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Characterization of yeast exopolysaccharides

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Abstract

In this thesis a method for quantification of the exopolysaccharides (EPS) of the yeast cell wall (YCW) has been under development. Such a method will be of interest for the research on the use of yeast in aquafeed to take advantage of the immunostimulative effect the carbohydrates in the YCW. The major carbohydrate components in the YCW are β-1,3-glucan, β-1,6-glucan, mannan oligosaccharides and chitin. In the method being developed, Microfluidizer^{TM} LM20 was used to the disrupt the yeast cells with shear forces. Centrifugation was then used to isolate the YCW in a carbohydrate-rich pellet and create a protein-rich supernatant. The isolated YCW was then hydrolyzed using both sulfuric acid, and specific enzymes for hydrolysis of defined bonds. Sulfuric acid hydrolysis gave the total β-glucan content. Exo-1,3-β-D-glucanase isolated from *Asperillus oryzae*, endo-1,3-β-D-glucanase isolated from barley, endochitinase C and chitobiase, both isolated from *Serratia marcescens* was used in one step to hydrolyse β-1,3-glucan and chitin into their respective monosaccharides, glucose and N-acetylglucoseamine. In another step α-mannanase and α-mannosidase was used to hydrolyze the mannan oligosaccharides. High-performance anion exchange chromatography with pulsed amperometric detection was used to detect the mono- and oligosaccharides released by hydrolysis. The amount of β-1,3-glucan found by enzymatic hydrolysis would be subtracted from the total β-glucan found by acid hydrolysis to give the amount of β-1,6-glucan. The motivation for using enzymatic hydrolysis in addition to acid hydrolysis, was to give higher yields for chitin to avoid underestimation of this components and to obtain a specific quantification of the components, including their specific linkages.

The results showed that disruption of the yeast cells by using Microfluidizer^{TM} LM20 did not give the clear separation into a carbohydrate-rich cell wall fraction and a protein-rich supernatant as expected, though most of the protein was determined to be in the supernatant. Even though we were able to estimate the amounts of 1,3 linked β-glucan using enzymes, the enzymatic conversion yield was low and further optimization of enzyme loadings and reaction conditions is needed to give a sufficient and reliable quantitative enzymatic hydrolysis.

Sammendrag

I denne oppgaven har en metode for kvantifisering av eksopolysakkaridene (EPS) av celleveggen til gjær (YCW) vært under utvikling. En slik metode vil være av interesse for forskningen på bruk av gjær i fiskefor for å utnytte den immunstimulerende effekten av karbohydrater i YCW. β-1,3-glukan, β-1,6-glukan, mannan oligosakkarider og kitin utgjør den største andelen av karbohydrater i YCW. I denne prosessen ble Microfluidizer^{TM} LM20 brukt for å lysere gjærcellene med skjærkraft(shear force). Sentrifugering ble deretter utført for å isolere YCW i en pellet med høyt karbohydratinnhold og en proteinrik supernatant. Den isolerte celleveggen ble så hydrolysert ved å bruke svovelsyre, og spesifikke enzymer for hydrolyse av definerte bindinger. Hydrolyse med svovelsyre ga det totale innholdet av β-1,6-glukan. Ekso-1,3-β-D-glukanase isolert fra *Asperillus oryzae*, endo-1,3-β-D-glukanase isolert fra bygg, endokitinase C og kitobiase, begge isolert fra *Serratia marcescens* ble brukt i et steg for å hydrolysere β-1,3-glukan og kitin til deres respektive monosakkaridenheter, glukose og N-acetylglukoseamin. I et annet steg ble α-mannanase og α-mannosidase brukt til å hydrolysere mannan oligosakkaridene. *High-performance anion exchange chromatography* med pulserende amperometrisk deteksjon ble brukt til å detektere mono-og oligosakkaridene frigjort ved hydrolyse. Mengden av β-1,3-glukan funnet ved enzymatisk hydrolyse ble subtrahert fra den totale β-glukan funnet ved syrehydrolyse for å gi mengden av β-1,6-glukan. Motivasjonen for bruk av enzymatisk hydrolyse i tillegg til syrehydrolyse var for å gi høyere grad av hydrolysering av kitin for å unngå underestimering av denne komponenten og for å oppnå en spesifikk kvantifisering av komponentene, inkludert deres spesifikke bindinger.

Resultatene viste at lyseringen av gjærcellene ved bruk av Microfluidizer^{TM} LM20 ikke førte til en tydelig separasjon til en karbohydratrik celleveggfraksjon og en proteinrik supernatant som forventet. Mesteparten av proteinet viste seg likevel å være i supernatanten. Selv om vi var i stand til å estimere mengden av 1,3-linket β-glukan ved bruk av enzymer, var det enzymatiske hydrolyseutbytte lite og ytterligere optimalisering av enzymdoser og reaksjonsbetingelser er nødvendig for å gi en tilstrekkelig og stabil kvantitativ enzymatisk hydrolyse.

Abbreviation

AEC	Anion exchange chromatography
BSA	Bovine Serum Albumin
CHB	Chitobiase
COS	chitooligosaccharides
DP	Degree of polymerization
EPS	exopolysaccharides
GE	Glucose equivalents
GlcNAc	N-acetyl-D-glucosamine
HPAEC-PAD	High performance anion exchange cromatography with pulsed amperometric detection
INM	Ivory nut Mannan
MOS	Mannan oligosaccharides
RB	Repeated Batch
SC-Man	α-Mannan purified from *Saccharomyces cerevisiae*
YCW	Yeast cell wall

Contents

1

Introduction

Foods of Norway, a Centre for Research-based Innovation, states at their homepage (https://www.foodsofnorway.net/) that Norway can no longer depend on imported plant ingredients for use in fish feed, due to decrease resources in the world and increase in prices. Plant ingredients are often used as a protein source, for example soybean meal and peas in aquafeed. However, these plant based protein sources can cause an inflammatory reaction in the distal part of the intestine of fish such as the Atlantic salmon (*Salmo salar L*), rainbow trout (*Oncorhynchus mykiss W.*) and common carp (*Cyprinus carpio L*). This inflammatory reaction is referred to as soybean meal induced enteropathy(SBMIE)(Grammes et al., 2013).

The need for new protein sources to be used in aquafeed has led to research on microbial ingredients, such as yeast and algae, with promising result. Oliva-Teles and Gonçalves (2001) found through their dietary trials on sea bass (*Dicentrarchus labrax*) juveniles indication of that brewer's yeast *Saccaromyces cerevisae* could replace 50% of fishmeal protein with no negative effect in the performance of the fish. In addition, they found that a replacement of fishmeal protein with up to 30% *S.cerevisiae* improved the feed efficiency. Grammes et al. (2013) found through their studies that inclusion of the yeast *Candida utilis* in the fish diet counteracted SBMIE in Atlantic salmon, and the yeast *Kluyveromyces marxianus* reduced SBMIE partially.

Candida species and *S.cerevisiae* are believed to have immunostimulatory properties due to the complex carbohydrate composition(Oliva-Teles and Gonçalves, 2001). The yeast cell wall (YCW), and carbohydrate components like β-glucan, mannan oligosaccharides (MOS), chitooligosaccharides (COS), galactooligosaccharids (GOS) and galactomannan are categorized as prebiotics. Prebiotics are defined as "non digestible food or feed ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Roberfroid (2007),Gibson and Roberfroid (1995)). β-glucan are proven to enhance the innate immune factors and are suggested to have a role in adaptive immune response after dietary immunostimulation in fish (Brufau et al., 2015). In carps, β-glucan is reported to affect the microial composition of the intestines and the morphology of the intestine (Brufau et al., 2015). MOS are reported to affect the microbial composition in the intestines, which lead to an improvement of the gut morphology and the epithelial brush border in salmonids (Brufau et al., 2015).

To further investigate these results, a greater understanding of the composition of the exopolysaccharides (EPS) in the yeast cell wall and the immunostimulating effects of the components are needed. **The aim** of this thesis was to develop a method that quantified the specific components in the EPS to give accurate and reliable results. Such a method would be a great

resource in the research on the possibly immunostimulating effects of the carbohydrates in the YCW and the research on including yeast in aquafeed.

2

Theory

2.1 Fermentation and yeasts

Fermentation is processes using microorganisms, like yeast, to convert components in the growth mediums to useful products by taking advantage of the metabolic activity of the microorganisms (Watkinson et al. (2016), Li et al. (2014)). The yeasts used for the experiments in this were fermented using both batch fermentation and repeated fed-batch fermentation. Batch and fed-batch processes are together one of the two general fermentation processes, the other being continuous/semi-continuous fermentation(Li et al., 2014). In batch fermentation, an original culture is cultivated on the growth medium. During the fermentation process the amount of nutrients from the growth medium decrease as the number of yeast cells increases, and the fermentation is stopped to harvest the products (Watkinson et al., 2016). Repeated fed-batch fermentation is cycles of fed-batch fermentations, in which most of the yeast is harvested after a specific time, and new growth medium is added to the remaining yeast, and the fermentation continues in a new cycle. These cylces can be repeated several times, before the products are harvested.

The yeast strain used in these experiments were *Candida utilis*, *Wickerhamomyces anomalus* and *Arxula adeninivorans*. The growth mediums used were YPD, consisting of bacteriological peptone, yeast extract and glucose (Sigma-Aldrich®, 2019b), and chicken meat hydrolysate and Borregaard advanced lignin (BALI sugar).

2.2 Composition of the exopolysaccharides (EPS) in the yeast cell wall

Descriptions of the composition of the yeast cell wall and its carbohydrates components are often based on research on *Saccharomyces cerevisiae*. So the description given below, is of the YCW of *S.cerevisiae*, if not stated otherwise.

15-30 % of the dry weight is made up by the cell wall (Aguilar-Uscanga and François (2003), Yin et al. (2007)). β1,3-glucan, β1,6-glucan, chitin and mannose covalently linked to protein, called mannoprotein, are the four main carbohydrate components (Orlean, 2012), making up the EPS in the YCW of.

β-glucan in yeast are described as consisting of glucose-residues linked by β-1,3 linkages or β-1,6 linkages for β-1,3-glucan and ,6-glucan, respectively. β-glucan compose 30-60% of the dry weight of the cell wall (Orlean, 2012). Three fraction of β-glucan exist in the yeast cell wall Orlean (2012). The largest fraction consists of β-1,3-glucan with a degree of polymerization (DP) of 1500. From this polymer, side chains of β-1,3-glucan initiated with β1,6-glucan creates branching. The non-reducing ends of β-1,3-glucan can again be linked to chitin. The second fraction has the same structure, but here is no linking to chitin. The third fraction is β-1,6-glucan with a DP of 140, and some of the β-1,6-glucose in the polymer have a side-branch consisting of β1,3-glucose (Orlean, 2012).

Chitin consists of β-1,4-linked N-acetylglucosamine (GlcNAc) with a DP of about 90 to 100 residues ()thearticle and is responsible of 1-2% of the dry weight of the yeast cell wall Orlean (2012). In the yeast cell wall chitin exists either as free chitin, but chitin can also be bound to β-1,3-glucan. Some chitin is bound to β-1,6-glucan, which is again linked to β-1,3-glucan and mannan in the cross-linking region between the four components (Cabib (2009), Cabib and Durán (2005)).

The mannoproteins consist of mannan oligosaccahries (MOS) linked to polypeptides. The MOS consist of a backbone consisting of mannose residues linked with α-1,6-linkages. Side chains of short α-1,2- and α-1,3-linked mannose residues branch this backbone(Lipke and Ovalle, 1998). Phosphorylation of the mannose residues, and β-1,2-linkages can also occur, for example in *Candida albicans* (Cuskin et al., 2015).

Kollár et al. (1997) found through their studies that especially β-1,6-glucan plays an important role in connecting the four cell wall components together. They determined that β-1,6-glucan is attached to the mannoprotein through a remnant of a glycosylphosphatidylinositol (GPI) anchor. The reducing end of chitin is bound to the β-1,3-glucan sidechain of the β-1,6-glucan chain. The reducing end of the β-1,6-glucan is then attached to non-reducing end of β-1,3-glucan.

The yeast cell wall of *Saccharomyces* and *Candida* species may be described as consisting of two layers (Gow et al., 2017). Mannoproteins is the main component in the outer layer, while chitin and the glucans compose the inner layer, as illustrated in figure 2.1.

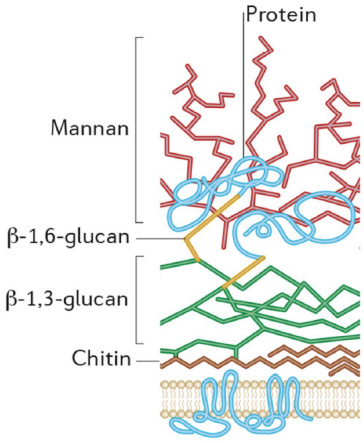

Figure 2.1: The figure from Gow et al. (2017) illustrates the structure of the cell wall of *Candida* species. The yeast cell wall consists of an outer layer of mannoprotein and an inner layer of β-glucan and chitin.

2.3 Method for quantification of the EPS

Several methods have been developed to analyze and estimate the carbohydrates in the yeast cell wall. Dallies et al. (1998) developed a method using sulfuric acid for hydrolysing polysaccharides into monosaccharides. In their study, commercial polysaccharides and YCW were first treated with 72% H_2SO_4 at room temperature for 3 hours. The solution was then diluted to 2N-H_2SO_4 for 4 hours at 100°C. But Schiavone et al. (2014) highlight some disadvantages with the use of only acid hydrolysis for the quantification of the carbohydrates in the yeast cell wall. As elaborated in 2.5, the monomers released in the acid hydrolysis may be degraded, leading to underestimation when quantifying the polysaccharides in the cell wall. Also, acid hydrolysis is not able to completely hydrolyze chitin, so the amount of this carbohydrate is underestimated. Another disadvantage when using acid hydrolysis, is that it is not possible distinguish between β-1,3-glucan and β-1,6-glucan.

Schiavone et al. (2014) found that the use of their combined acid and enzymatic hydrolysis released more chitin. Schiavone et al. (2014) used acid hydrolysis to find mannan. Then chitinases in addition to endo- and exo-β-1,3-glucanases were used first to find chitin and β-1,3-glucan, then endo-β-1,6-glucanase with β-glucosidase were used to find β-1,6-glucan. They provided a method combining sulfuric hydrolysis and enzymatic hydrolysis that could quantify β-1,3-glucan and β-1,6-glucan without needing fractionation like the method developed by Fleet and Mannan (1976) or radiolable and dialysis processes like in the method developed by Magnelli et al. (2002), estimate chitin without underestimate it and avoid degradation of monosaccharides during acid hydrolysis to some extent, ending up with a more effective and accurate quantification of the carbohydrates in the yeast cell wall.

Inspired by Schiavone et al. (2014), we developed a method. In this method sulfuric acid hydrolysis will be used to quantify the total β-glucan content in the EPS yeast cell wall. Endo chitinase C and N-acetylglucosaminidase (CHB) isolated from *Serratia marcescens* BJL200, endo-1,3-β-D-glucanase from barley and exo-1,3-β-D-glucanase from *Asperillus oryzae* will be used in one step to hydrolyse chitin and β-1,3-glucan to the corresponding monosaccharides N-acetylglucosamine and glucose, respectively. Then α-1,2-mannosidase 99B isolated from *Bacteroides thetaiotaomicron* will be used to hydrolyze off the side chain, to estimate the mannan in the side chain. Then α-1,6-mannanase 76B isolated from *Bacteroides thetaiotaomicron* will hydrolyze the α1,6-mannan backbone chain. The glucose released from β-1,3-glucan will then be subtracted from the total β-glucan content found by sulfuric acid hydrolysis, giving the amount of β-1,6-glucan. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) will be used to detect the released mono- and oligosaccharides released from the acid and enzymatic hydrolysis. The steps in the method is illustrated in Figure 2.2

2.4 Cell wall preparation

2.4.1 Disruption and isolation of yeast cells

Bzducha-Wróbel et al. (2014) examined several disruption method, including autoclaving, thermally-induced autolysis, homogenization in a bead mill, sonication and combinations of these, to find a method that was suitable for cell wall preparation for β-glucan isolation. They

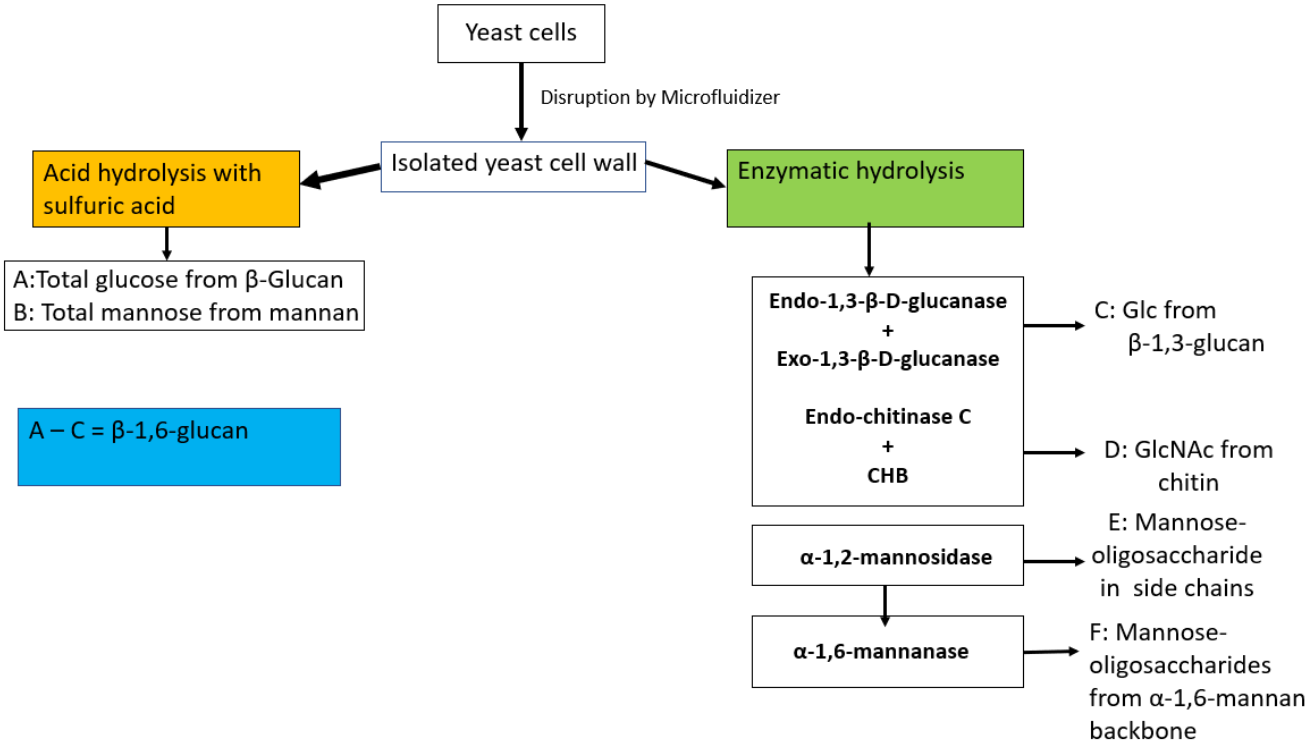

Figure 2.2: The figure illustrates the idea of the method, based on Schiavone et al. (2014) combining acid and enzymatic hydrolysis to quantify the EPS in yeast cell wall. Isolated yeast cell wall will first be hydrolyzed in a two step process. First the samples will be incubated with 72% H_2SO_4 at 30°C for 1 hour. The the samples will be diluted to get a final concentration of 4% H_2SO_4 before the samples will be autoclaved at 121°C for 1 hour. exo/endo-1,3-β-D-glucanases combined with endocitinase C and chtiobiase in a mixture will release glucose and N-acetylglucosamine form β-1,3-glucan and chitin, respectively. α-1,2-mannosidase will be used to hydrolyze off the side chain. Then α-1,6-mannanase will hydrolyze the α-1,6-mannan backbone chain. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) will be used to quantify the released mono- and oligosaccharides. The glucose released from β-1,3-glucan will then be subtracted from the total β-glucan content found by acid hydrolysis, giving the amount of β-1,6-glucan. The use of specific enzymes makes it possible to quantify the carbohydrates in the EPS, including their specific linkages and obtain higher yields for chitin compared to the yields obtained by acid hydrolysis.

evaluated the content of total saccharides, β-glucan and protein in the cell wall preparation, and concluded that cell homogenization with zircoium-glass beads (0.5 mm ib diameter) was the most effective method giving the cell wall preparation with the highest degree of purification of β-glucan. But this method is more difficult to scale up. It has been shown that disruption at 20 000 psi gave the highest order of disruption, compared to freeze drying and autolysis (internal unpublished data), indicating that yeast needs a high shear force for sufficient disruption.

Based on this we decided to use Microfluidizer^{TM} LM20 for disruption of the intact yeast cells. Microfluidizer^{TM} LM20 converts high fluid pressure into shear forces capable of disruption the yeast cell with pressure up to 30 000 psi. Constant pressure ensures that the samples is disrupted by with the same force, giving reliable results (Microfluidics^{TM}, 2019). Microfluidizer^{TM} LM20 also provides the possibility for up-scale processes giving the Microfluidizer^{TM} LM20 a more practical aspects for down-stream processing of yeast.

After the cells are disrupted, the slurry from collected from the Microfluidizer^{TM} LM20 will be centrifuged. It is then expected that the intracellular components such as protein will be in the resulting supernatant, while the pellet will contain the yeast cell wall, thus creating a protein-rich supernatant and and a carbohydrate-rich pellet.

2.4.2 Freeze drying

Freeze-drying is a gentle drying process, avoiding high temperature for liquid removal. The process used for drying in a freeze dryer is sublimation. The samples are placed in the freeze dryer in frozen condition, and the liquid is removed from the samples directly from solid phase to gas phase under vacuum by addition of thermal energy. Due to the vacuum, melting of the samples is prevented when thermal energy is added, assuring that the liquid is removed directly from solid to gas phase. This form for drying preserve the biological properties of the samples, and the samples are unchanged for qualitative and quantitative analysis (Christ, 2013).

2.5 Acid hydrolysis

The acid hydrolysis with sulfuric acid (H_2SO_4) preformed in this thesis is based on National Renewable Energy Laboratory (NRLE) procedure "Determination of structural carbohydrates and lignin in biomass" (A.Sluiter et al., 2012). It has been shown to give reproducible and reliable determinations for the hydrolysis of structural carbohydrates in lignin adn in biomass, but was not intended for hydrolysis if yeast EPS. Optimization of the method may therefore be necessary. The procedure is based on two steps. In the first step, strong H_2SO_4 (72%) is used to swell the biomass, before the strong acid is diluted to 4% using water and the polysaccharides are hydrolyzed to monosaccharides in the second step. During the hydrolysis, the released monosaccharides can degrade further into furfural and 5-hydroxymethylfurfural, for pentoses and hexoses respectively. For this reason sugar recovery standards are used to account for this degradation. A 90% initial dry matter content is required to sustain reliable result. A higher water content could dilute the acid concentration resulting in insufficient hydrolysis. Smaller particle size provides larger surface area compared to volume, so the hydrolysis reaction occur at more of the particle, compared to bigger particles. The particles should also be uniform, to reduce the possibility for inhomogeneous samples and giving results that are more reproducible. For dried yeast sample, this would imply that crushing of the dried yeast into a

more homogenized powder would be beneficial.

Dallies et al. (1998) tested several acids for hydrolysis of yeast cell wall , and concluded that sulfuric acid resulted in the most complete hydrolysis of the main polysaccharides, β-glucan, chitin and mannan, present in the cell wall and was successfully used to quantify these components in cell walls from *Saccharomyces cerevisiae* and genetically well-characterized mutants of the yeast, verifying the acid hydrolysis method used, though low yield for chitin was obtained.

When the polysaccharides present in the yeast cell wall are hydrolyzed, glucose is released from β-glucan, mannose from mannan, and gluosamine from chitin, as the N-acetyl residue in N-acetyl-glucosamine is acid-labile, and is removed during the acid hydrolysis.

