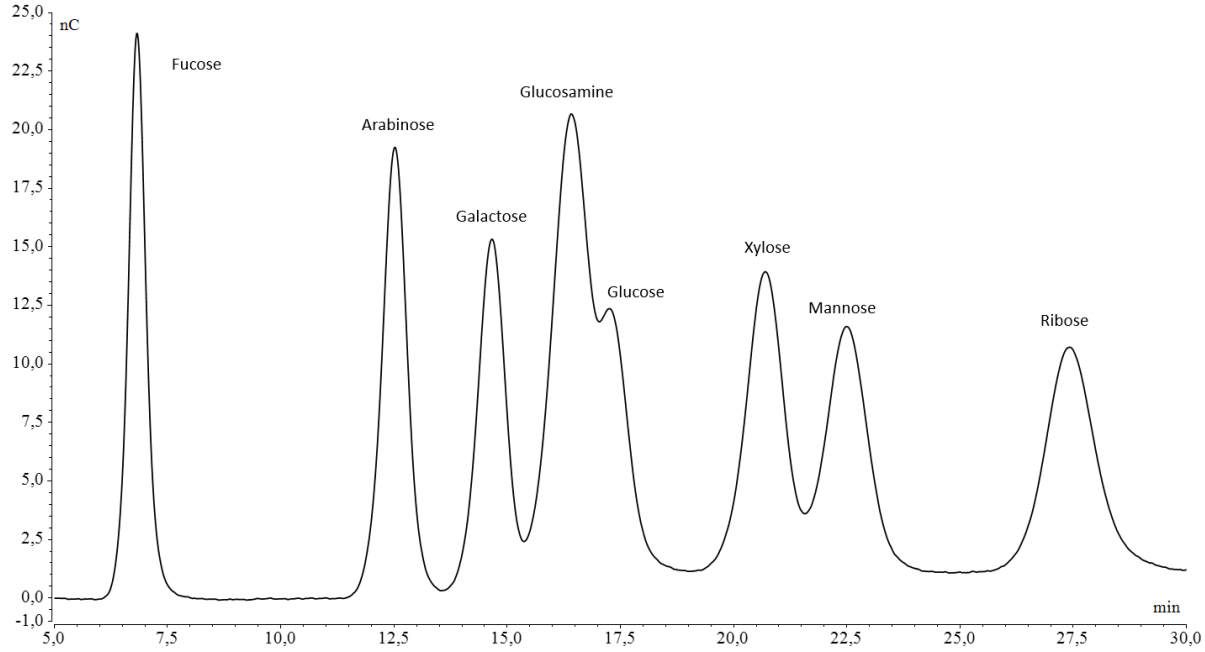

Figure A: Chromatogram showing the standards of fucose, arabinose, galactose, glucosamine, glucose, xylose, mannose and ribose used un the monosaccharide composition analysis.

Appendix B

$$C_{strc.carb} = \frac{C_{HPLC} \cdot D \cdot V_{sample} \cdot P_{hex/pent}}{R_f \, m_{DMload}/1000}$$

Figure B: Formula used in the calculations in the monosaccharide composition analysis.

Appendix C

Table C: Estimated recovery during the acid hydrolysis used in the monosaccharide compositional analysis based in % recovery of the sugar recovery standards.

Estimated recovery based on theoretical values	
Monosaccharide	Recovery (%)
Glucose	1
Mannose	0,82
N-acetyl-glucosamine	1