2.6 Carbohydrate analysis

2.6.1 High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The use of high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) for carbohydrate analysis is a well known method, and provides separation with high resolution and sensitive detection using PAD (**?**).

Anion exchange chromatography (AEC) is a separation technique that separate anions by using anion exchange resins with positively charged functional groups on their surface. When the anions to be separated are applied to the column they bind to the resins with different affinities depending on their net negative charge, exchanging the negative counter-ions on the resin. The degree of affinity will determine how long the anions stay in the columns, the retention times, and the anions are separated due to different affinities (Miller, 2005).

The HPAEC-PAD system used for the carbohydrate analysis in this thesis is Dionex ICS (Thermo Fisher Scientific) equipped with CarboPac PA1 column(Thermo Fisher Scientific). The resin in the column consists of pellicular polystyrene/divinyl benzene substrate aggomerated quaternary ammonium functionalized latex microbeads (Jagger, 2012b). Quaternary amine groups provide a strong anion-exchanger. This resin gives short diffusion path lengths resulting in separations with high resolutions. The stationary phase comprised of these resin is able to tolerate the high pH in the mobile phase necessary for deprotonation of the carbohydrates (**?**).

At high pH (> 12) the hydroxyl carbohydrates are deprotonated to oxyanions (**?**), obtaining a negative charge. The oxyanion form of a carbohydrate is therefore possible to separate using AEC if a high pH is maintained in the column.

The anion-exhange process that occurs on the resin in the column can be represented by the equillibrium in equation 2.1, where A^{n-} represent the carbohydrates separated on the column.

$$-NR_3{}^+OH^- + A^{n-} \rightleftharpoons (-NR_3{}^+)_n A^{n-} + nOH^- \tag{2.1}$$

Monosaccarides have similar chemical properties giving similar affinities to the stationary phase,

so the spatial configuration of the hydroxyl groups affects the separation of monosaccharides. The eluting conditions also needs to be weaker compared to oligosacchardies, to be able to distinguish between the small differences among the monosaccharides (Jagger, 2012a). Generally, mobile phases with only hydroxide, not NaOAc, is used for separations of monosaccharides. The chemical similarities of monosaccharides also require a column with a stationary phase that is able to provide separation with high resolution (**?**).

Oligo- and polysaccharides will get more negative charges corresponding to the length, and get higher affinities to the stationary phase, leading to longer retention times. Separation of oligo- and polysaccharides therefore almost always use gradient run combining NaOH and NaOAc as eluents. NaOAc has a higher eluting strength, as the amount of negative counter ions is increased in the mobile phase, and this shortens the retention times. NaOH is important for maintaining the high pH necessary for high sensitivity (Jagger, 2012b). NaOAc shorten the retention time maintaining the high selectivity and without interfering with the detection. When using gradient elution with NaOH and NaOAc, it is recommended that the concentration of NaOH is kept constant. Since acetate exihibit no buffer capacity at the high pH in the system, a constant NaOH concentration will keep the baseline stable.

The column hardware consists of polyether ether keton (PEEK) for the reason than this material does not shed of metal ions in the basic environment, preventing contamination in the column and interference during detection (**?**).

If the pH is too low it will decrease the sensitivity. The high ionic strength caused by the hydroxide ions may increase the sensitivity, but this will reduce the resolution. Sensitivity will be lower at 1mM KOH, but this will give the resolution needed for separation of the monosaccharides in the samples (Jagger, 2012b).

The separation in the column is affected by carbonate. Carbonate can be dissolved in the eluent if it is exposed to CO_2. Carbonate, being an anion, binds to the stationary phase, often stonger than the analyte. This reduces the column capacity and resolution (Jagger, 2012b).

Pulsed amperometric detection (PAD) is used for detection after the separation of mono- and oligosaccharides. This is a direct detection method, where no derivatization of the analytes are needed before or after they are separated on the column. PAD only detects the compounds that have a functional group that is oxidized at the specific voltage employed to the gold electrode. This makes the detection method specific and selective for carbohydrates. The carbohydrates are oxidized at the surface of the gold electrode, and the electrical current that is generated is measured and the carbohydrates are detected. To remove the oxidized product from the electrode, four consequent potentials are applied to the electrode. After the current created by the oxidized carbohydrates is measured, the potential is changed to a high negative potential. This is to "flush" off the oxidized products (Cataldi et al., 2000). The potential is then raised to oxidize the gold surface and the rest of the oxidized products desorpts from the surface. Finally the potential is lowered, reducing the surface back to gold. Four potentials are thus applied to the electrode with fixed duration Rohrer (2013).

2.6.2 Ion exclusion chromatography with UV-detection

Ion exclusion chromatography is a separation process for separating ions from weakly ionized or natural compounds. Analytes with negative charge are separated using a cation-exchanger with negative functional groups, while analytes with positive charge are separated using an

anion-exchanger with positive functional groups (Haddad, 1990). When a cation-exchanger is used, anions are repelled (excluded) and elute first, while cations and neutral compounds elute later.

Dionex Ultimate 3000 RSLC equipped with Rezex^{TM} RFQ-Fast Acid H^+ (8%) column is used for ion exclusion chromatography with UV-detection for detection of N-acetylglucosamine (GlcNAc). Rapid separation liquid chromatography (RSLC) make it possible to use small particle columns to obtain fast separations with high resolution Scientific (2019). The stationary phase consists of sulfonated styrene divinyl benzene, with a negative charge and sulfuric acid (H_2SO_4) is used as mobile phase. The GlcNAc can receive a proton from the acid and get a positive charge. The now positive GlcNAc has affinity for the negative stationary phase, and is retained in the column, separated from other species present in the sample.

The acetyl group in GlcNAc absorb light at wavelength 195 nm (Jagger, 2016), so UV-detection with this wavelength is used to detect if GlcNAc is present in the sample.

2.6.3 Matrix-assisted laser desorption/ionization - time of flight mass spectrometry (MALDI-ToF MS)

Matrix-assisted laser desorption/ionization is a mild ionization technique that causes no or little fragmentation if the analyte molecule during ionization. MALDI is an ionization technique that occurs in two steps. In the first step the samples that are being analyzed are dissolved in a matrix. Several matrices are available including 2,5-Dihydroxy-benzoic acid (DHB),α-Cyano-4-hydroxycinnamic acid and 3,5-Dimethoxy-4hydroxycinnamic acid, where DHB is commonly used for oligosaccharides (de Hoffmann and Stroobant, 2007). The matrix and sample mixture is then dried removing the liquid solvent that was used in sample preparation. The analyte molecules are embedded in the matrix being completely isolated from each other, preventing clustering of sample molecules that can inhibit appearance of molecular ions. In the second step, the matrix embedded with analyte molecules is shot at with laser pulses in vacuum in the ion source inside the mass spectrometer. The matrix molecules have strong absorption at the wavelength of the laser, and this intense irradiation causes excitation of the matrix molecules. The large amounts of energy caused by the excitation is accumulated in the condensed phase, results in a rapid heating of the crystals in the matrix. This causes localisation sublimation of the the matrix crystals and a portion of the crystals at the surface is ejected and this portion consisting of intact analyte molecules surrounded by matrix molecules and salt ions. Cationization of the analyte molecule can happen when an alkali molecule is attached to the analyte (**?**). Cationization by sodium cation (Na^+ creates the characteristic $[M+Na]^+$, that is produced when DHB is used as matrix Sigma-Aldrich® (2019a).

Since the matrix absorbs most of the energy from the laser pulses, this minimize the damage to the analyte molecules. It also increase the efficiency of the transfer of energy to the analyte, increasing the sensitivity.

Time of flight mass analyzer is suited for MALDI ion source,

After the molecular ions $[M+Na]^+$, are produced and expelled from the ion source, they are accelerated towards a flight tube due to different potential between an electrode and extraction grid. This potential difference gives all the molecular ions the same kinetic energy. When the molecular ions then enters a field-free region, the flight tube, after the acceleration region, they

are separated due different velocity the different molecular ions with different mass have. An ion that is accelerated by a potential V when having a total charge $q = ze$ and mass m will use a time t to cover the distance L to the detector. This can be represented by equation 2.2. This equation shows that m/z value of the molecular ion can be found by measuring time of detection.

$$t^2 = \frac{m}{z}(\frac{L^2}{2eV}) \tag{2.2}$$

Reflectrons are used to to improve the mass resolution. The relectron is often positioned opposite of the ion source, and then the detector is positioned at the ion source side. A reflectron consists of a retarding field and correct for the kinetic dispersion, where molecular ions with same m/z ration have different kinetic energy. Ions that have higher kinetic energy in the acceleration region, will penetrate the retarding field deeper, and spend more time in the reflectron before it is reflected. Ions with lower kinetic energy will spend less time in the retarding field, due too less penetration. This ensures that molecular ions with same m/z, but different kinetic energy will reach the detector at the same time, and the increased mass resolution is obtained. The reflectron however, decrease the number of ions that reach the detector, so the transmission, is decreased, resulting in lower sensitivity. Transmission is defined as "the ratio of number reaching the the detector and the number of ions entering the mass analyzer" (de Hoffmann and Stroobant, 2007).

2.6.4 Determination of reducing sugar with 3,5-dinitrosalisylic acid

3,5-dinitrosalisylic acid (DNS) is a reagent that is able to react with the reducing end of sugars and can be used in the estimation of concentration of reducing sugars. The reagent used consist of sodium hydroxide, 3,5-dinitrosalisylic acid and potassium sodium tartrate ($KNaC_4H_4O_6$*$4H_2O$) dissolved in water.

Under alkaline conditions the free carbonyl group at the reducing end reduce 3,5-dinitrosalisylic acid to 3-amino-5-nitrosalicylic acid (Miller, 1959). These reactions happens in an equimolar fashion. The reduction of 3,5-dinitrosalisylic acid to 3-amino-5-nitrosalicylic acid changes the absorption of light at wavelength 540 nm, and this change is proportional to the amount of reducing carbohydrates. If glucose is used as standard, the measured amount is often given in glucose equivalents. Similarly, if mannose is used as standard the measured amount could be given in mannose equivalents.

Miller (1959) examined the challenge of loss of reducing sugar during the analysis. He concluded that a concentration of 1 % of NaOH is the optimal as this concentration gives the highest color intensity without contributing to loss of reducing sugars. A concentration of 1% of DNS was also found to be the optimal concentration by Miller (1959), as this DNS concentration gave the highest color intensity. DNS was found to not contribute to loss of reducing sugar. Potassium sodium tartrate is necessary to stabilize the color developed by the reduction of DNS.

2.7 Protein analysis

2.7.1 The Kjeldahl method

Kjeldahl is a method for determining organic nitrogen. Protein contain approximately the same percentage of nitrogen, and for this reason, the percentage of protein can be multiplied with a factor to give the protein concentration (in %) in sample. 6.25 is often the factor used to estimate the protein content in food material, and was used for analysis of yeast protein by Schiavone et al. (2014) and Bzducha-Wróbel et al. (2014).

When a samples is analyzed using the Kjeldahl method it is first decomposed using heated concentrated sulfuric acid (H_2SO_4), transforming the bounded nitrogen in the sample to ammonium ions. The samples is then cooled and diluted, before NaOH is added to make the sample basic. The ammonium ions are then converted to ammonia. A distillation of the ammonia in the basic solution then occurs. The ammonia is collected in an acidic solution and by a neutralization titration, the organic nitrogen concentration is determined (Skoog et al., 2014).

The first decomposition step converting bound nitrogen to ammonia can be time consuming. To catalyze this reactions a neutural salt such as potassium sulfate could be added. This addition increase the boiling point of H_2SO_4 making a higher reaction temperature possible. Selenium is another substance that catalyze decomposition of organic compounds, like proteins (Skoog et al., 2014). Kjeldahl tablets, like Kjeltabs Auto-AA11 (Thompson & Capper®), contain both potassium sulfate and selenium, and is added to catalyze the reaction during the protein analysis.

2.7.2 The Bradford method

The Bradford method is a method for determining the protein concentration in samples. In this method the dying agent Coomassie Brilliant Blue G-250 binds to the proteins. Coosmassie Brilliant Blue G-250 has originally an red color with absorption maximum at 465 nm, but when the dying agent is bound to a protein the absorption maximum is changed to 595 nm and a blue color is observed (Bradford, 1976). The absorbance can be measured at this wavelength to determine the protein content by using protein standards with known concentrations. This method have several advantages. It is rapid, needing approximately 10 minutes to complete the binding of the dying agent to the protein. The color developed is then stable for up to 1 hour. Compounds like sodium, potassium or carbohydrates causes no interference effecting the protein determination. The complex consisting of the dye and protein have a high extinction factor (ε) giving a high sensitivity since high absorption values (A_{595}) is measured for low protein concentrations.

3

Methods and materials

3.1 Materials

3.1.1 Chemicals

Chemicals	Supplier
α-chitin	In-house stock
Bovine Serum Albumin	SIGMA-ALDRICH®
β-glucan (Barley)	Megazyme
Curdlan (1,3-β-D-glucan)	Megazyme
L-(-)-Fucose	SIGMA®
D-(-)-Fructose	VWR chemicals
D-(+)-glucosamine hydrochloride	SIGMA®
D-(+)-glucose, anhydrous	VWR Chemicals
D-(+)-Galactose	SIGMA-ALDRICH®
Hydrochloric acid	
Laminarin (1,3-β-D-glucan)	SIGMA-ALDRICH
Mannan (ivory nut)	Megazyme
Mannan (*Saccharomyces cerevisiase*)	SIGMA®
D-(+)-mannose	SIGMA®
N-acetyl-D-glucosamine	SIGMA®
Protein assay Dye reagent concentrate	Bio-Rad
Pustulan (1,6-β-D-glucan)	Elicityl OligoTech®
Sodium acetate, anhydrous	VWR Chemicals
Sulphulric Acid 96%	VWR Chemicals
Trisma®base	SIGMA-ALDRICH®
D-(+)-Xylose	SIGMA-ALDRICH®

3.1.2 Yeasts

An overview of the yeasts used is given in tabel 3.1

Table 3.1: Description of the different yeast strain, fermentations and growth mediums used

Yeast strain	Supplier	Growth medium	Fermentation
C.utilis LYCC 7549	Lallemand	YPD	Batch
		Chicken meat hydrolysate and BALI sugar	Batch
		Chicken meat hydrolysate and BALI sugar	Repeated fed batch
W.anomalus J121	SLU	YPD	Batch
		Chicken meat hydrolysate and BALI sugar	Batch
		Chicken meat hydrolysate and BALI sugar	Repeated fed batch
A.adeninivorans LS3	SLU	YPD	Batch
		Chicken meat hydrolysate and BALI sugar	Batch
		Chicken meat hydrolysate and BALI sugar	Repeated fed batch

3.2 Methods

3.2.1 Time estimation to obtain 95% cell lysis with Microfluidizer™ LM20

For *C.utilis*, *W.anomalus* and *A.adeninivorans* from batch fermentation culitvated on YPD, a 10 % (w/v) slurry was made by dissolving 3.0 g of freeze-dried yeast into 30 mL of milli-Q water. The slurry was transferred to the Microfluidizer™ LM20. Before starting the microfluidizer™ LM20 500 µL of the slurry was transferred to an Eppendorf tube and stored on ice. This was repeated after 2.5, 5.0, 7.5, 10, 12.5 and 15 minutes after starting the microfluidizer™ LM20. For *A. adeninivorans* the Microfluidizer™ LM20 was only run for 10 minutes. The microfluidizer™ LM20 ran in loop-mode at 30 000 psi. The intact cells were counted using a microscope (Leitz, Laborlux K) magnifying the sample 400 times and using a counting chamber (Bürker). The number of living cells were plotted against microfluidizer™ LM20 operating time, and the time needed for 95 % cell death was estimated.

3.2.2 Cell wall preparation

For all yeasts listed in Table 3.1 a 12 % (w/v) slurry was made by dissolving 4.0 g, 5.0 g or 6.0 g of freeze-dried yeast into 33.33, 41,67 or 50 ml of milli-Q water, respectively. 6.0

g was preferably used, but when not enough dry yeast was available, 4.0 g or 5.0 g were used, maintaining the 12% (w/v) loading. The slurry was transferred to the Microfluidizer™ LM20 and 150 µL of Glanapon DB 870 antifoam (Busetti) were added. Before starting the microfluidizer™ LM20, 100 µL of the slurry was transferred to an Eppendorf tube and stored on ice. The microfluidizer™ LM20 ran for 45 minutes in loop-mode at 30 000 psi. 100 µL was transferred to an Eppendorf tube after 45 minutes. Then all of the remaining slurry was transferred to tubes. The microfluidizer™ LM20 was also washed with milli-Q water to collect as much of the original slurry as possible. To determine how many cells disrupted by the microfluidizer™ LM20, the cells collected after 0 and 45 minutes was counted using a microscope (Leitz, Laborlux K) magnifying the sample 400 times and using a counting chamber (Bürker) to see if 95% of the cells were lyzed.

To test the reproducibility of the disruption caused by the microfluidizer™ LM20, a triplicate of *W.anomalus* from batch fermentation produced on chicken meat hydrolysate and BALI sugar was prepared and disrupted by Microfluidizer™ LM20 as described above.

All slurry and water used for washing were collected and centrifuged with an Avanti™ J-25 centrifuge (Beckman Coutler), JA-10 rotor at 16 000 *g* at 4 °C for 15 minutes. The supertantant was collected and stored at 4 °C for later analysis of protein content. The pellet was washed with milli-Q water two times using the same centrifuge conditions.

The pellets and supernatants (after protein analysis) were then freezed at -80 °C over night and freeze dried using Christ ALPHA 2-4 LD plus freeze dryer. The dried material was weighed and stored in freezer.

3.2.3 Dry weight analysis

Dry matter analysis of a selection if freeze dried pellets and supernatants were done by drying triplicates of each sample at 105°C for 20 hours. Samples were cooled down to room temperature in a vacuum excicator before the samples were weighed. The vaccum exciator had a water withdrawing material (Sicapent containing phosphatepentoxide) to prevent moisture to enter the samples during cooling.

3.2.4 Analysis of protein concentration using the Bradford method

To estimate the protein concentration, Biophotometer D30 (eppendorf) was used. A blank sample consisting of 20 mM Tris-HCl buffer at pH 7.5 and a standard consisting of 5 µL 500 µg/mL BSA in 795 µL 20 mM Tris-HCl buffer at pH 7.5 was used. Either 10 µL of sample in 790 µL Tris-HCl-buffer or 5 µL in 795 µL Tris-HCl-buffer was used, to get the right dilution so the measured value is in the linear area of the standard curve. To the blank, standard and samples, 200 µL Protein assay Dye reagent concentrate (Bio-Rad) was added. After stabile color was achieved, approxiamterly after 5 minutes, the prepared samples was transferred to cuvettes and the A_{595} was measured. An internal standard curve (concentration = 25.663*A_{595} - 0,06207, R^2 = 0,9971) was used to estimate the protein concentration. The internal standard curve was made with six BSA-standards (two replicate of each) with concentrations 0, 1.25, 2.50, 5.00, 7.50 and 10.00 µg/ mL.

3.2.5 Analysis of total protein

The protein content in the supernatants collected after centrifugation of yeast slurry collected after the microfluidizer^{TM} LM20, was estimated as described in section 3.2.4. One replicate of each supernatant was measured. The estimated protein concentration (mg/mL) was multiplied with the total volume of supernatant to find the total protein concentration in the supernatants.

3.2.6 Analysis of protein concentration using the Kjeldahl method

To estimate the protein concentration in the pellets and supernatants, the micro Kjeldahl method was used. Duplicates of approximately 0.2 g (exact weight recorded) crushed freeze dried pellet or supernatant were prepared in Kjeldahl tubes. A catalyzing Kjeldahl tablet (Kjeltabs Auto-AA11), was added to each tube before 3 mL of 96% H_2SO_4 was added. The tubes containing sample, Kjeldahl tablet and sulfuric acid was then heated at 450 °C for one hour using 3001 Prot Feed AOAC on an autodigestor (FOSS, Tecator, Hoganas) for acid digestion. The nitrogen content was then measured using Foss Kjeltec^{TM} 8400 (FOSS, Tecator, Hoganas). The results were given as % protein by multiplying the total nitrogen by a factor of 6.25. The protein concentration was then converted to g protein/ g pellet or g protein/g supernatant by multiplying the exact weighed mass of the samples with the protein concentration given in % protein.

3.2.7 H_2SO_4-hydrolysis of pellets and supernatants

Triplicates of 10 mg of pellet and 5 mg supernatant were prepared in Eppendorf tubes, and 25 µL of 72% H_2SO_4 was added. The tubes were then placed on a water bath (Julabo 5A ED) at 30 °C for 60 minutes. Then 700 µL of milli-Q water were added to obtain 4% H_2SO_4. Sugar recovery standards (SRS) were made with glucose, mannose and N-acetyl-D-glucosamine, with the following concentration 12, 11 and 1 mg/mL, respectively, mimicking the actual concentrations in yeasts. Two replicates of SRS with volume 500 µL were prepared and 11.4 µL of 96% H_2SO_4 was added to obtain 4% H_2SO_4. Standards of SC-Man, INM, α-chitin, β-glucan, laminarin and pustulan were prepared with final concentration of 0.2% in a total volume of 500 µL. As with the SRS, 11.4 µL of 96% H_2SO_4 were added, to get a final concentration of 4% H_2SO_4. All the samples, SRS and standards was autoclaved at 121°C for 60 minutes with CertoClav Labor-Autoklav. The samples were then centrifuged using eppendorf Centrifuge 5418 R at 16 900 *g* for 5 minutes, or filtered using micro plate filter (Nunc^{TM} 96-Well Filter Plates) depending on the sample. The samples were then stored at 4°C. To the standards, DNS-analysis was carried out as described in section 3.2.20.1 to find the yield from the acid hydrolysis of the given standards. The pellets and supernatants were analyzed using HPAEC-PAD as described in section 3.2.22.

To take the difference in volume into account, the concentration given by the DNS-analysis or HPAEC-PAD was multiplied with the volume (either 511.4 µL or 725 µL). The value was also multiplied with the dilution factor. Further more, due to increase in mass equivalent to water when a polysaccaride is hydrolysed into monosaccharides, the values were adjusted with the right polymer correction factor. Recovery factors calculated from the SRS were used to make up for any degradation of monosaccharides during the acid hydrolysis. This calculation is summerized in Formula A.1 given in Appendix A.1, which shows how the content of the

different carbohydrates is calculated as % of dry mass (DM)

3.2.8 H_2SO_4-hydrolysis of *C.utilis* and *W.anomalus* with and without pretreatment with 72% H_2SO_4

Two triplicates of 10 mg of *C.utilis* and *W.anomalus*, both from batch fermentation and cultivated on YPD, were prepared in Eppendorf tubes. To one of the triplicates 25 µL of 72% H_2SO_4 were added and stired using a toothpick to ensure complete wetting of the yeast sample. The tubes were then placed on water bath (Julabo 5A ED) at 30 °C for 60 min. Then 700 µL of water was added to obtain 4% H_2SO_4. To the other triplicate 500 µL of milli-Q water and 11.4 µL of 96 % H_2SO_4 were added to obtain 4% H_2SO_4. Two replicates of SRS were made with glucose, mannose and GlcNAc, in the same way as described in section 3.2.7. The samples and SRS were autoclaved at 121 °C for 60 minutes using CertoClav Labor-Autoklav. The samples were then centrifuged or filtered using micro plate filter (Nunc^{TM} 96-Well Filter Plates) depending on the sample, before stored at 4°C. A DNS-analysis was carried out as described in section 3.2.20.1, except that the analysis was carried out straight into the micro plate as described in section 3.2.20. Formula 1 in Appendix 1 was then used to calculate the yield of the two treatments.

3.2.9 Enzymes

An overview of the enzymes used is given in Table 3.2.

Table 3.2: Description of enzymes used for enzymatic hydrolysis.

Name	Origin/supplier	pH optimum	Temperature optimum (°C)
endo-1,3-β-D-glucanase	Barley/Megazyme	5.0[1]	40[1]
exo-1,3-β-D-glucanase	*Asperillus oryzae*/Megazyme	5.0[2]	40[2]
Endochitinase C	*Serratia marcescens* BJL200	3.9 (but active at pH 5)[3]	N/A
N-acetylglucosaminidase CHB	*Serratia marcescens*BJL200	6-8[4]	52[4]
α-mannosidase 99B	*Bacteroides thetaiotaomicron*/nzytech	5.5-7.5[5]	37[5]
α-mannanase 76B	*Bacteroides thetaiotaomicron*/nzytech	7.0[6]	37[6]

[1]Megazyme (2019a), [2]Megazyme (2019b), [3]Synstad et al. (2008), [4]Tews et al. (1996), [5], [6],

3.2.10 Test of the enzymatic activity of Chitinase-cocktail and endo-1,3-β-d-glucanase in Tris and NaOAc buffer at pH5

A chitinase cocktail consisting of 90% (w/w) chitinaseC with protein concentration 1.9 mg/ml and 10% (w/w) chitobiase with protein concentration 2.8 mg/mL was prepared to a final protein concentration of 2 mg/mL. endo-1,3-β-D-glucanase with enzymatic activity of 0.02 U/µL was

used. The chitinase-cocktail and endo-1,3-β-D-glucanase were tested in 50 mM Tris-HCl buffer and 50 mM NaOAc-buffer, both at pH 5. The chitinase-cocktail was tested with α-chitin as substrate. endo-1,3-β-D-glucanase was tested on both laminarin (mainly 1,3-β-D-glucan) and pustulan (1,6-β-D-glucan) to test the specificity of the enzyme. α-chitin was used with a final concentration of 1% (w/v), laminarin with a final concentration of 0.5% (w/v) and pustulan with a final concentration of 0.1% (w/v), all in a final volume of 200 µL. 15 µL chi-cocktail and 1 µL of the laminarinase were used. An enzyme control, with no substrate, and a substrate control, containing no enzyme was also prepared with same amounts to a final volume of 200 µL. The Eppendorf tubes were placed in a Thermomixer (Eppendorf) at 40°C and 1000 rpm over night.The Eppendorf tubes were boiled for 15 minutes straight after, to stop the enzymatic reaction. The samples were then analyzed using both DNS-analysis as described in section 3.2.20. The samples testing the activity of endo-1,3-β-D-glucanase were also tested using MALDI-ToF and HPAEC-PAD, as described in sections 3.2.21 and 3.2.23, respectively.

3.2.11 Progression curve for endo-1,3-β-d-glucanase

In a 50 mM Tris-HCl buffer at pH 5, 1% (w/v) curdlan (1,3-β-D-glucan) was hydrolyzed by endo-1,3-β-D-glucanase. Duplicates with 1% (w/v) curdlan in 50 mM Tris-HCl buffer was prepared and 2 µL of laminarinase with enzymatic activity 0.02 U/mL was added to a final volume of 400 µL. An enzyme control, with no substrate and a substrate control, containing no enzyme were also prepared. The tubes were placed in a Thermomixer at 40°C and 1000 rpm. 50 µL were withdrawn and transferred to Eppendorf tubes after 30 minutes, 1 hour, 3 hours, 5 hours and 24 hours. The Eppendorf tubes were boiled for 15 minutes straight after, to stop the enzymatic reaction. The Eppendorf tubes were then stored at 4°C. A DNS-analysis was carried out as described in section 3.2.20. The values for the control samples were withdrawn from the samples. The progression curve was made by plotting the concentration of GE against the respective reaction time.

3.2.12 Progression curve for Chitinase-cocktail

In a 50 mM Tris-HCl buffer at pH 5, a chitinase cocktail consisting of 6 µL of ChitinaseC (protein concentration 4,6 mg/mL) and 2 µL of chitobiase (enzyme concentration 2.8 mg/mL) was tested with 1% (w/v) α-chitin as substrate. Duplicates with 1% (w/v) α-chitin in 50 mM Tris-HCl buffer were prepared and the chitinase-cocktail was added to a final volume of 400 µL. An enzyme control, with only enzyme and a substrate control, containing only substrate were also prepared. The tubes were placed in a Thermomixer (Eppendorf) at 40°C and 1000 rpm. 50 µL were withdrawn and transferred to Eppendorf tubes after 30 minutes, 1 hour, 3 hours, 5 hours and 24 hours. The Eppendorf tubes were boiled for 15 minutes straight after, to stop the enzymatic reaction. The Eppendorf tubes were then stored at 4°C. A DNS-analysis was carried out as described in section 3.2.20. The values for the control samples were withdrawn from the samples. The progression curve was made by plotting the concentration of GE against the respective time of outtake.

3.2.13 Preliminary experiments on α-mannanase and endo-α-mannosidase

To test the amount necessary of α-mannanase and endo-α- mannosidase, a preliminary experiment was performed, testing the two enzymes on SC-Man and INM as substrates with a final concentration of 1% (w/v). The assays were carried out in NaOAc-buffer with pH 7. The final buffer-concentration was 50 mM. Three different enzyme-concentrations were tested of both enzymes; 0.0005, 0.001 and 0.0025 mg/mL, in a total volume of 200 µL. The enzymes were mixed with the substrate in NaOAc-buffer in Eppendorf tubes and placed in a Thermomixer (Eppendorf) at 37°C, 1000 rpm for 5 hours. The samples were then boiled for 15 minutes and then cooled prior to DNS-analysis was then carried out as described in section 3.2.20. The GE concentrations were found and the enzyme concentration necessary was evalueted .

Another experiment tested α-mannanase and endo-α-mannosidase on 1% (w/v) SC-Man, both separately and in a mixture together in 50 mM NaOAc-buffer in a total volume of 200 µL. The enzyme concentration for each enzyme was 0.005 mg/mL, both separately and in the mixture. The Eppendorf tubes containing the reaction mix were placed in a Thermomixer (Eppendorf) at 37°C, 1000 rmp for 24 hours. The samples were then boiled for 15 minutes to stop the enzyme reactions and stored at 4°C. Oligosaccharide analysis was carried out as described in section 3.2.23. The activity of α-mannanase and endo-α-mannosidase was evaluated.

Since α-1,2-mannobiose was available, endo-α-mannosidase was tested on this disaccharide with a final substrate concentration of 0.01% (w/v). endo-α-mannosidase had a final enzyme concentration of 0.005 mg/mL in a total volume of 300 µL 50 mM NaOAc-buffer pH 7. The Eppendorf tubes containing the reaction mix were placed in a Thermomixer (Eppendorf) at 37°C, 1000 rpm for 24 hours. The samples were then boiled for 15 minutes to stop the enzyme reactions and stored at 4°C. Later a monosaccharide and oligosaccharide analysis was carried out as described in section 3.2.22 and 3.2.23, resepectively. The activity of endo-α-mannosidase on α-1,2-mannan was then evaluated.

3.2.14 Test of the specificity of the enzymatic activity of exo-1,3-β-d-glucanase

To test the specificity of the exo-1,3-β-D-glucanase, the enzyme was tested with pustulan as substrate. In 50 mM NaOAc-buffer 10 µL of 0.0025 U/µL of the exo-1,3-β-D-glucanase was added. According to the description (Megazyme, 2019b), BSA was added to a final concentration of 1.0 mg/mL. Pustulan was used with a final concentration of 0.1% (w/v). All were mixed in eppendorf tubes to a final volume of 200 µL. The tubes were placed in a thermomixer at 40°C, 1000 rpm for 24 hours. The samples were then boiled for 15 minutes, then stored at 4°C. A monosaccharides analysis was carried out as described in section 3.2.22.

3.2.15 Test of exo-1,3-β-d-glucanase and endo-1,3-β-d-glucanase

A mix of exo-1,3-β-D-glucanse and endo-1,3-β-D-glucanse was tested in a 50 mM NaOAc-buffer. Curdlan, with a final concentration of 1% (w/v) was used as a substrate. 10 µL of 0.002 U/µL endo-1,3-β-D-glucanase and 10 µL of 0.0025 U/µL exo-1,3-β-D-glucanase was added in a total volume of 200 µL in Eppendorf tubes. BSA was also added, accordingly to description

(Megazyme, 2019b), to a final concentration of 1.0 mg/mL. The tubes were then placed in a Thermomixer at 40°C and 1000 rpm for 24 hours. The tubes were then boiled for 15 minutes, before stored in the fridge. The samples were analyzed for oligosaccharides as described in section 3.2.23. A MALDI-analysis was also carried out as described in section 3.2.21.

3.2.16 Activity of chitinase- and glucanase-cocktail tested on pellets

Duplicates of about 5 mg of crushed, freezed dried pellet from *C.utilis*, *W.anomalus* and *A. adeninivorans* were prepared in Eppendorf tubes. The pellets were dissolved on 50 mM NaOAc buffer pH5, and endochitinase C, CHB, endo-1,3-β-D-glucanse and exo-1,3-β-D-glucanse were added. Endochitinase C and CHB had final enzymes concentration of 0.23 mg/mL and 0,056 mg/mL, resepctively. endo-1,3-β-D-glucanse and exo-1,3-β-D-glucanse had final enzyme activity of 0.0001 U/mL and 0.000125 U/mL. BSA was also added, according to description (Megazyme, 2019b), to a final concentration of 1.0 mg/mL. The final volume was 200 µL. The Eppendorf tubes were placed in a Thermomixer at 40°C and 1000 rpm for 24 hours. After 24 hours, the samples boiled for 15 minutes and stored at 4°C. The samples were then analyzed for monosaccharides as described in section 3.2.22 and the activity of the enzymes on the pellets was analyzed.

3.2.17 Effect of buffer concentration on the activity of chitinase- and glucanase-cocktail hydrolyzing intact yeast

To test if the buffer concentration had any effect on maintaining the pH and keeping the reaction conditions optimal for the enzymes, two triplicates of 5 mg of crushed freeze dried intact *C.utilis*, *W.anomalus* and *A. adeninivorans* were prepared in Eppendorf tubes. One of the triplicates was dissolved in 200 mM NaOAc buffer pH 5 and the other triplicate was dissolved in 50 mM NaOAc pH 5. Endochitinase C, CHB, endo-1,3-β-D-glucanse and exo-1,3-β-D-glucanse were added with the following final concentrations, 0.23 mg/mL, 0.056 mg/mL for the chitinases respectively and the glucanases had final activities of 0.0001 U and 0.000125 U for the endo-1,3-β-D-glucanse and exo-1,3-β-D-glucanse, resepctively. BSA was also added, according to description (Megazyme, 2019b), to a final concentration of 1.0 mg/mL. The final volume was 200 µL for both 200 mM and 50 mM NaOAc-buffer. In addition, a substrate and enzyme control were prepared. The Eppendorf tubes were placed in a Thermomixer(eppendorf) at 40 °C and 1000 rpm. 75 µL were withdrawn after 24 hours and boiled for 15 minutes before stored at 4°C. The rest were removed from the Thermomixer and boiled for 15 minutes after 48 hours and stored at 4°C. A monosaccharide analysis was carried out as described in section 3.2.22, and the effect of buffer concentration was analyzed.

In addition, another experiment was carried out to test if the concentration of buffer had any effect on maintaining the pH in the reaction. Two duplicates of 5.0 mg of intact, freeze dried and crushed *A.adeninivorans* were prepared in Eppendorf tubes. One of the duplicates was dissolved in 200 mM NaOAc buffer pH 5, while the other duplicate was dissolveed in 50 mM NaOAc buffer pH 5. Endochitinase C, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase were added, with the same enzyme concentration or activity as described above in a total volume of 200 µL. BSA was also added, according to description (Megazyme, 2019b), to a final concentration of 1.0 mg/mL. An enzyme and substrate control were prepared for both buffer concentrations. The pH was measured with Sentron SI series pH meter, in all samples including enzyme and

substrate blank and just buffer. The samples were placed in Thermomixer (Eppendorf) at 40°C and 1000 rmp for 24 hours. The pH was measured in all the samples again, before the samples were boiled for 15 minutes and stored at 4°C. The changes in pH were evaluated to see if the intact yeast had any effect on the pH.

3.2.18 Effect of heat on intact yeast

To test the effect of heat on the intact yeast, duplicates of 5.0 mg intact, freeze dried and crushed *C.utilis*, *W.anomalus* and *A. adeninivorans* were prepared in Eppendorf tubes. The yeast were dissolved in 200 µL 200 mM NaOAc buffer pH 5. Each duplicate were either placed 24 hours at 40°C, 50°C, 60°C or 70 °C, boiled for 15 minutes or just dissolved in 200 mM NaOAc buffer pH 5 right before the samples were prepared for monosaccharide analysis. All the samples were analyzed for monosaccharides as described in section 3.2.22, and the effect of heat on intact yeast was analyzed.

3.2.19 Three different enzymatic hydrolyses of intact *C.utilis*

Triplicates of 5.0 mg intact freeze dried *C.utilis* were prepared in Eppendorf tubes. One of the triplicates was treated with a mix of α-mannanase and endo-α-mannosidase in 200 mM NaOAc buffer pH 7 at 37°C in a Thermomixer at 1000 rpm for 24 hours. The final volume was 200 µL and the enzyme concentration was 0.05 mg/ml for both α-mannanase and endo-α-mannosidase. The second replicate was treated with endochitinase C, CHB, endo-1,3-β-d-glucanase and exo-1,3-β-D-glucanase in 200 mM pH 5 at 40°C in a Thermomixer(Eppendorf) at 1000 rpm for 24 hours. BSA was also added, according to description (Megazyme, 2019b), to a final concentration of 1.0 mg/mL. The final concentrations were 1.15 mg/mL and 0.28 mg/mL for endochitinase C and CHB, respectively. The final enzyme activity was 0.00125 U/mL and 0.001 U/mL for exo-1,3-β-d-glucanse and endo-1,3-β-D-glucanse, resepectively. The final volume was 200 µL. The third triplicate was preincubated with a mix of α-mannanase and endo-α-mannosidase for 4.5 hours at 37°C in a Thermomixer at 1000 rpm with the same buffer and enzyme concentrations as described above for the triplicate treated with only α-mannanase and endo-α-mannosidase. After 4.5 hours, the pH was adjusted to pH 5 using HCl. The pH was measured using Sentron SI series pH meter. Then Endochitinase C, CHB, endo-1,3-β-D-glucanase and exo-1,3-β--glucanase were added giving the same enzyme concentration as stated above for these four enzymes. In addition BSA was added to a final concentration of 1.0 mg/ml, following the descriptions given for exo-1,3-β-D-glucanase (Megazyme, 2019b). The final volume was 310 µL, changing the buffer concentration to 129 mM. The tubes were then placed in a Thermomixer (Eppendorf) at 40°C, 1000 rpm for 24 hours.

All the samples were boiled for 15 minutes after the 24 hours and stored at 4°C until a monosaccharide analysis was carried out on all the samples as described in section 3.2.22. The effects of the different enzyme hydrolyses were evaluated and compared.

3.2.20 DNS-analysis

If not stated otherwise, the analysis was carried out in micro plate (Nunc®Microwell™) wells. The samples were diluted with Milli-Q water to a final volume of 50 µL to get an A_{540}-value

in the linear area of the standard curve. A 1:1 relation was used between sample and DNS-reagent. After the samples and DNS-reagent were applied, the micro plate was covered with a plastic film, and placed in a Thermomixer (Eppendorf) at 100°C and 300 rpm for ten minutes. The micro plate was then cooled, before the plastic film was removed carefully, making sure no sample was attached to neither the film nor at the top of the wells. The A_{540} was measured using Multiscan FC (Thermo Scientific). Standards with the following glucose concentrations 0, 0.3, 1.0, 3.0 and 4.0 mM were always used, and a standard curve was made by plotting the A_{540}-value against the glucose concentration in the respective standards and then using linear regression to plot the curve. By using the standard curve and the measured A_{540}-value, the concentration of glucose equivalents was calculated.

3.2.20.1 DNS-analysis of samples from hydrolysis

1 µL of sample, 48 µL of water, 1 µL of 19.1 M NaOH (to neutralize the acid) were mixed with 50 µL DNS-reagent in an Eppendorf tube. All the samples, including standards were boiled for 5 minutes, before transferred to a micro plate (Nunc®Microwell™) and the A_{540} was measured. From the standard curve the concentration of glucose equivalents was found in the samples measured, as described in section 3.2.20. The yield of the SRS was also calculated.

3.2.21 MALDI-ToF/ToF analysis

For the MALDI-ToF/ToF analysis DHB-matrix consisting of 4.5 mg (± 1.5 mg) 2,5-Dihydroxybenzoic acid (DHB), 150 µL acetonitrile (ACN) and 350 µL milli-Q water was used. To each spot on the target plate, 2 µL of DHB-matrix and 1 µL of sample were added. The applied sample was then completely dried using hot air flow, before the target plate was placed in the MALDI-ToF/ToF instrument (ultraflextreme, Bruker) operating in reflectron mode and analyzed with Nitrogen 337 nm laser beam.

3.2.22 Monosaccharide-analysis using HPAEC-PAD

The hydrolysed samples were analyzed with using Dionex ICS3000 (Thermo Fisher Scientific) with an eluent generator separating the monosaccharides on a CarboPac PA1 column 250 mm x 4 mm (Thermo Fisher Scientific), using an isocratic run with 1 mM KOH at a flow rate of 0.250m mL/min at 30°C. In addition, A Dionex AminoTrap™ BioLC™ Guard 2 mm X 50 mm was used to prevent interference from amino acids. For detection PAD was used.

Qualitative standards of glucose, mannose, N-acetyl-D-glucosamine and D-(+)-glucosamine with concentration 0.1 mg/mL, and xylose, ribose, fructose, fucose, rhamnose and arabionose with concentration 0.01 mg/mL was made, to identify peaks in the samples with unknown monosaccharide composition.

The results led to the use of a standard mix consisting of glucose and mannose with the following concentrations 0.1, 0.01, 0.005 and 0.001 mg/mL and galactose, glucosamine, xylose and ribose with concentrations of 0.05, 0.005, 0.0025 and 0.0005 mg/mL. Fucose with a final concentration 0.05 mg/mL was used as internal standard. The same amount of internal standard (10 µL) was always added to the vials. The samples to be analyzed were centrifuged or filtered to

remove any particles present, before diluted to give measured values within the linear area of the standard curves.

The column was washed regularly with 1 M NaOAc in 0.1 M NaOH, to maintain the high resolution making it possible to separate especially glucosamine and glucose.

3.2.23 Oligosaccharide-analysis using HPAEC-PAD

For oligosaccharide analysis an ICS system from Dionex (Thermo Fisher Scientific) with a CarboPac PA1 column 250 mm x 4 mm (Thermo Fisher Scientific) were used. The separation of the oligosaccharides was achieved using a gradient run with 0.1M NaOH (eluent A) and 1M NaOAc in 0.1M NaOH (eluent B) at 30°C. Starting with 100% eluent A, the concentration of eluent B increased to 10% after 10 minutes, to 14% after 15 minutes and to 30% after 16 minutes before reaching 100% after 18 minutes, gradually increasing the eluation strength. The concentration was then changed back to 100% eluent A after 18.1 minutes, to recondition the column the last 9 minutes before the next injection. The flow rate was constant at 0.250 ml/min throughout the run.

The only standards available (in-house) used in the oligosaccharide HPAEC-PAD analysis were cellooligoes (1,4-linked glucose units) with length 1-6. They were used with concentration 0.1, 0.01 and 0.001 g/L to verify that the system was working as expected. The samples analyzed have β1,3- or β1,6-linkages eluting differently, so the standard are not suitable for indicating retention times, except for the monosaccharide. Standards with the correct linkages should be used, and could for example be be isolated from the product from the reactions with endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase, or they could be bought if possible.

3.2.24 Ion exclusion chromatography

To verify if the peaks in the chromatogram of the acid hydrolyzed samples was ribose and not GlcNAc, all the samples were analyzed using ion exclusion chromatography using Dionex Ultimate 3000 RSLC set up with a Rezex^{TM} RFQ-Fast Acid H^+ (8%) 100 x 7.8 mm. As mobile phase, 5mM H_2SO_4 was used isocratically with a flow rate of 1.00 mg/mL at 85°C. By using a UV detector at wavelenght 195 nm, the acetyl group in GlcNAc would be detected if present. Standard of the monosaccharide GlcNAc and the diasaccaride $[GlcNAc]_2$ with concentrations 50mM and 100mM were used.

4

Results

4.1 Yeast cell disruption with Microfluidizer™ LM20 and yeast cell wall isolation

To estimate the operating time needed to obtain 95% cell lysis, a 30 mL 10 % (w/V) slurries of the yeasts *C.utilis*, *W.anomalus* and *A.adeninivorans* were disrupted by Microfluidizer™ LM20, and samples were withdrawn before start and after 2.5, 5.0, 7.5, 10, 12.5 and 15 minutes. The intact cells were then counted using microscopy and the number of intact cells were plotted against the operating time. The result is shown in Figure 4.1.

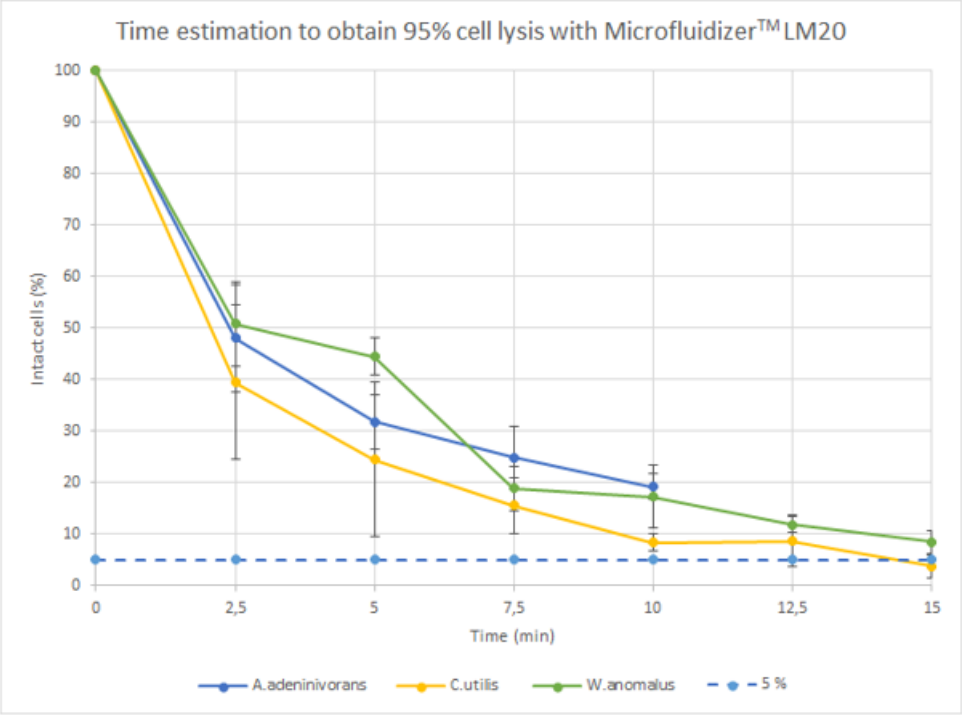

Figure 4.1: The figure shows the average percentage of intact yeast cells after a operating time of 2.5, 5.0, 7.5, 10, 12.5 and 15 minutes on the Microfluidizer™ LM20. The Microfluidizer™ LM20 was run at 30 000 psi in loop mode disrupting yeast cells in a 30 mL 10% (w/V) slurry. *A.adeninivorans* was only disrupted for 10 minutes.

As shown in Figure 4.1 95% of the cells of *C.utilis* were disrupted after a operating time of 15

minutes on the Microfluidizer^{TM} LM20, and *W.anomalus* got close to 95 % cell disruption after 15 minutes. But it is also clear that a operating time of 10 minutes was not enough to disrupt 95% of the cells of *A.adeninivorans*.