Appendix D

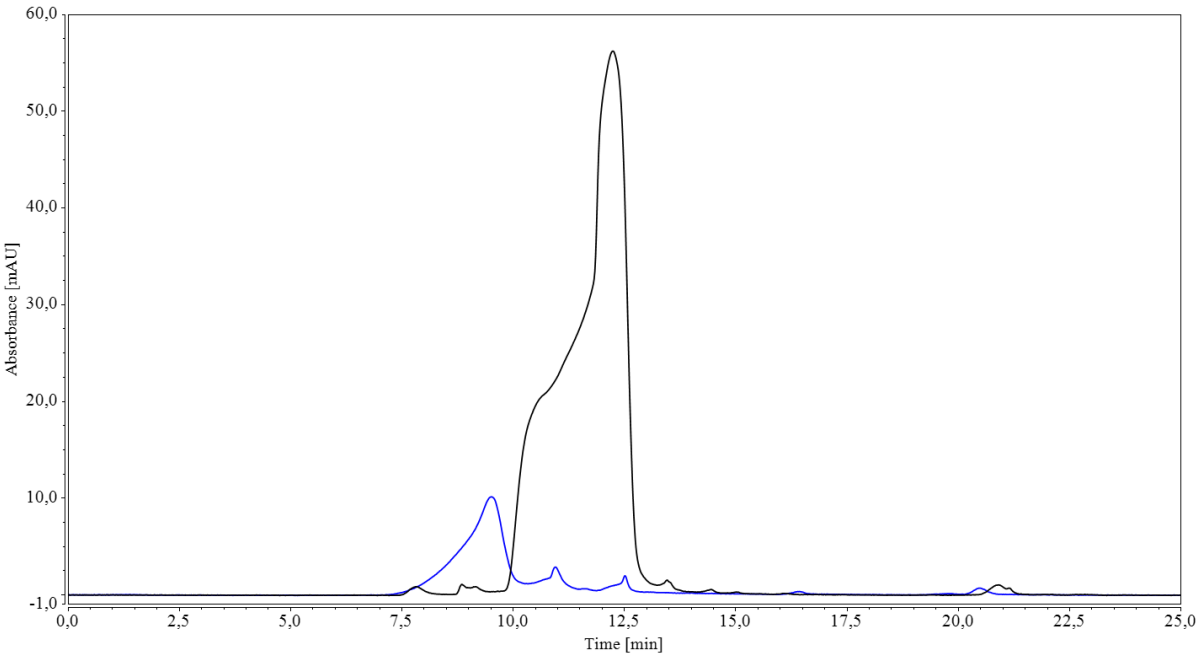

Figure D: UV chromatogram of *Saccharomyces cerevisiae* mannan (blue line) and Bovine Serum Albumin (black line) used in the size exclusion chromatography.