During initial experiments for preparations of YCW, a operating time of 20 and 30 minutes was used for disrupting yeast cells using Microfluidizer^{TM} LM20. When counting intact cells using microscopy before and after the 20 or 30 minutes, the results showed that this operating time was not enough to obtain 95 % cell lysis. Therefore, for preparation of YCW the operation time of the Microfluidizer^{TM} LM20 was set to 45 minutes, to ensure 95% cell disruption. For all of the YCW preparations made for further composition analyse, 95 % cell lysis was achieved.

To isolate the YCW, the slurries collected after each run on the Microfluidizer^{TM} LM20 were centrifuged, and separated into two fractions, a pellet and a supernatant. It was expected that the disruption of the yeast cell by the microfluidizer^{TM} LM20 would release intracellular proteins from the cells, and that the majority of these proteins would end up in the supernatant, while the YCW would precipitate into the pellet, and in this way creating a protein-rich supernatant and a carbohydrate-rich cell wall fraction. In further results and discussion when mentioning pellet or supernatant, it is these two fractions that is referred to.

For the batch fermentations samples of *C.utilis*, *W.anomalus* and *A.adeninivorans* grown with YPD used for initial experiments, the protein contents in the supernatants were measured using the Bradford method as described in section 3.2.5. The measured protein contents are shown in Table 4.1. *C.utilis* had the highest protein content in the supernatant. 13.1 % (w/w) of the intact dry yeast was estimated to be protein in the supernatant. The supernatant from *A.adeninivorans* had the lowest protein content with just 0.8 % (w/w) of the dry intact yeast.

Table 4.1: The table shows the total protein content in the supernatants prepared from 6.0 g intact *C.utilis*, *W.anomalus* and *A.adeninivorans* in initial experiments. The supernatants were collected from the centrifugation step after the disruption on the Microfluidizer^{TM} LM20. The protein content was found by the Bradford method performed as described in section 3.2.5

Yeast strain	Total volume of the supernatant (mL)	Total protein content in supernatant (mg)	% (w/w) of dry intact yeast
C.utilis	247	758	13.1
W.anomalus	585	187	3.1
A.adeninivorans	258	50	0.8

The pellets and supernatants were freeze dried and the weights of the dried material were measured. A summary of the weights for all samples is given in Table 4.2. The pellets were measured to be about 25-50% (w/w) of the dry mass of the intact yeast.

From Table 4.2 it can also be seen that some dry material is lost during the disruption and drying process. The loss in dry material vary between 0 for *C.utilis* from batch fermentation cultivated on chicken meat hydrolysate and BALI sugar,and 75.7% (w/w) for *C.utilis* from batch fermentation cultivated on YPD. Most samples lie in the range 18-28% (w/w) dry material loss.

Table 4.2: The table shows the amount in grams disrupted by the Microfluidizer$^{TM\ LM20}$ and the weights recorded for the pellets and supernatant after freeze dried, and also the %(w/w) loss of dry material.

Yeast strain	Fermentation	Growth medium	Amount disrupted (g)	Sample	Weight freeze dried (g)	Total weight freeze dried (g)	Dry material lost (%)
A.adeninivorans	Repeated fed batch 20 L	Chicken hydrolysate and BALI sugar	6.0	Pellet	2.57	4.89	18.5
				Supernatant	2.32		
	Batch	Chicken hydrolysate and BALI sugar	6.0	Pellet	2.32	4,33	27.9
				Supernatant	2.01		
	Batch	YPD	4.0	Pellet	0.98	1.84	54.0
				Supernatant	0.86		
C.utilis	Repeated fed batch 1.5 L	Chicken hydrolysate and BALI sugar	6.0	Pellet	3.07	4.77	20.5
				Supernatant	1.70		
	Batch	Chicken hydrolysate and BALI sugar	6.0	Pellet	3.00	6.04	0
				Supernatant	3.04		
	Batch	YPD	5.0	Pellet	1.12	1.22	75.7
				Supernatant	0.10		
W.anomalus	Repeated fed batch 1.5 L	Chicken hydrolysate and BALI sugar	6.0	Pellet	1.52	3.38	43.6
				Supernatant	1.86		
	Batch	Chicken hydrolysate and BALI sugar	6.0	Pellet 1	2.08	4.64	22.7
				Supernatant 1	2.55		
	Batch	Chicken hydrolysate and BALI sugar	6.0	Pellet 2	3.69	4.79	20.3
				Supernatant 2	1.10		
	Batch	Chicken hydrolysate and BALI sugar	6.0	Pellet 3	1.89	2.87	52.1
				Supernatant 3	0.98		
	Batch	YPD	6.0	Pellet	3.25	4.36	27.3
				Supernatant	1.11		

In addition to use the freeze dryer to obtain dry material, a selection of the samples were dried at 105°C for 20 hours after the freeze dryer. The weight of the material was recorded before and after the second drying process, to measure the water content in the samples after the freeze dryer. These results are given in Table 4.3. The result shows that the supernatant from the replicate 3 of *W.anomalus* produced on chicken meat hydrolysate and BALI sugar has the highest average water loss of 8.13 ±0.723% (w/w). For the other samples tested the average water loss vary between 1.87% (w/w) to 4.84%(w/w).

Table 4.3: The table shows the average water loss ± standard deviation recorded after a selection of the samples were dried at 105 °C for 20 hours after the freeze dryer.

Strain	Growth medium	Sample	Water loss (% (w/w))
A.adeninivorans	Chicken hydrolysate and BALI sugar	Pellet	1.87 ± 0.100
C.utilis	Chicken hydrolysate and BALI sugar	Pellet	3.60 ± 0.010
		Supernatant	2.83 ± 0.384
W.anomalus	Chicken hydrolysate and BALI sugar	Pellet 1	2.77
		Supernatant 3	8.13 ± 0.723
	YPD	Supernatant	4.8428 ± 0.3266

4.2 Acid hydrolysis using H_2SO_4

To determine the yield from acid hydrolysis with sulfuric acid H_2SO_4, relevant carbohydrate standards, mimicking the components in YCW, were hydrolyzed as described in section 3.2.7.

As shown in Figure 4.2 α-chitin had the lowest yield after acid hydrolysis with H_2SO_4, with a yield of approximately 70%. β-glucan had also a lower yield, just under 80%. For all the other carbohydrate standards, the yield were over 90%.

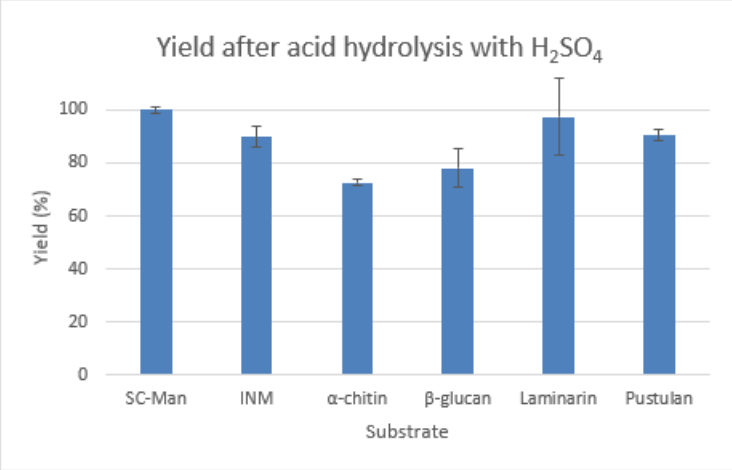

Figure 4.2: The figure shows the hydrolysis yield, i.e. the ability to break down a polysaccharide chain to monomer units of α-mannan purified form *S. cerevisiae*, Ivory nut Mannan, α-chitin, β-glucan, laminarin and pustulan. The standards were hydrolyzed with mild acid hydrolysis using 4% H_2SO_4 (incubated at 121°C for 1 hour). DNS-analysis was performed and the resulting GE concentrations were adjusted with sample volume and the correct polymer correction factor before the yields were calculated.

To test whether the pre-treatment with 72% H_2SO_4 was necessary when hydrolysing yeast pellet, hydrolysis of the preliminary pellets from *C.utilis* and *W.anomalus* with and without this pre-treatment was performed. The results from the DNS-analysis performed to analyze these results are shown in figure 4.3. The result shows that the the pellets that were first treated with 72% H_2SO_4 had higher GE values compared to the samples that were not treated with the strong acid before hydrolysis with 4% H_2SO_4.

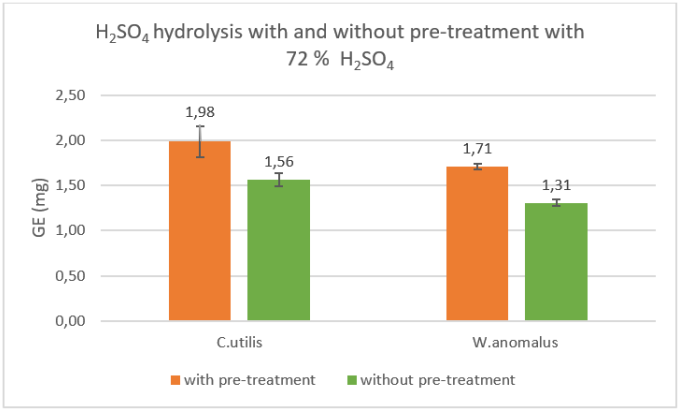

Figure 4.3: The figure shows the average amount of GE found by DNS-analysis after acid hydrolysis with 4% H_2SO_4 (autoclaved at 121°C for 1 hour) of pellets from *C.utils* and *W.anomalus*, with and without pre-treatment with 72% H_2SO_4 at 30°C for 1 hour. The resulting GE concentrations were adjusted with the sample volume and polymer correction factor.

The freeze dried pellet and supernatants were hydrolyzed using H_2SO_4 as described in section 3.2.7. After acid hydrolysis, a monosaccharide analysis was performed using HPAEC-PAD, as described in section 3.2.22. The monosaccharides released during acid hydrolysis were identified based on corresponding retention time to respective standards. The determined values were adjusted as described in section 3.2.7 and calculated with the formula given in Appendix A.1. The micro-Kjeldahl method (section 3.2.6) was used to measure the protein concentration in these samples. A summary of the carbohydrate composition determined, as well as the protein concentration is given in Table 4.4, 4.5 and 4.6. These tables show the composition after fermentation and growth medium. The result (not shown) from the ion exclusion chromatog-

raphy (section 3.2.24) showed no peaks corresponding to GlcNAc, indicating that ribose was the monosaccharide detected when using HPAEC-PAD.

Table 4.4 shows the measured concentration of carbohydrate and protein in the pellets and supernatants of the yeast from repeated batch fermentation with chicken protein and BALI sugar as growth medium. As shown in Table 4.4, the protein content varied between about 30 and 50% depending on the yeast strain and whether the sample was pellet or supernatant. *A.adeninivorans* stood out, with a bigger difference in protein concentration between pellet and supernatant. Also in carbohydrate composition *A.adeninivorans* was different from *C.utilis* and *W.anomalus*. Galactose was detected in *A.adeninivorans*, but not in *C.utilis* and *W.anomalus*. *A.adeninivorans* also had a higher chitin content compared to the two others. Ribose was detected in both pellet and supernatant, while in *C.utilis* and *W.anomalus*, ribose were only detected in the supernatant. The supernatant of *C.utilis* from this repeated batch fermentation stood out in the way that this was the only sample, from all analyzed samples, where xylose was detected.

Table 4.5 shows the protein content and carbohydrate composition measured for *C.utilis*, *W.anolamus* and *A.adeninivorans* from batch fermentation with YPD as growth medium. The protein content varied between 8.7 and 64.8% among the different samples and yeast strain. Generally, the protein content was higher in supernatants. Galactose was detected in *A.adeninivorans*, both pellet and supernatant, but not in*C.utilis*, *W.anolamus*. *A.adeninivorans* had lower values for β-glucan and mannan compared with the two other yeast strains, but more chitin. The pellet from *C.utilis* was the only sample in which ribose was not detected.

The carbohydrate composition of the yeast strains from batch fermentation cultivated on chicken meat hydrolysate and BALI sugar are given in Table 4.6. The protein concentration for the different samples of *C.utilis*, *W.anomalus* and *A.adeninivorans* was between 20.7 and 52.7%. *A.adeninivorans* was the only strain in which galactose and ribose were detected in both the pellet and supernatant. It was also the strain with the highest percentage of chitin of the three. There was variation between the three replicates of *W.anomalus* in both protein concentration and carbohydrate composition. The first replicate had lower values for the carbohydrates, but higher protein concentrations.

Figures 4.4, 4.5 and 4.6 gives a visualization of the composition of the different yeast strains based on fermentation and growth mediums. The figures is made with the values given in Table 4.4, 4.5 and 4.6.

Table 4.4: The table gives a summary of carbohydrate and protein content in the pellets and supernatants from *C.utilis*, *W.anolamus* and *A.adeninivorans* from repeated batch fermentation cultivated on chicken meat hydrolysate and BALI sugar. Triplicates of pellet or supernatant were incubated with 72% H_2SO_4 at 30°C for 1 hour, before the samples were diluted to a final concentration of 4% H_2SO_4 and autoclaved at 121°C for 1 hour. The carbohydrate contents in the hydrolyzed samples were found using HPAEC-PAD. The protein content in a duplicate was found using micro-Kjeldahl. The results are presented as average percentage (w/w) of dry mass (DM) ± standard deviation. Sample refers to whether the content is measured in the pellet or supernatant, originating from the centrifugation step after cell disruption by Microfluidizer™ LM20

Repeated batch fermentation Chi&BALI								
	C.utilis							
	Sample	Protein content (% of DM)	Polymeric galctose(% of DM)	β-glucan (% of DM)	Mannan (%DM)	Chitin (% of DM)	Polymeric xylose (% of DM)	Polymeric ribose (% of DM)
	Pellet	41.4 ± 1.08	0 ± 0	38.03 ± 6.98	14.7 ± 3.32	0.4 ± 0.26	0 ± 0	0 ± 0
	Supernatant	42.9 ± 0.28	0 ± 0	21.7 ± 1.00	11.7 ± 0.98	0 ± 0	0.6 ± 0.12	0.6 ± 0.12
	W.anomalus							
	Sample	Protein content (% of DM)	Polymeric galactose (% of DM)	β-glucan (% of DM)	Mannan (%DM)	Chitin (% of DM)	Polymeric xylan (% of DM)	Polymeric ribose (% of DM)
	Pellet	42.6 ± 0.83	0 ± 0	26.8 ± 2.74	15.9 ± 1.85	0.3 ± 0.10	0 ± 0	0 ± 0
	Supernatant	46.8 ± 0.72	0 ± 0	1.4 ± 0.45	9.0 ± 0.40	0 ± 0	0 ± 0	0.4 ± 0.18
	A.adeninivorans							
	Sample	Protein content (% of DM)	Polymeric galactose (% of DM)	β-glucan (% of DM)	Mannan (%DM)	Chitin (% of DM)	Polymeric xylose (% of DM)	Polymeric ribose (% of DM)
	Pellet	29.6 ± 0.17	5.6 ± 0.70	25,8 ± 3.89	18.0 ± 2.12	7.19 ± 1.19	0 ± 0	0.9 ± 0.25
	Supernatant	50.8 ± 0.51	1.1 ± 0.17	1.4 ± 0.55	8.8 ± 1.82	0 ± 0	0 ± 0	0.3 ± 0.03

Table 4.5: The table gives a summary of carbohydrate and protein content in the pellets and supernatants from *C.utilis*, *W.anolamus* and *A.adeninivorans* from batch fermentation cultivated on YPD. Triplicates of pellet or supernatant were incubated with 72% H_2SO_4 at 30°C for 1 hour, before the samples were diluted to a final concentration of 4% H_2SO_4 and autoclaved at 121°C for 1 hour. The carbohydrate contents in the hydrolyzed samples were found using HPAEC-PAD. The protein content in a duplicate was found using micro-Kjeldahl. The results are presented as average percentage (w/w) of dry mass (DM) ± standard deviation. Sample refers to whether the content is measured in the pellet or supernatant, originating from the centrifugation step after cell disruption by Microfluidizer™ LM20

Batch fermentation YPD								
	C.utilis							
	Sample	Protein content (% of DM)	Polymeric galactose (% of DM)	β-glucan (% of DM)	Mannan (%DM)	Chitin (% of DM)	Polymeric xylose (% of DM)	Polymeris ribose (% of DM)
	Pellet	8.7*	0 ± 0	76.6 ± 5.31	40.8 ± 1.83	1.5 ± 0.13	0 ± 0	0± 0
	Supernatant	63.4 ± 0.20	0 ± 0	26.6 ± 3.89	11.8 ± 1.97	0 ± 0	0 ± 0	0.9 ± 0.20
	W.anomalus							
	Sample	Protein content (% of DM)	Polymeric galactose (% of DM)	β-glucan (% of DM)	Mannan (%DM)	Chitin (% of DM)	Polymeric xylose (% of DM)	Polymeric ribose (% of DM)
	Pellet	18.6 ± 0.37	0±0	56.8 ± 0.90	35.9 ± 1.36	2.3 ± 0.16	0 ± 0	0 ± 0.0
	Supernatant	64.8 ± 0.20	0 ± 0	29.1 ± 2.28	12.9 ± 1.34	0 ± 0	0 ± 0	0.7 ± 1.97
	A.adeninivorans							
	Sample	Protein content (% of DM)	Polymeric galactose (% of DM)	β-glucan (% of DM)	Mannan (%DM)	Chitin (% of DM)	Polymeric xylose (% of DM)	Polymer ribose (% of DM)
	Pellet	35.7 ± 0.49	3.1 ± 0.48	24.1 ± 2.10	14.4 ± 2.20	6.9 ± 1.23	0 ± 0	1.1 ± 0.52
	Supernatant	58.1 ± 0.12	1.7 ± 0.19	17.5 ± 2.42	10.9 ± 1.63	0 ± 0	0 ± 0	0.7 ± 0.21

Table 4.6: The table gives a summary of carbohydrate and protein content in the pellets and supernatants from *C.utilis*, *W.anolamus* and *A.adeninivorans* from batch fermentation cultivated on chicken meat hydrolysate and BALI sugar. Triplicates of pellet or supernatant were incubated with 72% H_2SO_4 at 30°C for 1 hour, before the samples were diluted to a final concentration of 4% H_2SO_4 and autoclaved at 121°C for 1 hour. The carbohydrate contents in the hydrolyzed samples were found using HPAEC-PAD. The protein content in a duplicate was found using micro-Kjeldahl. The results are presented as average percentage(w/w) of dry mass (DM) ± standard deviation. Sample refers to whether the content is measured in the pellet or supernatant, originating from the centrifugation step after cell disruption by Microfluidizer™ LM20. Pellet 1,2,3 and supernatant 1,2,3 indicate which replicate of *W.anomalus* it originated form. *indicate that only one replicate was measured in the Kjeldahl analysis. For the other samples duplicates were used.

Batch fermentation Chi&Bali	*C.utilis*							
	Sample	Protein content (% of DM)	Polymeric galactose (% of DM)	β-glucan (% of DM)	Mannan (% of DM)	Chitin (% of DM)	Polymeric xylose (% of DM)	Polymeric ribose (% of DM)
	Pellet	46.0 ± 0.15	0 ± 0	31.3 ± 1.90	12.1 ± 0.86	0.3 ± 0.02	0 ± 0	0 ± 0
	Supernatant	52.7 ± 0.07	0 ± 0	35.9 ± 2.68	11.9 ± 1.01	0 ± 0	0 ± 0	0.8 ± 0.17
	W.anomalus							
	Sample	Protein content (% of DM)	Polymeric galactose (% of DM)	β-glucan (% of DM)	Mannan (% of DM)	Chitin (% of DM)	Polymeric xylose (% of DM)	Polymeric ribose (% of DM)
	Pellet 1	55.8 ± 1.25	0 ± 0	5.3 ± 1,86	4.3 ± 1.15	0 ± 0	0 ± 0	0 ± 0
	Supernatant 1	45.1*	0 ± 0	38.2 ± 3.14	13.4 ± 0.81	0 ± 0	0 ± 0	0.6 ± 0.60
	Pellet 2	20.8*	0 ± 0	54.7 ± 2.83	35.6 ± 1.68	1.2 ± 0.13	0 ± 0	0 ± 0
	Supernatant 2	51.3 ± 0.03	0 ± 0	39.1 ± 2.14	14.9 ± 1.29	0 ± 0	0 ± 0	0.6 ± 0.03
	Pellet 3	27.8*	0 ± 0	45.8 ± 4.26	26.7 ± 5.34	0.9 ± 0.30	0±0	0 ±0
	Supernatant 3	49.3 ± 0.83	0 ± 0	38.5 ± 5.79	15.9 ± 2.63	0 ± 0	0± 0	0.5 ± 0.08
	A.adeninivorans							
	Sample	Protein content (% of DM)	Polymeric galactose (% of DM)	β-glucan (% of DM)	Mannan (%DM)	Chitin (% of DM)	Polymeric xylose (% of DM)	Polymeric ribose (% of DM)
	Pellet	34.4 ± 0.12	3.1 ± 0.22	21.5 ± 0.34	13.9 ± 0.33	5.6 ± 0.22	0 ± 0	1.0 ±0.03
	Supernatant	43.4 ± 3.76	1.4 ± 0.13	16.9 ± 1.36	8.0 ± 0.60	0 ± 0	0 ± 0	0.3 ± 0.05

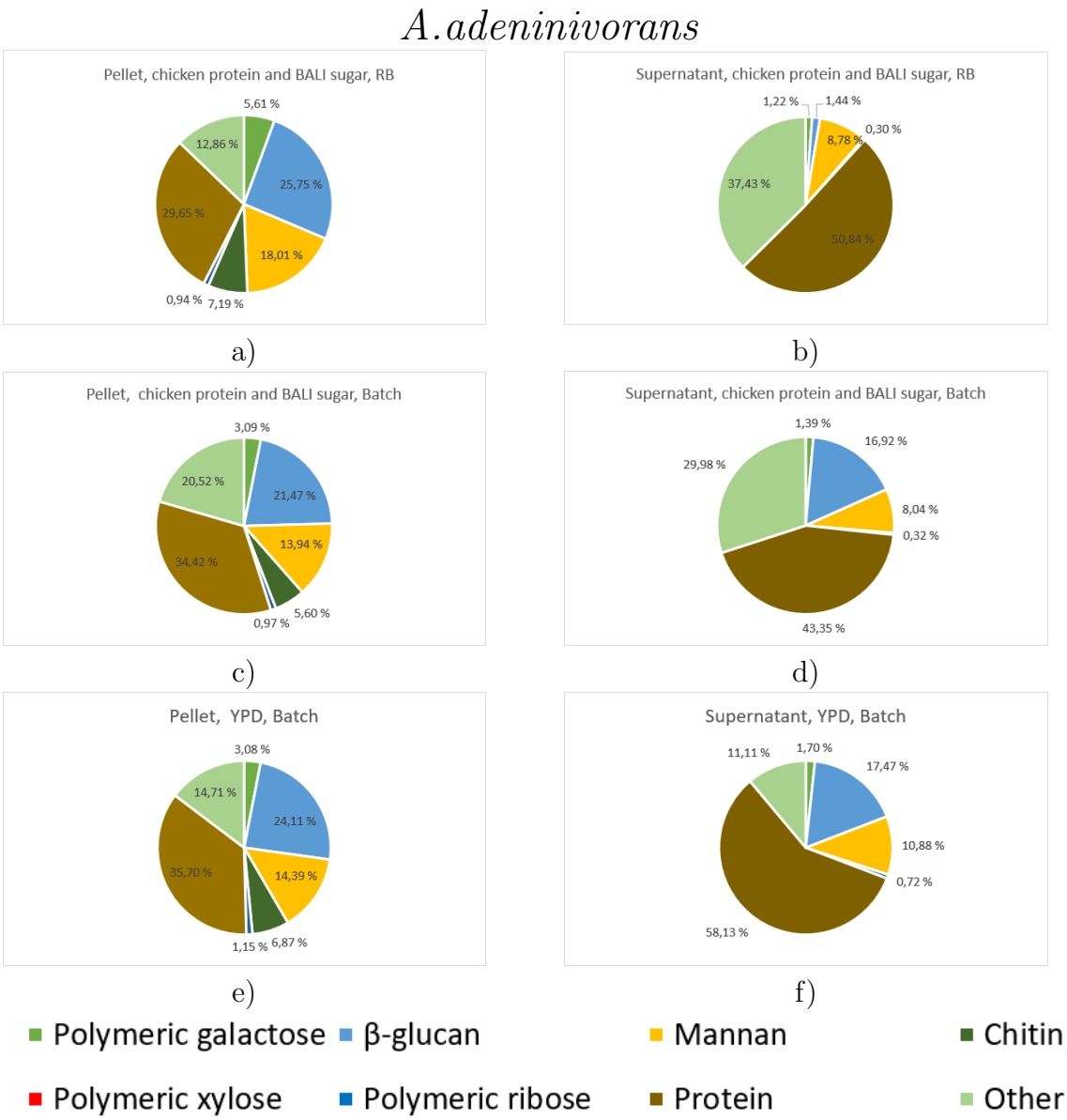

Figure 4.4: The figure shows the distribution of carbohydrates and protein in the different samples of *A.adeninivorans* as percentage of dry mass of the sample. HPAEC-PAD was used to measure the carbohydrate content and micro-Kjeldahl was used to measure the protein concentration.

Figure 4.4 shows that the protein concentration was higher in the supernatant of *A.adeninivorans* compared to the pellet for all fermentations and growth mediums. The biggest difference in protein concentration was observed when YPD was growth medium. The carbohydrate concentrations was on the other hand higher in the pellet.

Figure 4.5, show that in *C.utilis* the protein concentration was quite similar between the pellet and supernatant cultivated on chicken meat hydrolysate and BALI sugar. But when YPD was used as growth medium, the protein concentration was higher in the supernatant. β-glucan make up the most of the carbohydrate fraction of the samples, and is as high as 76.65% for the pellet from *C.utilis* fermented with YPD as growth medium.

Figure 4.6 shows the composition of *W.anomalus* that was either repeated batch fermented or batch fermented using YPD or chicken meat hyrdolysate as growth medium. The difference between the first replicate shown in Figure 4.6 e) and f), and the two other replicates can immediately be seen, especially for the pellet. The first replicate had lower concentration in β-

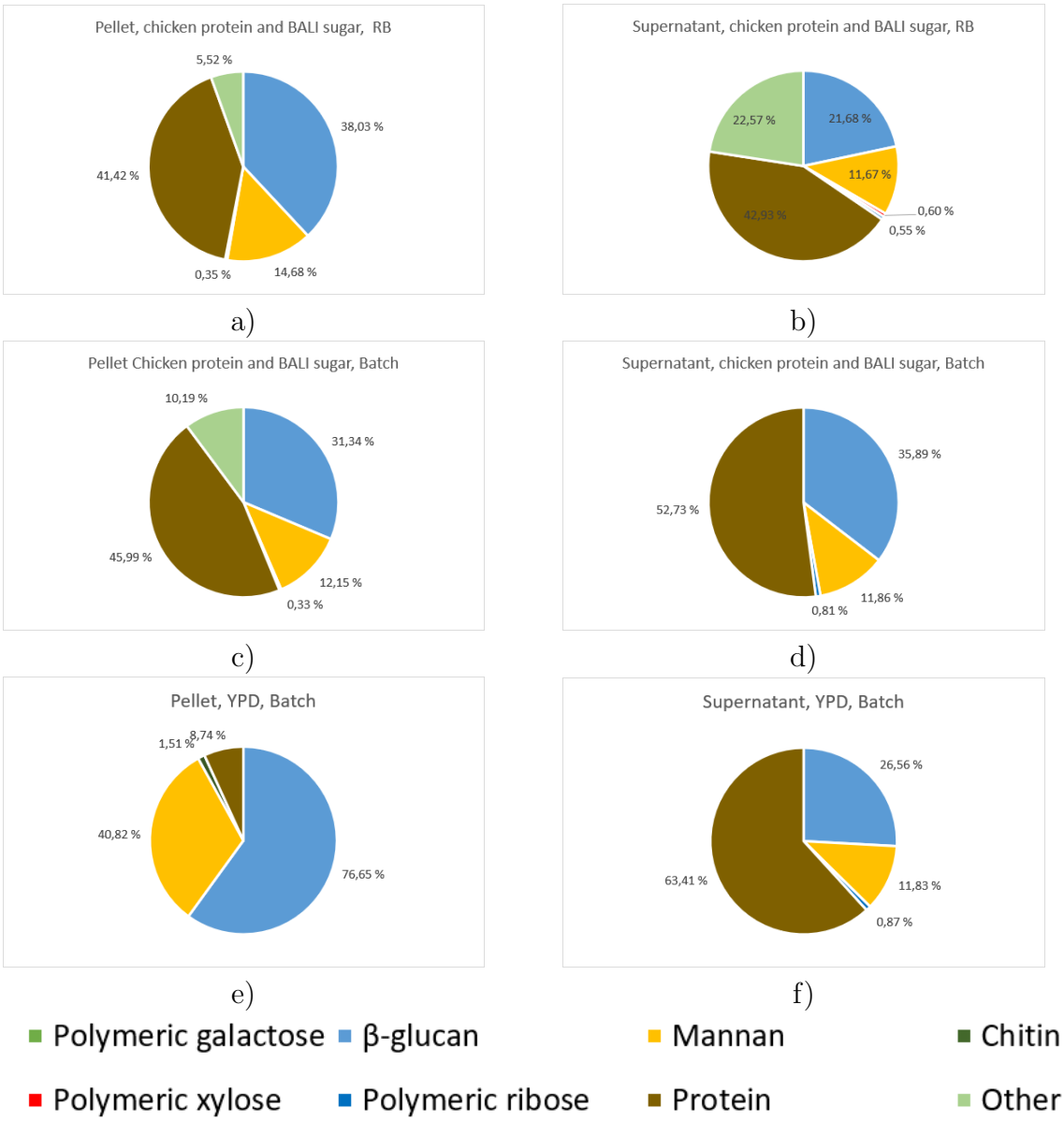

Figure 4.5: The figure shows the distribution of carbohydrates and protein in the different samples of *C.utilis* as percentage of dry mass. HPAEC-PAD was used to measure the carbohydrate content and micro-Kjeldahl was used to measure the protein concentration.

glucan and mannan and no chitin was found to be in this pellet. The protein on the other hand was higher in the pellet of the first replicate compared to the two other. For the supernatant, the composition was more similar between the triplicates, though some other components is present in the first replicate.

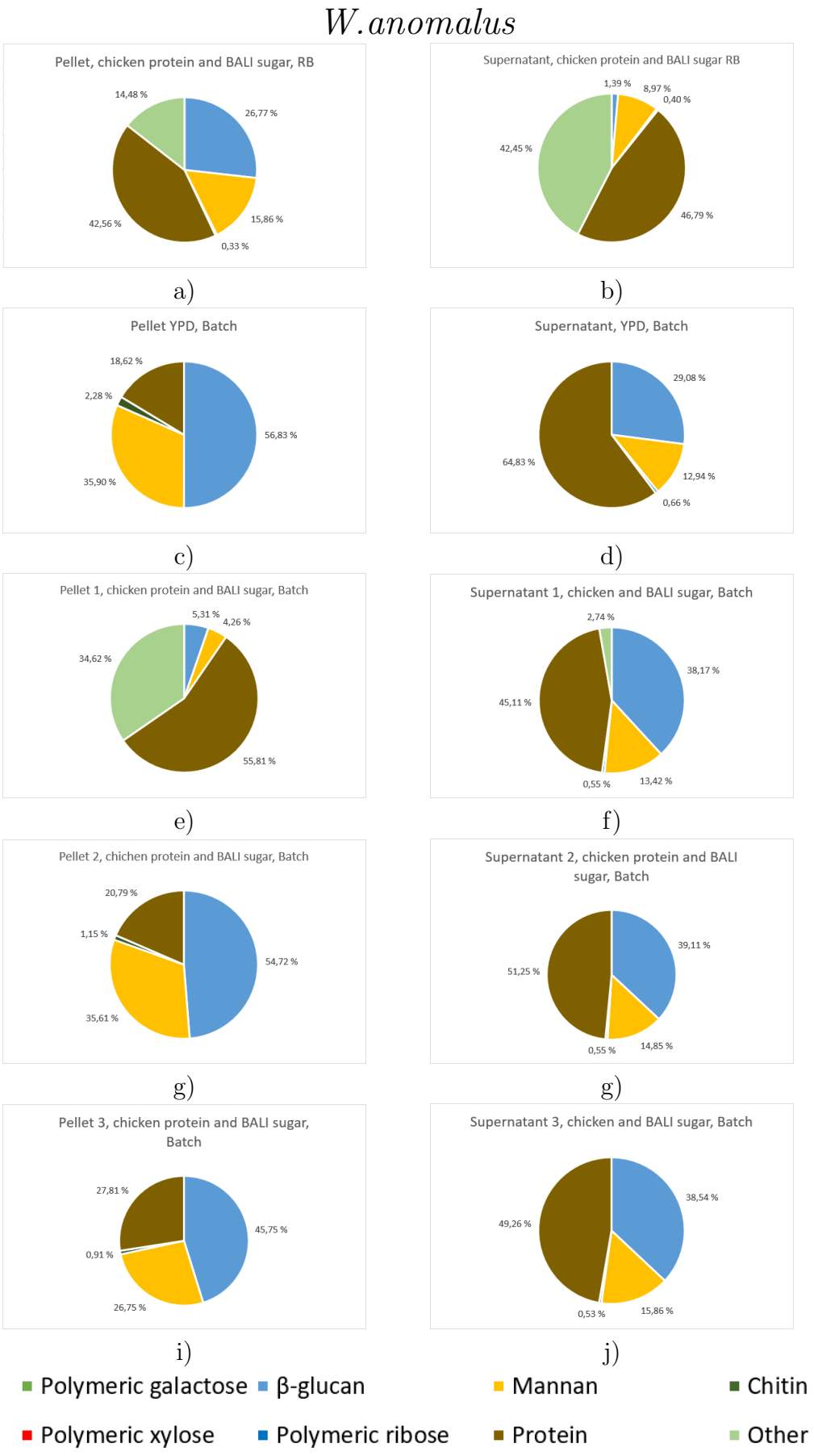

Figure 4.6: The figure shows the distribution of carbohydrates and protein in the different samples of *W.anomalus* as percentage of dry mass. Pellet 1,2,3 and Supernatant 1,2,3 indicate which replicate of *W.anomalus* it originated form

4.3 Enzymatic hydrolysis

As a preliminary experiment the activity of the endo-1,3-β-D-glucanase and the chitinase cocktail consisisting of 90% (w/w) chitinaseC and 10% (w/w) CHB were tested in both NaOAc-buffer and Tris-HCl buffer as described in section 3.2.10. The results form the DNS-analysis is shown in Figure 4.7.

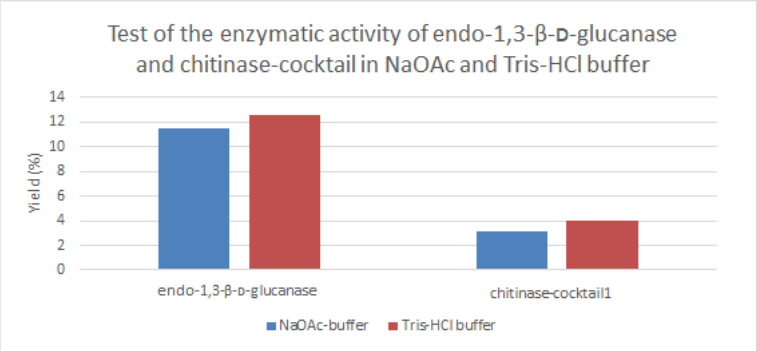

Figure 4.7: The figure shows the yield measured by DNS-analysis (see section 3.2.20) after the activity of endo-1,3-β-D-glucanase and a chitinase cocktail consisisting of 90% (w/w) chitinaseC and 10% (w/w) CHB were tested in both NaOAc-buffer pH 5 and Tris-HCl buffer pH 5. 0.5% (w/v) laminiarin was used as a substrate for endo-1,3-β-D-glucanase, while 1% (w/v)α-chitin was used as substrate for the chitinase-cocktail. The measured GE concentration was corrected with sample volume and the correct polymer correction factor before calculating the yield.

Later experiments performed with the first described chitinase-cocktail1, indicated that the activity of this cocktail was not stable. So the rest of the experiments were carried out with a new chitinase-C mixed with the same CHB (no longer a 1:9 relation).

Progression curve for this mixture with the new chitinase C and CHB, was made as described in section 3.2.12. The progression curve is given Figure 4.8.

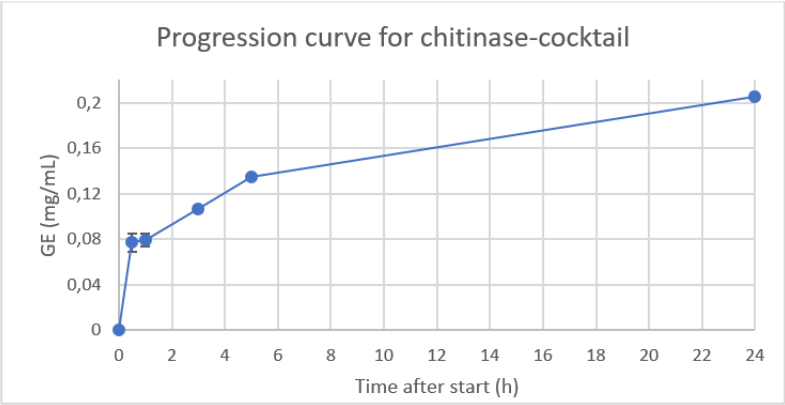

Figure 4.8: The figure shows the progression curve for the chitinase cocktail when α-chitin was used as substrate. The α-chitin and enzymes were mixed in 50 mM Tris-HCl buffer at pH 5 and incubated at 40°C. Samples were withdrawn 30 minutes, 1 hour, 3 hours, 5 hours and 24 hours after reaction start. By DNS-analysis(see section 3.2.20, the concentration of glucose equivalents (GE) was measured. The GE concentration was plotted against the respective time of withdraw to plot the progression curve.

The progression curve for endo-1,3-β-D-glucanase were made as described in 3.2.11 and is showed in Figure 4.9.

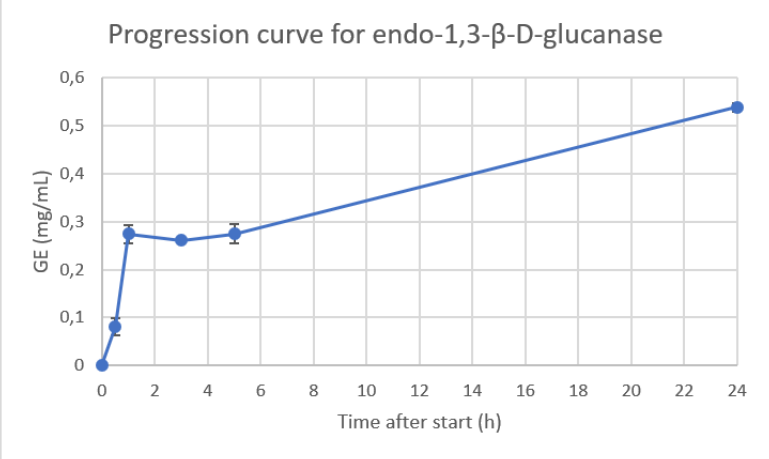

Figure 4.9: The figure shows the progression curve for endo-1,3-β-D-glucanase when curdlan was used as substrate. The substrate and enzymes were mixed in 50 mM Tris-HCl buffer at pH 5 and incubated at 40°C. Samples were withdrawn 30 minutes, 1 hour, 3 hours, 5 hours and 24 hours after reaction start. By DNS-analysis(see section 3.2.20, the concentration of glucose equivalents (GE) was measured. The GE concentration was plotted against the respective time of withdraw.

The specificity of endo-1,3-β-D-glucanase was also tested using pustulan with β-1,6 linkages in the glucose chain. The chromatogram in Figure 4.10 shows that no difference was detected between the sample where pustulan was hydrolyzed using endo-1,3-β-D-glucanase and the control sample.

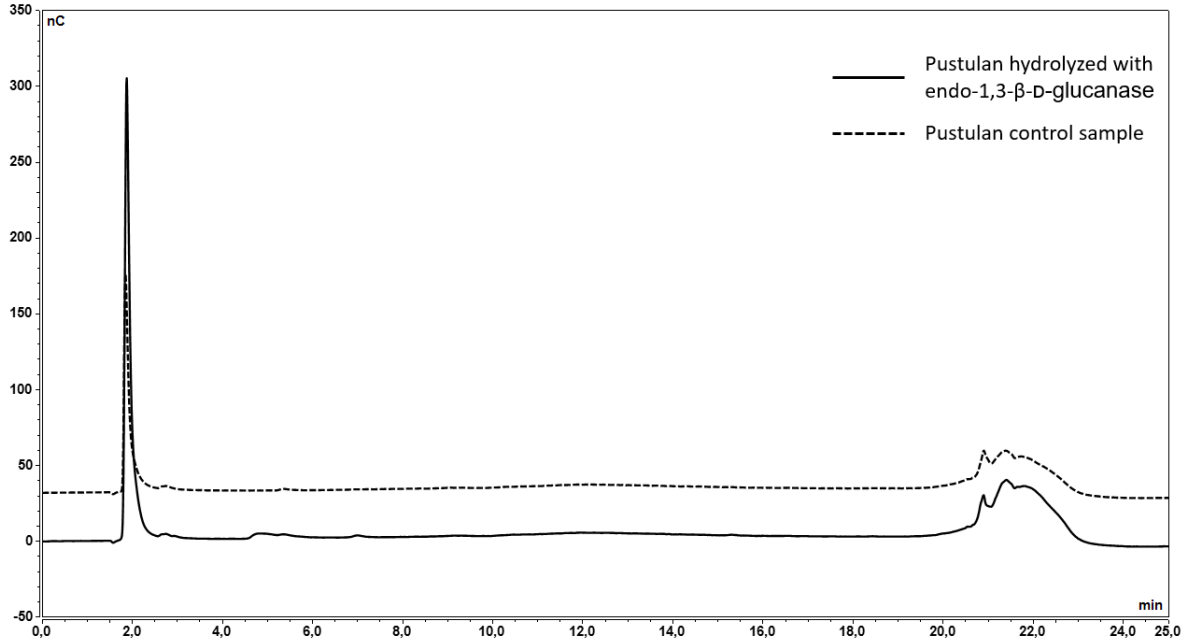

Figure 4.10: The figure shows the resulting chromatogram form oligosaccharide analysis using HPAEC-PAD, after samples with pustulan were hydrolyzed by endo-1,3-β-D-glucanase in 50 mM NaOAc buffer at pH 5 and incubated at 40°C.

The mass spectra in Figure 4.11 show the resulting spectra after laminarin and pustulan were hydrolyzed using endo-1,3-β-D-glucanase. As shown in the spectra peaks with m/z values indicating different oligosaccarides of glucose, like m/z 1013, 851 and 527, was present in mass spectrum showing the result from the hydrolysis of laminarin by endo-1,3-β-D-glucanase. But when pustulan was hydrolyzed using endo-1,3-β-D-glucanase none of these peaks were present, as shown in the last spectrum in Figure 4.11.

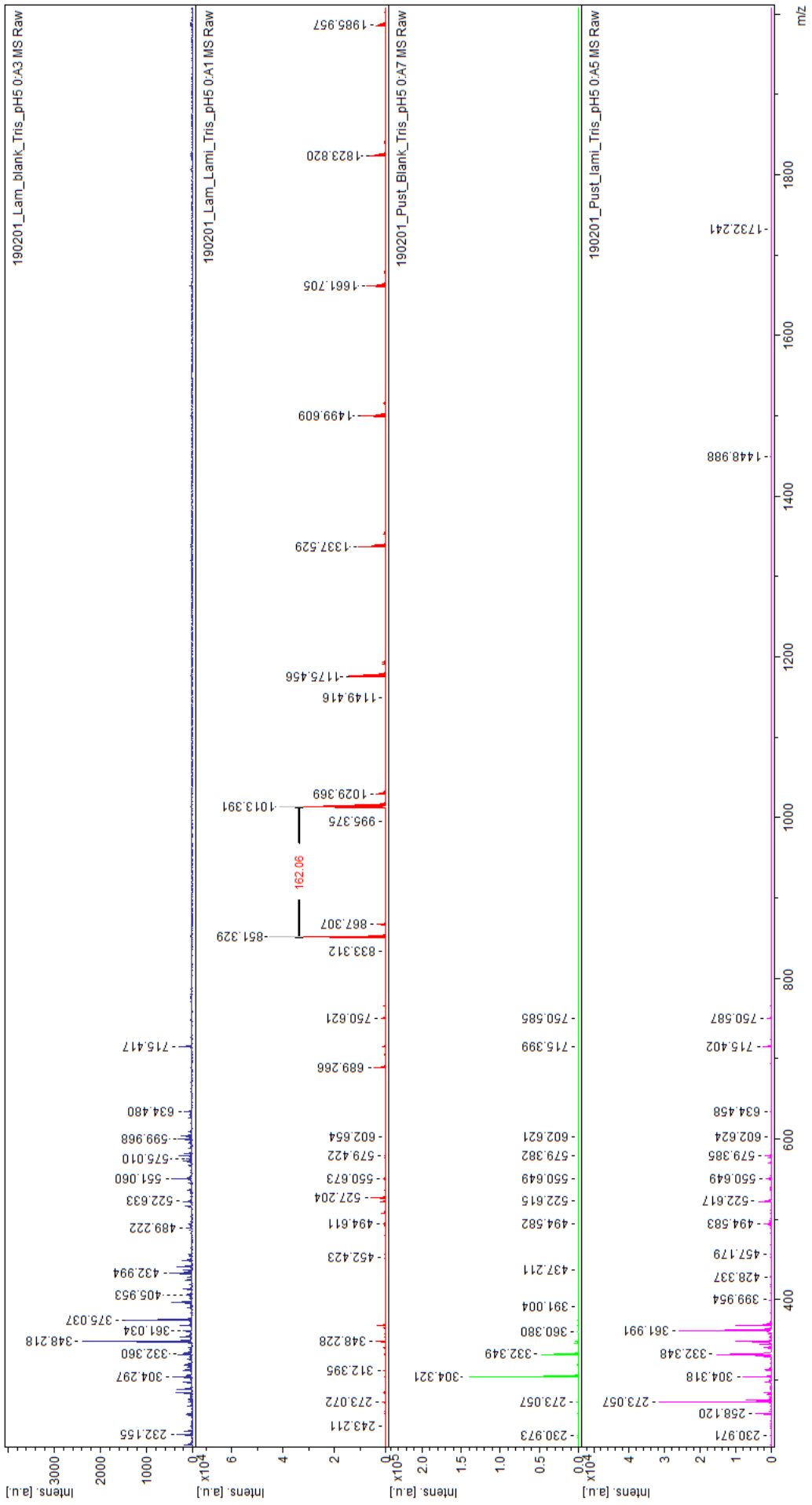

Figure 4.11: The figures show the mass spectra from the analysis using MALDI-TOF after laminarin and pustulan were hydrolyzed by endo-1,3-β-D-glucanase. The first mass spectrum shows the control sample of laminarin where no endo-1,3-β-D-glucanase was added. The second shows the result when laminarin was hydrolyzed by the enzyme. The third mass spectra shows the control sample of pustulan and the last mass spectrum shows the spectrum resulting from the hydrolysis of pustulan with endo-1,3-β-D

To test the specificity of exo-1,3-β-D-glucanase, pustulan was hydrolyzed with exo-1,3-β-D-glucanase. The results from this experiments are shown in the chromatogram in Figure 4.12. The figure shows that no difference was detected between the sample where pustulan was hydrolyzed using exo-1,3-β-D-glucanase and the control sample.