Appendix E

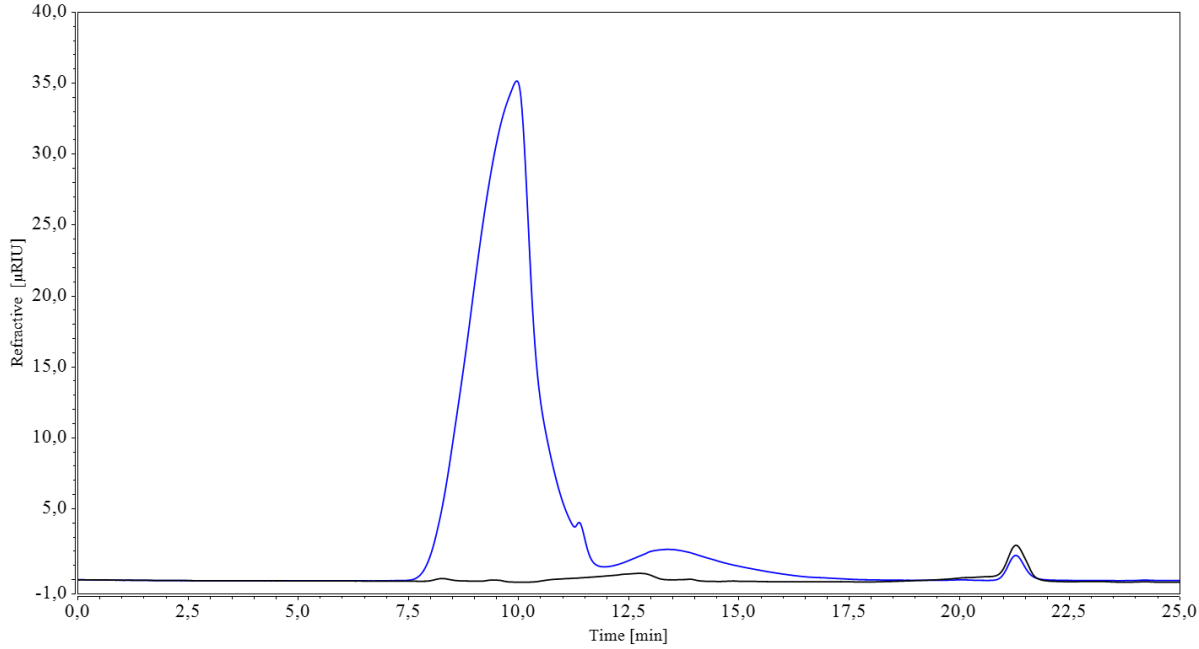

Figure E: Refractive index (RI) chromatogram of *Saccharomyces cerevisiae* mannan (blue line) and Bovine Serum Albumin (black line) used in the size exclusion chromatography analysis.

Appendix F

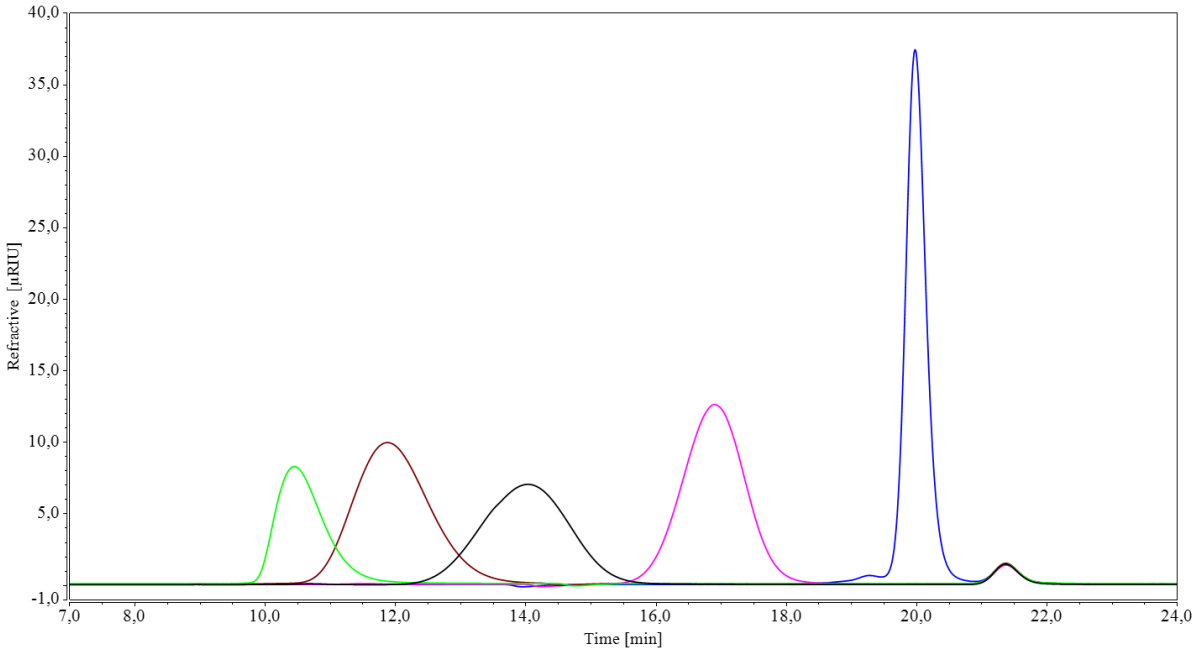

Figure F: Refractive index (RI) chromatogram presenting the pullulan molecular weight standards used in the size exclusion chromatography analysis. Green line: 113 kDa. Red line: 48,8 kDa. Black line: 21,1 kDa. Pink line: 6,2 kDa. Blue line: 0,0,0342 kDa.

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