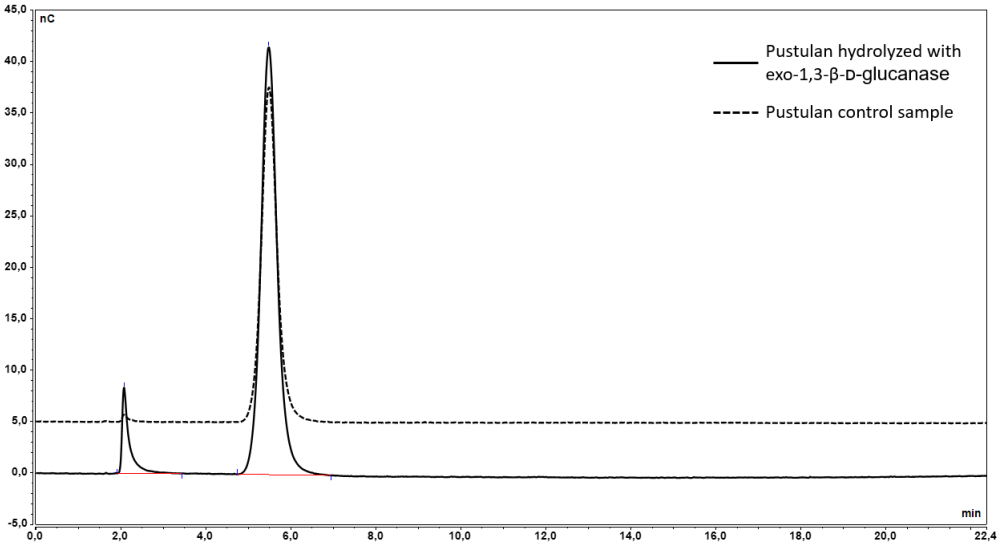

Figure 4.12: The figure shows the resulting chromatogram from the monosaccharide analysis by HPAEC-PAD analysis on the samples made to test the specificity of exo-1,3-β-D-glucanase. Pustulan were hydrolyzed by exo-1,3-β-D-glucanase in 50 mM NaOAc buffer at pH 5 and incubated at 40°C. The peak around 6 minutes, it the internal standard (0.5 mg/mL fucose).

A mix of exo-1,3-β-D-glucanase and endo-1,3-β-D-glucanase was used to hydrolyze curdlan as describe in section 3.2.15. As described in this section, endo-1,3-β-D-glucanase was also used alone to hydrolyze curdlan. The result from this experiment is presented through the chromatograms in Figure 4.13 and the mass spectra in Figure 4.14 and Figure 4.15.

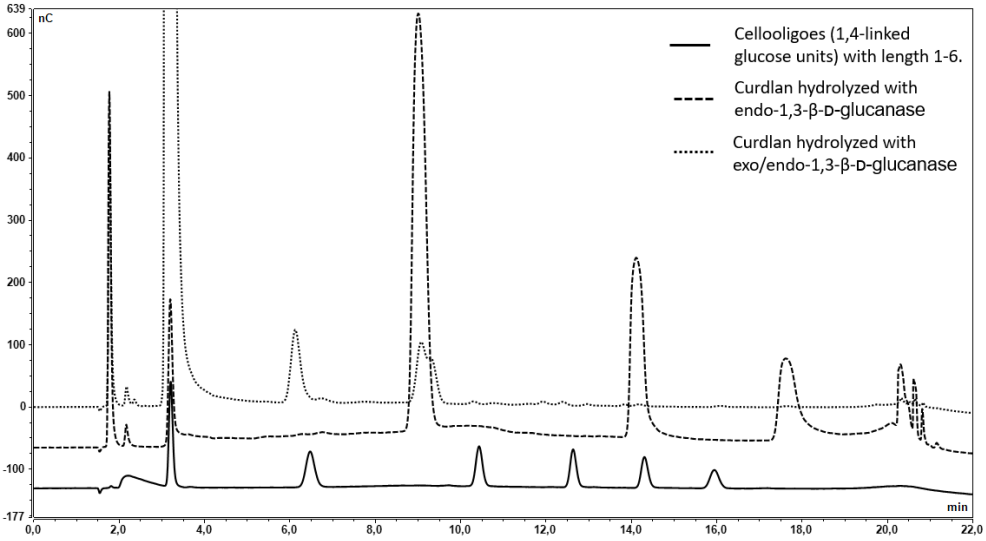

Figure 4.13: The figure shows the chromatograms after HPAEC-PAD was used to analyse the samples where a mix of exo-1,3-β-D-glucanase and endo-1,3-β-D-glucanase and endo-1,3-β-D-glucanase alone were used to hydrolyze curdlan. The standard used were cellooligoes (1,4-linked glucose units), while the samples analyzed had β-1,3 linkages eluting differently, so the standard were not suitable for indicating retention times, except for the monosaccharide.

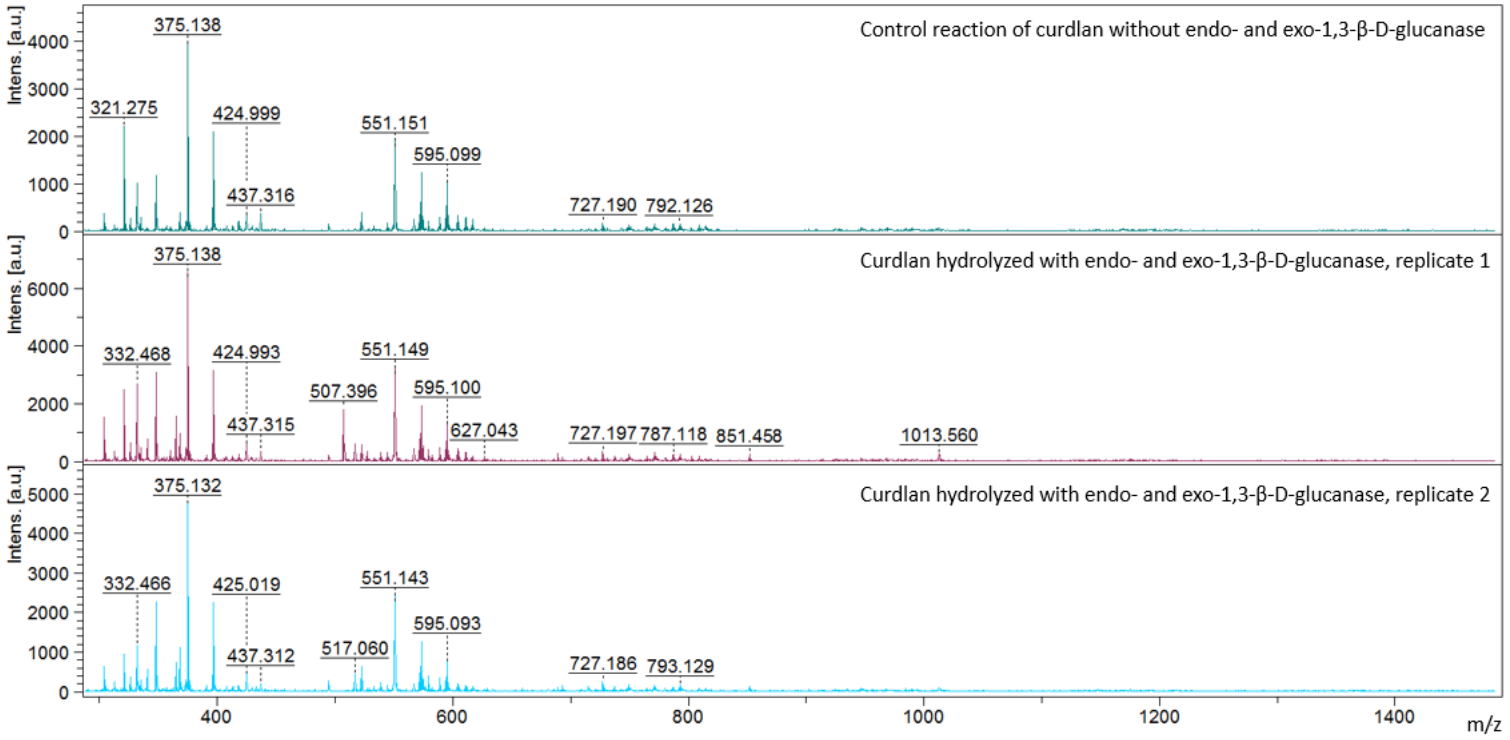

Figure 4.14: The figure shows the mass spectra after MALDI-TOF/TOF was used to analyse the samples where a mix of exo-1,3-β-D-glucanase and endo-1,3-β-D-glucanase was used to hydrolyze curdlan. The first mass spectrum is the result from the control of curdlan without exo-1,3-β-D-glucanase and endo-1,3-β-D-glucanase. The two last mass spectra show the results from when the two glucanses were used in mix.

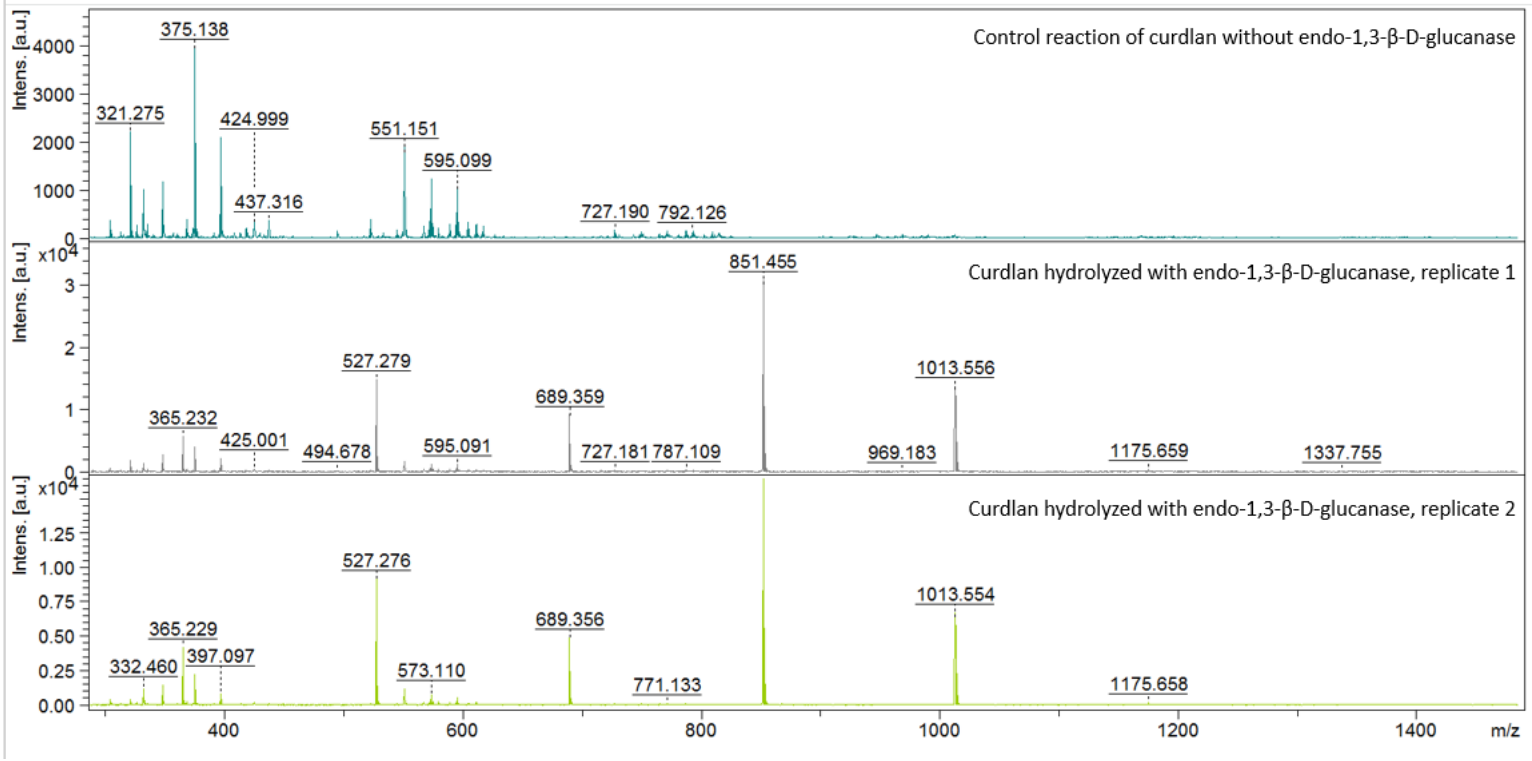

Figure 4.15: The figure shows the mass spectra after MALDI-TOF/TOF was used to analyse the samples endo-1,3-β-D-glucanase was used to hydrolyze curdlan. The first mass spectrum is the result from the control of curdlan without endo-1,3-β-D-glucanase. The two last mass spectra show the results from when curdlan is hydrolysed with endo-1,3-β-D-glucanase.

As shown in figure 4.13, a minor peak for glucose, and most likely peaks corresponding to the dimer, trimer and tetramer was observed, when curdlan was hydrolyzed with only endo-1,3-β-D-glucanase. When curdlan was hydrolyzed with a mixture of exo-1,3-β-D-glucanase and endo-1,3-β-D-glucanase one peak for glucose dominates.

The sepctra in Figure 4.15 shows that peaks with m/z values indicating different oligosaccarides of glucose, like m/z 1013, 851 and 527, was present in mass spectra showing the result from the hydrolysis of curdlan by endo-1,3-β-D-glucanase. But when curdlan was hydrolysed using both exo-1,3-β-D-glucanase and endo-1,3-β-D-glucanase none of these peaks are present, as shown in spectra in Figure 4.14.

The results from the preliminary experiment of testing the enzyme loading needed of α-mannanase and endo-α-mannosidase to hydrolyse SC-Man and INM described in section 3.2.13 are shown in Figures 4.16 and 4.17, for SC-Man and INM, respectively.

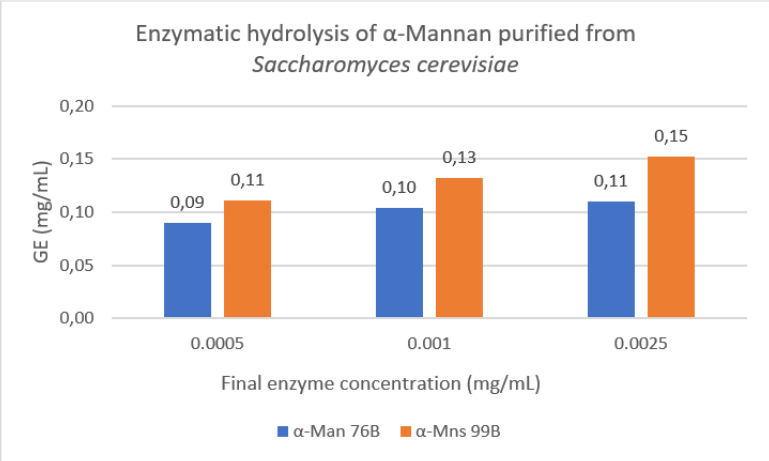

Figure 4.16: The figure show the result from the preliminary experiment testing the enzyme concentration of α-mannanase (α-Man 76B) and α-mannosidase (α-Mns 99B) needed to hydrolyze α-mannan purified form *S.cerevisiae*. 1% (w/v) SC-Man was incubated at 37°C in 50 mM NaOAc buffer pH 7 for 5 hours.

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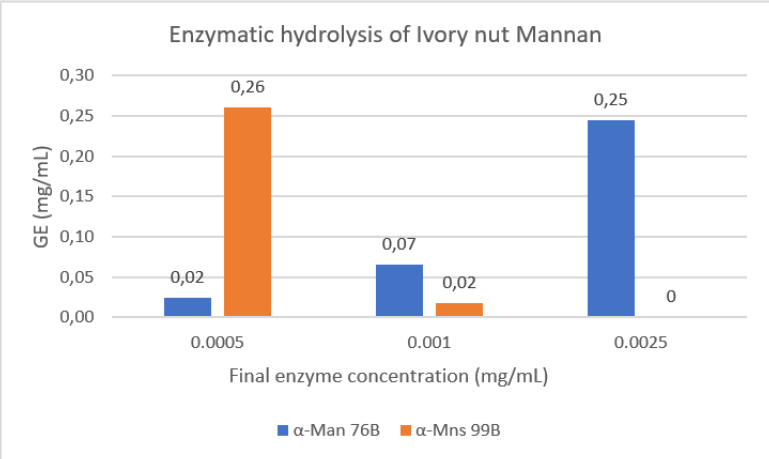


Figure 4.17: The figure show the result from the preliminary experiment where three enzyme concentration of α-mannanase (α-Man 76B) and α-mannosidase (α-Mns 99B) were used to hydrolyze α-mannan from Ivory nut (INM). 1% (w/v) INM was incubated at 37°C in 50 mM NaOAc buffer pH 7 for 5 hours.

Figure 4.16 shows that the highest enzyme concentration released most monosaccharides from SC-Man for both enzymes. Figure 4.17 shows opposite results for INM. Here α-mannosidase released most monosaccharides from INM at low enzyme concentration, but released less monosaccharides when using a higher enzyme concentration. α-mannanase showed hydrolytic activity on INM at the highest enzyme loading tested.

The samples from the experiment where SC-Man were hydrolyzed using α-mannanase and α-mannosidase both separately and in mix, were analyzed using oligosaccharide analysis. The resulting chromatograms showed no difference between the control samples of SC-Man and the samples where SC-Man where hydrolyzed with α-mannanase and α-mannosidase (result not shown).

The result from the experiment where α1,2-mannobiose was hydrolyzed with α-mannosidase, are shown in the chromatograms presented in Figure 4.18. The peak at around 5 minutes was assumed to be α1,2-mannobiose. As shown no difference was observed regarding this peak.

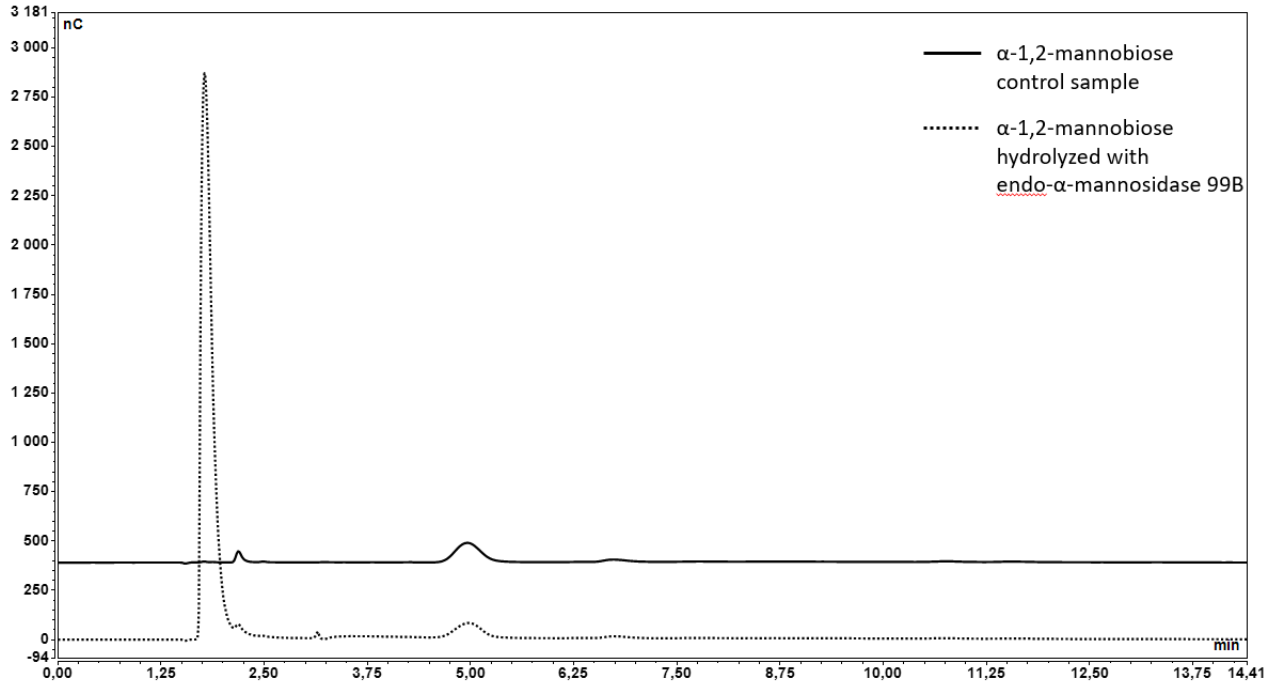


Figure 4.18: The figure shows the chromatograms from the HPAEC-analysis done after α1,2-mannobiose hydrolyzed with α-mannosidase 99B in 50 mM NaOAc-buffer pH 7 for 24 hours.

In section 3.2.16 it is described how the activity of the four enzymes chitinaseC, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase together were tested on the pellets prepared from *C.utilis*, *W.anomalus* and *A.adeninivorans*. The results are shown in Figures 4.19. No chitin was determined to be in the pellet from *C.utilis* and *W.anomalus*, whereas 0.9% (w/v) of the dry pellet was determined to be chitin in *A.adeninivorans*. The determined β-1,3-glucan content varied between 0.56 and 3.32% (w/w)of the dry pellets from the three yeast strains. A big standard deviation was observed for the average β-1,3-glucan content determined.

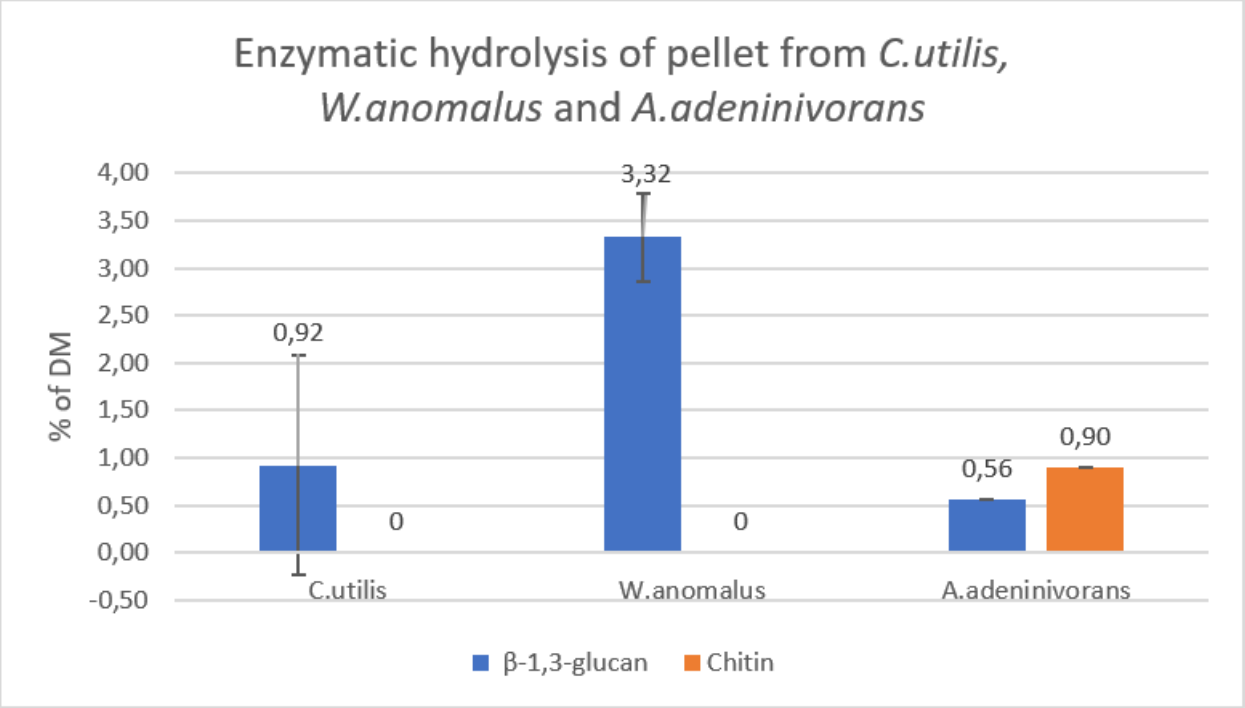
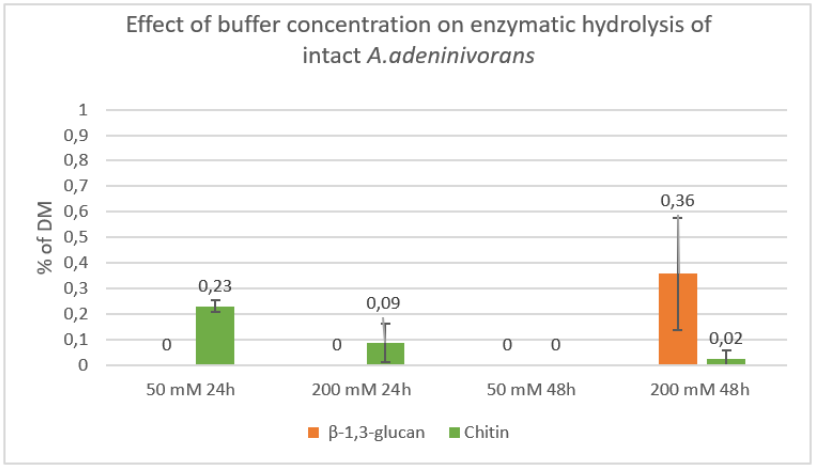
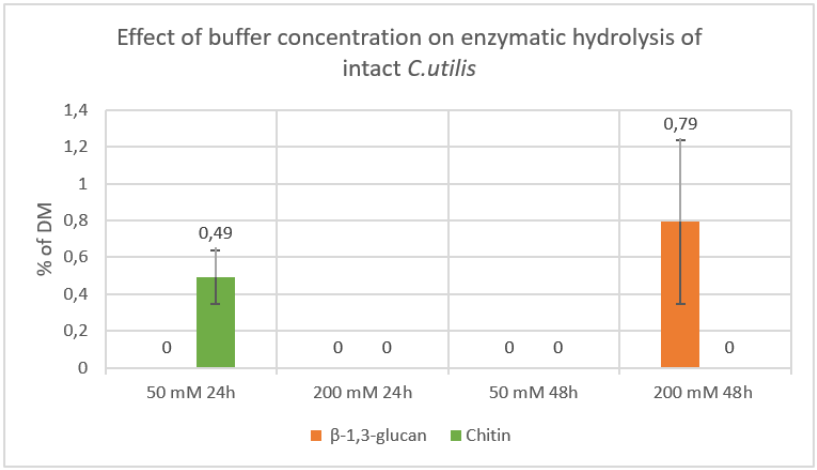


Figure 4.19: The figure shows the results from the HPAEC-PAD analysis of the pellets form *C.utilis*, *W.anomalus* and *A.adeninivorans* that were hydrolyzed with chitinaseC, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase in 50mM NaOAc-buffer pH5. The samples were incubated at 40°C for 24 hours. The values are the average of two duplicates. One of the replicates of *A.adeninivorans* failed, so this result was only based one sample.

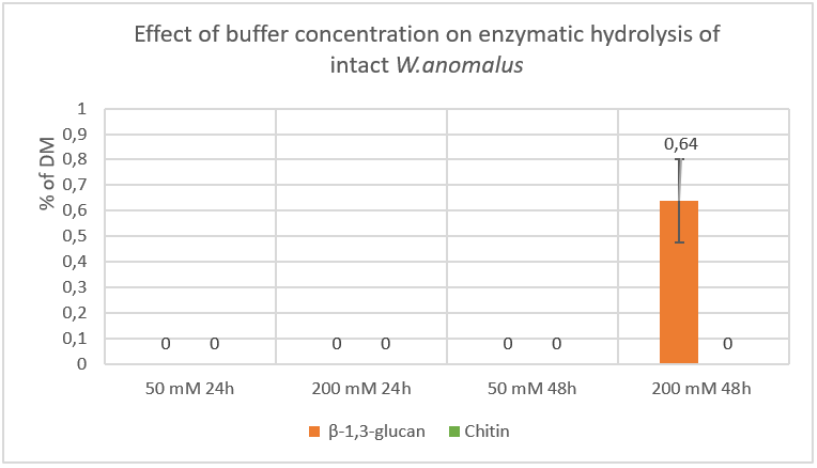
To see if the buffer concentration had any effect on maintaining the optimal pH for the enzymes during hydrolysis, intact *C.utilis*, *W.anomalus* and *A.adeninivorans* were hydrolyzed with chitinase C, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase in a mixture as described in section 3.2.17. The results from this experiment is shown in Figure 4.20. The determined content of chitin and β-1,3-glucan were under 1% (w/w) of the dry yeast. For both *A.adeninivorans* and *C.utilis* chitin were determined to be in the sample incubated for 24 hours in 50 mM, but no chitin was determined to be in the sample after 48 hours.



a)



b)



c)

Figure 4.20: The figure shows the results from the HPAEC-PAD analysis of intact *C.utilis*, *W.anomalus* and *A.adeninivorans* hydrolyzed with chitinaseC, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase together using two different buffer concentrations (50mM and 200 mM) for 24 hours and 48 hours.

The effect of the buffer was also tested by measuring the pH in samples of *A.adeninivorans* before and after enzymatic hydrolysis with chitinaseC, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase in both 50 mM and 200 mM NaOAc buffer pH 5, as described in section 3.2.17. The pH values measured before and after the enzymatic hydrolysis is shown in Table 4.7

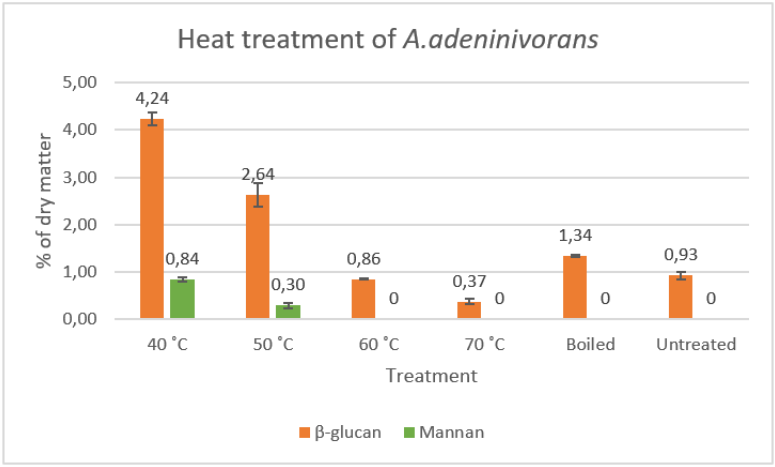
Table 4.7: The table gives the measured pH values before and after the enzymatic hydrolysis of intact *A.adeninivorans* with chitinaseC, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase in both 50 mM and 200 mM NaOAc buffer pH 5.The pH in the 200 mM buffer was 5.04 and in the 50 mM buffer the pH was 5.09.

Buffer concentration	Sample	pH before	pH after
200 mM	Replicate 1	5.08	5.06
	Replicate 2	5.08	5.06
	Control without enzyme	5.08	5.05
	Enzyme control without yeast	5.03	5.01
50 mM	Replicate 1	5.27	5.31
	Replicate 2	5.27	3.31
	Control without enzyme	5.22	5.20
	Enzyme control without yeast	5.11	5.12

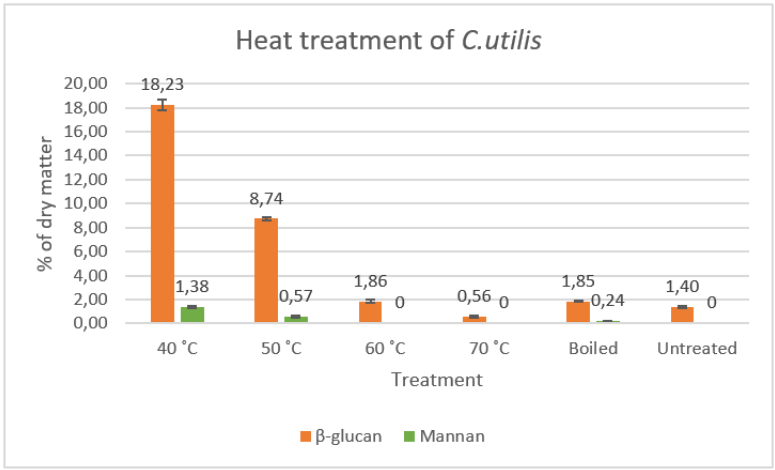
Table 4.7 shows that for the the NaOAc-buffer with concentration 200 mM, the pH is measured to be 5.0 both before and after the enzymatic hydrolysis lasting 24 hours. For the 50 mM buffer, the pH was measured to 5.09 in the buffer. When the intact *A.adeninivorans* and enzymes had been added the pH had increased to 5.27. If only yeast was dissolved in the buffer (control sample), the pH increased to 5.22, and for the enzyme control the pH increased to 5.11.

The effect the incubation temperature, and hence induction of autolysis in the reaction tube, was tested on intact *C.utilis*, *W.anomalus* and *A.adeninivorans*. Samples with intact yeast dissolved in 200 mM NaOAc buffer pH5 were incubated at 40°C, 50 °C, 60°C and 70°C. In addition, some samples were boiled for 15 minutes. Control samples which were not incubated in any tempereature or boiled were also prepared. In Figur 4.21 the determined content of β-glucan, mannan and chitin are given.

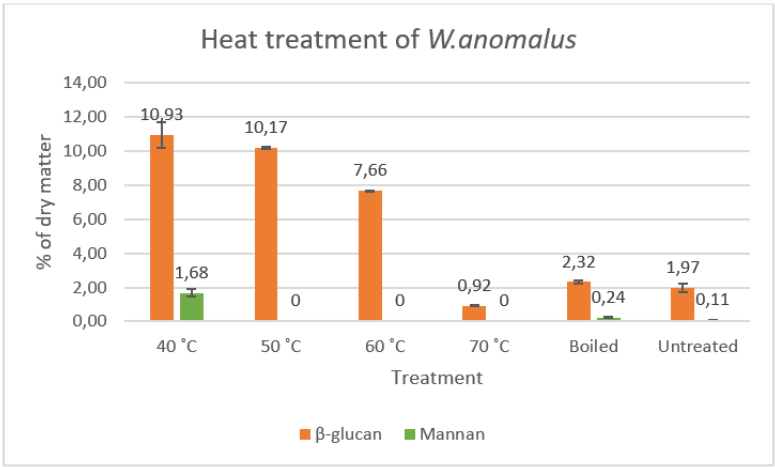
The results shows that the determined values for β-glucan and chitin decreased when the incubation temperature increased. *W.anomlus* had the highest determined values for both β-glucan and chitin. *A.adeninivorans* showed values generally lower than the two other yeast strains.



a)



b)



c)

Figure 4.21: The figure shows the amounts of β-glucan and mannan measured to be in the samples of *C.utilis*, *W.anomalus* and *A.adeninivorans* found by HPAEC-PAD after incubation in 200 mM NaOAc pH 5 at 40°C, 50°C, 60°C and 70°C. Some samples were only boiled for 15 minutes and some control samples were neither incubated nor boiled.

ChitinaseC, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase, in addition to α-mannanase and α-mannosidase, were used to hydrolyze intact *C.utilis* as described in section 3.2.19. The result from this enzymatic hydrolysis is given in figure 4.22.

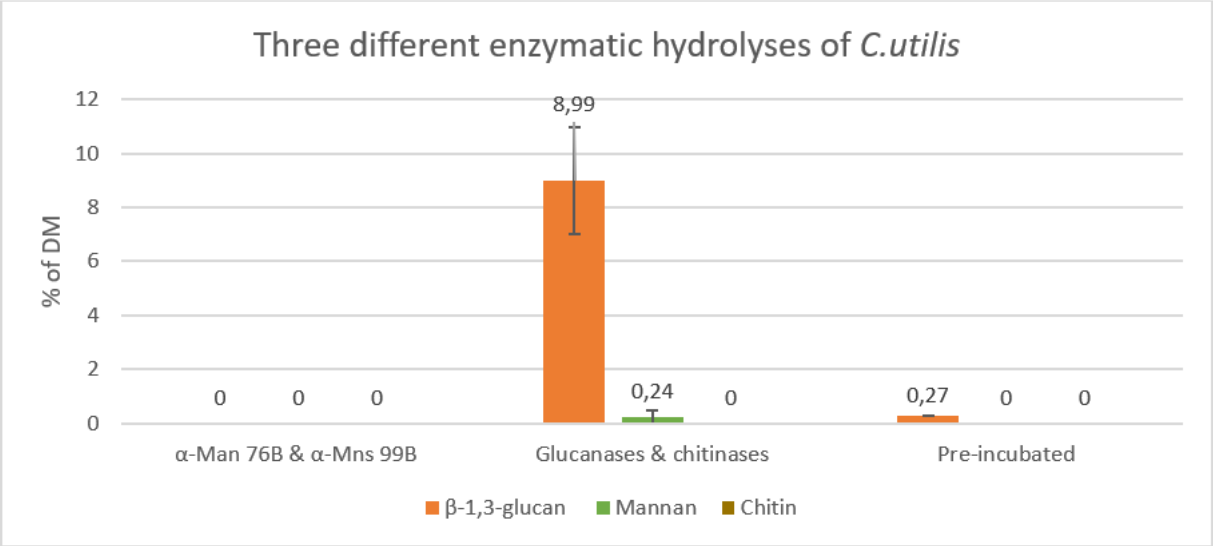


Figure 4.22: The figure shows the result of the three different enzymatic hydrolysis tested on *C.utilis*, namely hydrolysis with 1.α-mannanase and α-mannosidase for 24 hours in 200 mM NaOAc buffer pH 7, 2.endochitinase C, CHB, endo-1,3-β-D-glucanse and exo-1,3-β-D-glucanse for 24 hours in 2 mM NaOAc buffer pH 5 and 3. pre-incubation with α-mannanase and α-mannosidase for 4.5 hours in 200 mM pH 7 followed by hydrolyse by endochitinaseC, CHB, endo-1,3-β-D-glucanse and exo-1,3-β-D-glucanse for 24 hours in 200 mM pH 5.

As shown in the figure, hydrolysis with only α-mannanase and α-mannosidase released no monosaccharides that were detected in the monosaccharide analysis with HPAEC-PAD. The highest content of β-1,3-glucan and chitin were determined to be in the samples hydrolyzed with the mixture of endochitinase C, CHB, endo-1,3-β-D-glucanse and exo-1,3-β-D-glucanse.

5

Discussion

The aim of this thesis was to develop a method that would quantify the EPS in the yeast cell wall in an accurate and reliable way. The first step involved cell disruption using Microfluidizer^{TM} LM20. To isolate the yeast cell wall the slurry collected after cell disruption was centrifuged and separated into two fractions, a pellet and a supernatant. This was done as it was expected that this would create a protein-rich supernatant and a carbohydrate-rich cell wall fraction. Both fractions were then freeze dried to obtain dry material. Acid hydrolysis using sulfuric acid was then performed, to break down the polysaccharides into monomers, that could be quantified using HPAEC-PAD. But previous research show that acid hydrolysis is not sufficient to hydrolyse chitin, and it is not possible to quantify β-1,3-glucan and β-1,3-glucan specifically.

For this reason, methods using enzymes in addition to or combined with acid hydrolysis have been developed. Schiavone et al. (2014) develop such a method, with successive results. Inspired by this, a method was tried being developed in this thesis based on their method. The outline of this method is shown in Figure 2.2.

5.1 Yeast cell disruption by Microfluidizer^{TM} LM20

45 minutes was chosen as operating time when the cell wall preparation were made by disrupting the yeast cells on the microfluidizer^{TM} LM20, to ensure that 95% cell disruption. Later cell counting performed, however, may indicate that less than 45 minutes was necessary to obtain 95% cell death, so the operating time could be further optimized. The concentration of the slurry (% (w/v)) and the use of loop mode versus several passages can effect the lysing process. Therefore, these aspects should be optimized for each yeast strain. When counting cells using a normal light microscope, as done in this case, it was difficult to separate fragments from lyzed cells and intact cells. In addition, areas with clotted cells and cell fragments were observed, making it difficult to count accurately. Another counting method, like using methylene blue colouloration as Schiavone et al. (2014) did, could be considered if available.

As previously mentioned, it was expected that the disruption of the yeast cell by the microfluidizer^{TM} LM20 would create a protein-rich supernatant and a carbohydrate-rich cell wall fraction. As Figure 4.4, 4.5 and 4.6 show, the majority of protein was found in the supernatant, as expected, but it was not such a clear cut as anticipated. 40-55% carbohydrates was also determined to be in the supernatant, indicating that the Microfluidizer^{TM} LM20 seemed to shred off some EPS

during cell disruption.

To test the reproducibility of the microfluidizer^{TM} LM20, three replicates of *W.anomalus* from batch fermentation cultivated on chicken meat hydrolysate were microfluidized separately. A triplicate was made from each replicate for acid hydrolysis. As shown in Figure 4.6, replicate 1 is different from the two other replicates, especially the pellet. For replicate 2 and 3 the sum of all components add up to more then 100%, so the quantification was not accurate (discussed in more detail in section 5.7), and a possible presence of other components, as found in replicate 1, could not be excluded. By analyzing these results, the reproducibility of Microfluidizer^{TM} LM20 may not seem to be as good as desired, and the use of this disruption method may not be adequate to obtain accurate quantification. Other reasons than inconsistent cell disruption by the Microfluidizer^{TM} LM20 could have caused this deviation between replicates. Insufficent acid hydrolysis did not seem reasonable, since the calculated standard deviations were relatively small among the concentrations calculated. A more likely explanation may be that the samples were inhomogeneous, and more crushing and mixing of the dry intact yeast before weighing out could give more corresponding results.

If the Microfluidizer^{TM} LM20 is to be used as method disruption, a way to isolate the EPS from the disrupted cell in one fraction should be developed. The results presented here show that the centrifugation of the slurry collected from the microfluidizer^{TM} LM20 was not sufficient to isolate the EPS. Nguyen et al. (1998) dissolved the yeast in phosphate buffer at pH 8.5 during disruption to inhibit endogenous glucanases, and conclude that the use of this buffer gave a result 5-10% higher compared to other results reported. So a buffer with a specific pH or composition inhibiting endogenous enzymes could be used to prevent formation of more soluble monosaccharides that are transferred to the liquid phase instead of precipitate during centrifugation the same way as less viscose and soluble poly- and oligosaccharides do. Several other studies, including Schiavone et al. (2014) used glass beads to disrupt the yeast cells, getting a protein content of about 14% in the isolated cell wall fraction. Bzducha-Wróbel et al. (2014) also concluded that this was the method that gave the purest cell wall prepartion with the highest β-glucan content, though they did not examine microfluidizing as a disruption method. Another possibility may be to hydrolyse intact yeast, without disruption. But as the results obtain in this study showed, it could be hard to obtain accurate results when using enzymes to hydrolyze the yeast cell wall of intact yeast. If this would be used, further optimization of enzyme loading is needed. Autolysis is a problem for specific quantification of the components in the yeast cell wall it it should be performed on intact yeast.

5.2 Generation of dry matter (DM)

The result from the dry weight analysis, given in Table 4.3 shows that only using the freeze dryer may not provide sufficient drying. This analysis showed that the water content could be considered taken into account to get a more accurate quantification. If the pellets and supernatants are not completely dried, the quantification of the EPS will be underestimated.

The dry weight analysis was carried out about a month after the samples was removed form the freeze dryer. The samples were stored in a freezer, but were kept at room temperature without being stored in an exicator for some short periods when samples were weighed out. So the humidity in the air could have increased the water content in the samples before the dry weight analysis was carried out. The tubes used during the analysis were not dried in advance, so the tubes may have attributed to the measured water loss, but most likely to a small extent. The

water loss found by the dry weight analysis was not taken into account when the carbohydrate and protein content were calculated in this case. This was due to that not all samples were analysed due to lack of dry matter, and because of the uncertainties in the analysis discussed above

From Table 4.2 it can be seen that some dry matter was lost during the disruption and drying process. The reason for the loss of dry matter could be because not all of the slurry is washed out of the microfluidizer^{TM} LM20. The microfluizer was washed with milli-Q water until the slurry was clear, so the loss of material in the microfluidizer^{TM} LM20 should be small. The slurry was first collected in tubes from the microfluidizer^{TM} LM20, and then transferred to centrifugation tubes before finally transferred to the tubes used for the freeze dryer, so loss of material could happen during the transfers. When dried, the consistency of the material is very light, so some dried material was lost when the material was weighed after the freeze drier. To minimize the loss of matter, the number of transfers between tubes should be kept at a minimum and precautions should be taken when handling the dried material. The loss of dry matter in the disruption and drying process will effect the quantification of the carbohydrates in the YCW.

For *C.utilis* from batch fermentation cultivated on chicken meat hydrolysate and BALI sugar the weight of the supernatant and pellet added up to 6.0388. Since the intact yeast was weighed in with no more than one decimal accuracy one cannot say if any dry matter was added, but compared to the other samples, where all samples lost material, it may be that this sample was not dried completely in the freeze drier. The freeze dryer had too low capacity for all the samples to be dried. Due to other users, the samples were sometimes removed and put back in, so it is likely that this have effected the drying process, and made less optimal.

For almost all samples the weight of the pellet were around 25-50% (w/w) of the intact yeast. In *S.cerevisiae*, the cell wall is determined to be responsible for 15-30% of the dry weight of the yeast Orlean (2012). If it was assumed that the YCW was isolated in a carbohydrate-rich pellet, the values reported here are somewhat higher for the *C.utilis*, *W.anomulus* and *A.adeninivorans*. But the results provided by monosaccharide analysis and the Kjeldahl analysis, a pure YCW fraction was most likely not achieved, explaining the higher values.

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5.3 Protein analysis

The results from the initial experiments where Bradford analysis were used to find the total protein content in the supernatants from *C.utilis*, *W.anomulus* and *A.adeninivorans* from batch fermentation cultivated on YPD before they were freeze dried, is given in Table 4.1. The protein % (w/w) found to be in the intact yeast based on the analysis of supernatants were low compared to for example the result reported by Chae et al. (2001). They determined the protein content in dry yeast (*Saccharomyces sp.*) to be 49.2 % using the Kjeldahl method. Though, the protein content can vary between yeast strain, the resulting values after the Bradford method indicated that a clear protein-rich supernatant was not obtaind as expected. This was further verified by the results from the Kjedahl analysis of pellets and supernatants, discussed in section 5.1.

Since the total organic nitrogen is determined when using the Kjeldahl method, it is possible that the nitrogen in GlcNAc in YCW-bound chitin is also decomposed and falsely determined

to be protein when the factor of 6.25 is used to estimate the protein content. However, the extent of this error should be low, due to the low yields of chitin by acid hydrolysis and since chitin is usully 1-2% of the YCW.

5.4 Acid hydrolysis with H_2SO_4

As shown in Figure 4.2, the yields for laminarin (β-1,3-glucan) and pustulan (β-1,6-glucan) were between 90-100%. A complete as possible hydrolysis of these polysaccharides was important to obtain, as acid hydrolysis was be used estimate the total β-glucan (i.e. β-1,3-glucan and β-1,6-glucan) in the method developed. Enzymatic hydrolysis with exo/endo-1,3-β-D-glucanase would then be used to estimate the amount of β-1,3-glucan, and this amount would be substracted from the total amount, giving the content of β-1,6-glucan. So if laminarin and pustulan were hydrolyzed with low yields, the quantification would be inaccurate. The lower acid hydrolysis yield of chitin was expected and described in the litterateur (Dallies et al., 1998), and is one of the reasons methods using enzymatic hydrolysis with chitinases being developed to quantify the EPS in the yeast cell wall. These results were obtained by a mild hydrolysis (4% H_2SO_4, without the pre-incubation step in 72% H_2SO_4. This was because the standards used were in solution, and the availability limited the use of powder form for this experiment.

The experiment testing if the step with pre-treatment of the material to by hydrolyzed with 72% H_2SO_4 was necessary, was performedormed to see if this step could be skipped and in this way minimize the degradation of monosaccharides that can occur during acid hydrolysis. From Figure 4.3 it can be seen that the step with pre-treatment with 72% H_2SO_4 gives a higher GE concentration for both *C.utilis*(27% increase) and *W.anomalus* (31% increase). From this result we concluded that the initializing step with strong 72% H_2SO_4 was necessary when using acid hydrolysis analysing yeast samples. The same conclusion was made by Dallies et al. (1998), after their studies on acid hydrolysis of yeast cell wall.

5.5 Carbohydrate composition in the pellets and supernatants

The monosaccharides detected when analysing the hydrolyzed pellets and supernatants were calculated into polymers (calculation shown in Appendix A.1). As described in the literature β-glucan, mannan and chitin are the most abundant polysaccharides present in the EPS. Therefore, we assumed that glucose, mannose and GlcNAc were released from these polysaccharides when the samples were hydrolyzed. Ribose, xylose and galactose were also calculated into polymers in the same way. It is not certain that the detected monosaccharides were released from the respective polysaccharides, so this should be further examined.

Figures 4.4, 4.5 and 4.6 show the carbohydrate composition of the yeast samples tested. β-glucan made up the largest fraction. Orlean (2012) reported that in *S.cerevisiae* β-glucan compose 30-60% of the dry weight of the cell wall. The result we obtained for *C.utilis*, *W.anomulus* and *A.adeninivorans* showed that the content of β-glucan in the pellets generally lie inside approximately the same range. The chitin content determined to be in the pellets from *A.adeninivorans* from all fermentation modes and growth mediums had higher values that what is reported for *S.cerevisiae*.In *A.adeninivorans* the chitin content was determined to be

5.60-7.19%, while 1-2% is reported for *S.cerevisiae* (Orlean, 2012). Mannan was also determined to one of the major carbohydrate components in the carbohydrate-rich pellet, in which the YCW was suppose to be isolated.

Galactose, xylose and ribose were detected using HPAEC-PAD, based on corresponding retention times. Ribose and GlcNAc elute at approximately the same time and cannot be separated using HPAEC-PAD. Since GlcNAc is degraded to glucosamine during acid hydrolysis, it was assumed that it was ribose that was detected in the samples of the acid hydrolyzed pellets and supernatants. The results from the analysis using ion exclusion chromatography and UV detection indicated that GlcNAc was not present in any samples, which strengthened the initial suggestion of ribose only based on the corresponding retention times.

Galactose was only detected in the samples from *A.adeninivorans* samples, with the highest average amount in the pellets. The galactose detected could originate from galactooligosaccharides. Dallies et al. (1998) detected galactose after acid hydrolysis of cell wall isolated form *Schizosaccgaromyces pombe*. They concluded that this was most likely from the galacto-mannan structure that is present in this yeast strain. So it is possible that A.adeninivorans contain such a galacto-mannan structure, similar to *S.pombe*.

Xylose was only detected in the the supernatant from *C.utilis* from repeated batch fermentation cultivated on chicken meat hydrolysate and BALI sugar in small amounts (0.60 % $\pm$ 0.12). Ribose was also detected in small amounts ranging from 0.30% to 1.14%. The pentose was detected in present in all samples, so this could originate from insufficient washing of the fermented yeast, but it could also originate from intracellular material which leaked out when the yeast cells were disrupted. It is possible that a smaller dilution of the samples from the acid hydrolysis could reveal xylose in other samples, giving more information to state where it originates from. A smaller dilution could also give a more accurate detection of ribose, xylose and chitin. So to make a more accurate quantification of the monosaccharides released from the EPS by acid hydrolysis, different dilutions of the samples should be used for quantification of the different components.

Whether the released glactose, ribose and xylose originate from the EPS or is a result of insufficient washing after fermentation needs to be further examined. It is also possible that the yeast have integrated the carbohydrates from the growth medium into the YCW.

For example, *A.adeninivorans* could have integrated galactose, since galactose is not detected in *C.utilis* or *W.anomalus*, and all were washed in a similar fashion. If it is due to insufficient washing, detection of galactose would be expected in these two as well. Galactose was detected in *A.adeninivorans* independently of growth medium, so the composition of the growth mediums should be analyzed, to tell if the galactose could originate from this. Further analysis to state whether the yeast, contain a galacto-mannan structure, if it could have integrated components from the growth medium or if it is debris left from the medium should be studied further.

A.adeninivorans had the most complex carbohydrate composition. Containing, β-glucan, mannan oligosaccharides and relatively high values for chitin, and possibly galactooligosaccharides and/or galactomannan structures. All of these components are described as prebiotics, indicating that the EPS in *A.adeninivorans* could have healt benefits if used in aquafeed.

5.6 Enzymatic hydrolysis

In the same way as for the acid hydrolyzed samples, the monosaccharides detected when analysing the hydrolyzed pellets and intact yeast were calculated into polymers (calculation shown in Appendix A.1, without recovery factor). It was also assumed that glucose, mannose and GlcNAc were released from β-glucan, mannan and chitin, respectively, though this is not certain and should be further examined.

5.6.1 Chitinases and 1,3-β-d-glucanases

Figure 4.7 shows that that yields of the enzymatic hydrolysis of laminarin and chitin was relatively low, and not higher than the yield obtained from acid hydrolysis, shown in Figure 4.2. The progression curves for the chitinase cocktail and endo-1,3-β-D-glucanase also showed low GE concentrations when hydrolyzing chitin and curdlan, respectively. An important aspect of developing an method using enzymes for hydrolysis of the YCW, was to obtain higher yields for hydrolysis of chitin than what was obtained by acid hydrolysis. So these low yield for chitin and laminarin was not promising. A reason for this low yield could be a too low enzyme loading or substrate inhibition of the enzymes that can happen when using model substrates. The progression curve for endo-1,3-β-D-glucanase was made with curdlan as substrate. As described in Megazyme (2019a), the enzyme has lower hydrolysis rate on this substrate, which could also contribute to the low GE concentration obtained.

endo-1,3-β-D-glucanase and the cocktail consisting of chitinase C and CHB gave higher yields when using NaOac-buffer pH 5, indicating higher activity in this buffer. Still the progression curves in Figure 4.8 and 4.9 were made with enzyme reactions in Tris-HCl buffer. The reason for this was insufficient interpretations of the results and somewhat rushed decisions due to a limited time frame. The later integration of exo-1,3-β-D-glucanase to the enzyme cocktail amplify the use of NaOAc-buffer, since this was the recommended buffer for this enzyme (Megazyme, 2019b).

As described, the method being developed in association with this thesis has the goal to use 1,3-β-D-glucanases in the enzymatic hydrolysis for quantification of β-1,3-glucan, and the difference between the estimated β-1,3-glucan and the total β-glucan found by acid hydrolysis to quantify 1,6-β-glucan, inspired by the study performed by Schiavone et al. (2014). Therefore, it was important that neither exo-1,3-β-D-glucanase nor endo-1,3-β-D-glucanase showed hydrolytic activity on 1,6-β-glucan.

Figure 4.10 and 4.11 verify that endo-1,3-β-D-glucanase has specific activity for 1,3-β-glucan. The chromatograms in Figure 4.10 shows that there was no difference between the control sample of pustulan and the sample where endo-1,3-β-D-glucanase was added. This was further verified by the mass spectra in Figure 4.11, where peaks with m/z values indicating different oligosaccarides of glucose, like m/z 1013, 851 and 527, was present when laminarin by endo-1,3-β-D-glucanase. But when pustulan was hydrolysed using endo-1,3-β-D-glucanase none of these peaks were present.

The monosaccharide analysis by HPAEC-PAD, results shown in figure 4.12, indicate no hydrolytic activity by exo-1,3-β-D-glucanase on pustulan, as no peaks corresponding to glucose was observed. Due to this, it was concluded that exo-1,3-β-D-glucanase was specific for 1,3-β-glucan.

The addition of exo-1,3-β-D-glucanase to the enzyme cocktail consisting of endo-1,3-β-D-glucanase and chitinase C and CHB made the quantification process easier, as only monosaccharide analysis was necessary. The results given in Figure **??** shows that when curdlan were only hydrolyzed with endo-1,3-β-D-glucanase the peaks with m/z values indicating glucose oligomers are present, while in Figure 4.14 the mass spectra shows that when curdlan is hydrolyzed with a mix of both exo-1,3-β-D-glucanase and endo-1,3-β-D-glucanase, these peaks are no longer present. The chromatograms shown in Figure 4.13 shows that when curdlan is only hydrolyzed using endo-1,3-β-D-glucanase, different peaks indicating oligomers of glucose is present, while only one peak indicating the monomer, glucose, dominates when exo-1,3-β-D-glucanase is also added. These results show that a mixture of exo-1,3-β-D-glucanase and endo-1,3-β-D-glucanase releases monosaccharide when curdlan is hydrolyzed.

Exo-1,3-β-D-glucanase was not tried to hydrolyze curdlan alone as the description states a low hydrolysis rate of curdlan by exo-1,3-β-D-glucanase (Megazyme (2019b) states < 0.0001 for CM-Curdlan (2.5 mg/mL)). Laminarin was not available, but if it was, exo-1,3-β-D-glucanase could be used on this substrate alone, to test if the enzyme is able to hydrolyze β-1,3-glucan into glucose.

5.6.2 Mannanase and mannosidase

The test of the enzyme amount needed for enzymatic hydrolysis of SC-Man by α-mannosidase 99B and α-mannanase 76B shows that both enzymes have activity on the substrate and that the highest enzyme concentration gives the highest GE concentration (see Figure 4.16). Figure 4.17 shows that α-mannosidase 99B and α-mannanase 76B have activity on INM, which was unexpected as INM is β-1,4-mannan (Megazyme, 2019c), and α-mannosidase 99B and α-mannanase 76B are described as 1,2-α-mannosidase (nzytech, 2019b) and endo-1,6-α-mannanase (nzytech, 2019a), respectively. The results from the DNS-analysis was also unexpected in the way that α-mannosidase 99B gives higher GE concentrations at the lowest enzyme concentration and non at the highest enzyme concentration. In hindsight, this experiment should have been redone. It is important that these mannananases are specific for the α-1,2 and α-1,6 linkages in the mannose-chain, if the quantification of the mannose in the side chain and in the α-1,6-mannose backbone is going to be specific. Therefore it should be verified that α-mannosidase 99B and α-mannanase 76B are not active on any other linkages.

Whether these results are due to Since the two enzymes are intended to be used in a mixture, NaOAc-buffer pH 7 was chosen as buffer, due to the greater difference between Tris-HCl buffer and NaOAc-buffer for α-mannosidase 99B.

α-mannosidase 99B showed no activity on α1,2-mannobiose. This can be concluded by the results given in the chromatogram in Figure 4.18, where no relevant difference was observed between the control sample and the sample where α-mannosidase 99B was added to α-1,2-mannobiose. The reason for this could be the fact that the enzyme is an endo-α-mannosidase (Thompson et al., 2012) and therefore not showing any activity in the disaccharide.

No hydrolysis of SC-Man using α-mannosidase 99B and α-mannanase 76B separately or in mix was observed (Results not shown).

The result that indicate no or little activity, in addition to the results indication that α-mannosidase 99B and α-mannanase 76B have activity on INM makes it necessary to further examine these enzymes. It is necessary to state their specificity and optimize the condition to

give a stable hydrolysis, before these two enzymes can be incorporated in the quantification method.

5.6.3 Enzymatic hydrolysis of pellets from *A.adeninivorans*, *C.utilis* and *W.anomalus*

The hydrolysis of pellets from *A.adeninivorans*, *C.utilis* and *W.anomalus* with the enzyme cocktail consisting of chitinase C, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase, gave inconsistent result, especially for *C.utils*, illustrated by the large standard deviation in Figure 4.19. The obtained β-1,3-glucan content for *C.utilis* and *A.adeninivorans* was under 1% of dry weight of the pellet, and 3.32% of the dry weight of the pellet from W.anomalus. Chitin was only determined to be in the pellet from *A.adeninivorans*. These values are much lower than the values obtained by acid hydrolysis, shown in Figure 4.4, 4.5 and 4.6. These results also indicate that further optimization of enzyme loading to obatain a sufficient and stable hydrolysis of the pellets, suggested to obtain the major fraction of the EPS.

Due to these inconsistent and low values, we did not try to subtract these concentrations from the concentrations found by acid hydrolysis to find the concentration of 1,6-β-glucan as originally suggested by the method being developed in association with this thesis.

5.6.4 Enzymatic hydrolysis of intact yeast

The results provided by the acid hydrolysis of pellets and supernatants showed that not all of the EPS in the YCW was isolated in the pellet. For this reason it was suggested that enzymatic hydrolysis of intact yeast could give a more accurate quantification of the EPS.

Intact yeast has a natural pH, and therefore a buffer with higher concentration was tested to see if this was necessary to maintain the pH needed for optimal conditions for the enzymes used for hydrolysis. NaOAc-buffer pH 5 with concentration 200mM and 50mM were compared. The measurements, given in Table 4.7 showed that the yeast increased the pH, but the additions of enzymes also contribute to a minor increase in pH. The result in Table 4.7 indicate that a higher buffer capacity gives a more stable pH when intact yeast is hydrolyzed, and could contribute to a more complete hydrolysis of the polysaccharides in the YCW.

As shown in Figure 4.20 the enzyme cocktail consisting of Endo-chitinase C, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase, seemed to have little or no effect on the intact yeast. The results were also somewhat inconsistent. This could be due to that only 1 µL of endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase were added, or that 75 µL of the samples was withdrawn after 24 hours. This is discussed further in section 5.7.

If the results obtained for the control samples in the experiment of enzymatic hydrolysis of intact yeast were examined, the majority of the released monosaccharides detected in the analysis using HPAEC-PAD seemed to be a result of autolysis by endogenous enzymes. As shown in Figure 4.21 higher concentration of β-glucan and chitin is found in the samples of intact yeast incubated at 40°C and 50°C compared to the samples hydrolyzed with the enzyme cocktail consisting of Endo-chitinase C, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase. In addition mannose was detected in the HPAEC-PAD of the samples incubated at 40°C and 50°C. At 60°C and 70°C, the detected carbohydrate concentrations are lower in the samples

from *A.adeninvorans* and *C.utilis*. For *W.anomalus* the concentration estimated at 60°C is still high, but at 70°C, the concentration is lower like the two other strains. The reason for the lower concentrations could be due to increasing inhibition of the endogenous enzymes with the increasing temperatures. The samples that were only boiled for 15 minutes also show some presence of released glucose from β-glucan and mannose from mannan. This could be due to lysis of some intact yeast cells during boiling, or due to activation of endogenous enzymes as the temperature increased in the samples, followed by inhibition when the temperature was too high. The yeast samples that were dissolved in buffer right before transfer to vials also show some release of glucose and mannose (only in *W.anomalus*). *A.adeninivorans* showed generally lower concentrations compared to C.utilis and W.anomalus, which could be due to a stronger cell wall or that this strain contain less active enzymes in the conditions tested.

Figure 4.22 shows the result from the last experiment performed where *C.utilis* was hydrolyzed with three different enzyme cocktails, with higher enzyme concentrations than for the other enzymatic hydrolyses. The samples hydrolyzed with only α-mannosidase 99B and α-mannanase 76B for 24 hours gave no release of glucose, mannose or GlcNAc that were detected in the monosaccharide analysis by HPAEC-PAD. This was expected as α-mannosidase 99B and α-mannanase 76B mostly yields oligosaccahrides of mannose residues. Ideally, a oligosaccharide analysis of these samples should be performedormed, to see if α-mannosidase 99B and α-mannanase 76B had a hydrolytic effect on the intact yeasts. These results show that no autolysis occur at this incubation temperature of 37°C in a buffer of pH7. The reason for this could be that the endogenous enzymes were not active at this temperature or pH.

The samples hydrolyzed with the enzyme cocktail consisting of chitinase C, CHB, endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase gave the highest estimated 1,3-β-glucan and chitin. As the control sample show, some of the released glucose is due to autolysis.The standard deviations for the controls is relatively big, and shows that the autolysis of the endogenous enzymes is inconsistent. This could be the reason for a different concentration found in these control sample compared to the sample incubated at 40°C in Figure 4.21. Interestingly, the samples that were pre-incubated with α-mannosidase 99B and α-mannanase 76B for 4.5 hours to see if these enzyme could remove the outer mannosylated layer of the YCW could make the β-glucan and chitin more accessible, gave lower concentrations of 1,3-β-glucan and no chitin. This could be due to the change in buffer concentration that occurs when HCl is added to reduce the pH from pH 7 to pH 5 and the addition of enzymes. In total, this changed the buffer concentration from 200 mM to 129 mM. This concentration may not provide a buffer capacity that is able to maintain a pH 5 and provide the optimal pH condition for the hydrolyzing enzymes.

The results from the enzymatic hydrolysis on intact yeast show that further testing and optimization is required to give accurate quantification of the carbohydrates in the EPS. Enzyme concentrations and reaction conditions that gives consistent and specific hydrolysis must be stated, in addition to optimization of the quantification of the released monosaccharide. A challenge would be to avoid autolysis during incubation. The autolysis makes a quantification of 1,6-β-glucan and 1,3-β-glucan inaccurate, as glucose from both could be released by autolysis.

5.7 Methodological considerations

The results from the Bradford analysis to find the protein content in supernatants (Table 4.1 were only based on just one replicate, so more replicates should be used to get a more representative result. The measured value for the 500 mg/mL BSA standard used was 732 mg/ml

when the measuring the protein concentration in *C.utilis* and *A.adeninivorans* and 486 mg/mL when measuring the protein concentration in *W.anomalus*. For this reason the measured result is not completely accurate. Most likely these deviations were due to heterogeneous material after freezing of the sample.

When performing the Kjeldahl method, no blank or standards like BSA were used. Due to this, the accuracy of the measurement cannot be evaluated and any possible attributes from a blank sample could not be taken into account. But the results presented give a representation of the distribution of proteins, though the values may not be accurate.

In the DNS analysis performed to give the results in Figure 4.2 and 4.3 only 1 µL of sample and NaOH were added, as described in 3.2.20.1. Also in the enzymatic hydrolysis of pellets, result given in 4.20, 1 µL of endo-1,3-β-D-glucanase and exo-1,3-β-D-glucanase were added. This is a small volume that could be hard to pipette accurately. A pipette with a range of 0.2-2 µL was used to get the most accurate volume, but to improve the accuracy a double reaction volume could be used, or a dilution of the original sample or enzymes could be made.

If the average concentrations of protein and the different carbohydrates, shown in Figure 4.6, 4.5 and 4.4, are added together, some of the compositions exceed 100%. This indicate that the quantification was not accurate. The inaccuracy is likely due to several reasons. As discussed above, no blank or standard were used during the Kjeldahl analysis, so it is possible that the protein concentrations were overestimated. Some samples also had measured amounts for glucose and mannose that exceeded the linear area of the standard curve, giving inaccurate quantification. This could have contributed to an overestimation of the polysaccharide content. For example, pellets originating from *C.utilis* and *W.anomalus* from batch fermentation cultivated on YPD had both estimated amounts for glucose and mannose that were outside the linear area of the standard curve, and they both had an estimated composition where the components exceed 100%. These samples should be analyzed again with a higher dilution, so that the measured amount will be in the linear area of the standard curve.

Neither chitin nor curdlan was soluble in the buffer, so when withdrawing samples at the specific times for making the progression curves in Figure 4.9 and 4.8, different amounts of substrates could have been removed from the samples. This could give inaccurate results, as the substrate concentration changed. This was also the case in the experiment where intact yeast was incubated with chitinase C, CHC, exo/endo-1,3-β-D glucanase in 50 mM and 200 mM for 24 and 48 hours. In this experiments 75 µL was withdrawn after 24 hours. To obtain more accurate results, duplicates could be made for each time of measurement to avoid withdraw of insoluble substrate. This was not done in the case with curdlan and chitin to minimize the use of substrates with limited availability.

When quantifying the samples that gave the result presented in the Figure 4.20, the signal from the internal standard fucose varied greatly. For all the samples withdrawn for after 48 hours, the signals for the internal standard were so different from what the standard showed, that we decided to quantify these samples without internal standard. The great variation in the signal introduced uncertainty in the quantification, and ideally an internal standard that gives stable signals should be used. Alternatively, experiments to find a relationship explaining the variation could be performed, making it possible to take the variation into account when quantifying. The reason for the variation in the signal from the internal standard when samples with intact yeast were analyzed using HPAEC-PAD is unclear. The signal was stable when pellets or supernatants were analyzed, so it must have been something that was present in the samples with intact yeast that interferred with fucose during the analysis.

6

Concluding remarks

The results provided by the experiments performed, indicate that the operating time and disruption mode (loop versus several passages) of the Microfluidizer^{TM} LM20 should be optimized for each yeast strain. Centrifugation of the slurry collected from the Microfluidizer^{TM} LM20 did not give the clear separation into a protein-rich supernatant and carbohydrate-rich yeast cell wall fraction as anticipated. Therefore, other disruption methods like the use of glass beads could be considered, or else a method to isolate most of the EPS into a purer fraction should be developed.

The enzymatic hydrolysis of pellets and intact resulted in low values for the content of 1,3-β-glucan, chitin and mannan, indicating low hydrolytic effects of the enzymes tested. Further optimization is therfore needed for enzyme loadings and reaction conditions to obtain an enzymatic hydrolysis that provide sufficient hydrolysis of the components in the EPS.

Appendix A

Appendix

A.1 Calculation of concentration of the structural polysaccharide

$$C_{\text{struc, carb(\% of dry matter)}} = \frac{C_{\text{monosacc, HPAEC}} \cdot V_{sample} \cdot D \cdot P_f}{m_{sample} \cdot R_f} \tag{A.1}$$

- $C_{struc,carb(\% \text{ of dry matter})}$ - concentration of the structural polysaccharide
- $C_{monosacc,HPAEC}$ - concentration of the monosaccharide determined by HPAEC-PAD
- V_{sample} - total volume
- D - dilution factor for the HPAEC
- m_{sample} - amount sample weighed out for each sample
- P_f - polymer correction factor. Corrects calculated value for the loss of one water molecule per monosaccharide when present as a polymer.
- R_f - the average recovery factor for replicate determination of the monosaccharide calculated based on SRS. It accounts for monosaccharide degradation during the dilute and hydrolysis.

The correction factor is 0.9, 0.88 and 0.92 for hexoses, pentoses and N-acetyl-glucosamine, respectively.

The determined recovery factor was determined to be 1 for glucose and N-acetylglucosamine, and 0.82 for mannose.

Since the standard used in quantification of the released glucoseamine from chitin was D-(+)-glucosamine hydrochloride, this was taken into account when the determined glucoseamine concentration was adsjused to N-acetyl-glucosamine (GlCNAc) concentration. This calculation is shown in the equation below.

$$C_{GlcNAc} = \frac{C_{\text{glucosamin, HPAEC}} \cdot Mm_{GlcNac}}{Mm_{\text{Glucosamine hydrocloride}}} \tag{A.2}$$

- C_{GlcNAc} - concentration GlcNAc estimated from the glucosamine concentration determined by HPAEC-PAD
- $C_{\text{glucosamine, HPAEC}}$ - concentration of glucosamine determined by HPAEC-PAD
- Mm_{GlcNac} - The molar mass of N-acetyl-glucosamine (221.21 g/mol)
- $Mm_{\text{glucosamine hydrochloride}}$ - the molar mass of glucosamine hydrochloride (215.64g/mol)

It is this calculated GlcNAc concentration that is inserted in the "$C_{\text{monosacc,HPAEC}}$" in the Formula A.1.

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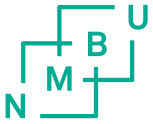

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