DETERMINATION OF 1,3/1,6 LINKED β-GLUCAN CONCENTRATION REMAINING IN THE CELL WALLS OF *SACCHAROMYCES CEREVISIAE* POST YEAST EXTRACTION PROCESSES

by

PHILLIP J. GLASS JR

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Phillip J. Glass Jr

Approved by:

Anne E. Vravick, Ph.D.	Date
Kathleen A. Boyle, Ph.D.	Date
Scott A. Schlipp, M.S.	Date
Tilak Nagodawithana	Date

Approved by the Dean for Natural & Health Sciences and Education:

Cheryl P. Bailey, Ph.D.	Date

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Abstract

β-glucans are a common type of polysaccharides that consist of glucose and are a main component in the structure of yeast cell walls. The major glucans within the *Saccharomyces Cerevisiae* cell wall are branched 1,3-β-glucan and 1,6-β-glucan. This material has biological activities in the form of dietary fiber, functionality as an antioxidant, and improve the health of gut microflora.

The efficient disruption of the yeast cell was a necessary step for cell wall preparations. The use of endogenous enzymes within the cell have been used historically in production of autolyzed yeast extracts. These endogenous enzymes reduce the yeast biomass associated with the cell wall through protein degradation activities. Modern yeast extract processing methods now target the protein degradation methods with the inoculation of exogenous enzymes that have been isolated from various microorganisms. The use of exogenous enzymes provides very efficient and targeted mechanisms that resulted in an increased amount of solubilized yeast biomass compared to traditional autolyzation methodologies. These increases are attributed to the lack of variability and activity that was associated with the content located in the vacuole within the yeast cell.

The identification and quantitation of 1,3-β-glucan and 1,6-β-glucan remaining in the cell wall fractions post yeast extract process can further determine the benefits of using exogenous enzymes in cell wall preparations. The 2% usage, dry solid basis, of exopeptidase on yeast cells resulted in higher combined 1,3/1,6-β-glucan concentration in the cell wall fractions then reported values of the same material after autolysis processing. The use of exogenous enzymes in the preparation of cell walls post yeast extraction processes can increase the remaining

concentrations of β-glucans and lessen the additional processing required to further concentrate these materials for use as a functional ingredient in food systems.

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Chapter 1. Introduction

1.1 Background

Yeast extracts are a staple in the savory category used to enhance our flavors in soups, sauces, spice blends, and topical seasonings. These products consist of a high concentration of small protein chains and other subtle molecules. Probably the most important of these to the flavoring community is glutamic acid, also known as Monosodium Glutamate (MSG). The taste contributed by MSG was identified by a scientist in Japan, Ikeda in 1908 (Yamaguchi and Ninomiya, 1999). This taste is called "Umami" and is considered the fifth basic taste along with sweet, sour, salty, and bitter. The term "umami" is often used synonymously with the description of the flavor impact provided by glutamic acid; however, inosine 5'-monophosphate (5'-IMP) and guanosine 5'-monophosphate (5'-GMP) also contribute to the umami taste, a synergistic enhancement (Cairoli et al., 2008). Both compounds can be derived from the enzymatic hydrolysis of yeast proteins and ribonucleic acid (RNA).

Traditionally, yeast extracts are manufactured using an autolyzation process which activates endogenous yeast enzymes located within the yeast cell vacuole. These endogenous enzymes then break down and solubilize yeast proteins and genetic materials. The typical protease type enzymes located in a *Saccharomyces cerevisiae* vacuole are identified in Table 1. The vacuole plays an important role in several functions including protein degradation. This allows the yeast cell to maintain protein homeostasis by degrading senescent, superfluous, and damaged proteins and organelles (Hecht et al., 2014). Of these seven enzymes, four have been identified to be key contributors in the autolyzation process for yeast extracts including Proteinase A, Proteinase B, Carboxypeptidase Y, and Carboxypeptidase S (Cook, 1958). The activation of these enzymes is also triggered during nutrient stress events and the yeast breaks

down internal proteins so the amino acids may be recycled to maintain vital functions (Thumm, 2000). It is this function that allows the manufacturing of yeast extracts. Once the cell dies and the vacuole can no longer keep these proteases isolated from the rest of the cell, widespread protein degradation occurs along with cell lysis. The RNA within the yeast is also targeted by these proteases and degraded.

Table 1. Saccharomyces cerevisiae vacuolar proteases (Hecht, 2014)

Protease	Activity	Function	Known P1 site amino acids
Proteinase A	aspartyl endoprotease	Initiator of protease activation cascade; protein degredation	Phe, Leu, Tyr, Trp, Thr, Asn, Gln, Glu, Lys, Ala, Ile
Proteinase B	serine endoprotease	protease activation; protease degredation	Leu, Arg, Phe, Tyr, Gln, Lys
Carboxypeptidase Y	serine carboxypeptidase	peptide degredation	Ala, Gly, Val, Leu, Ile, Met, Phe
Carboxypeptidase S	Zinc metalloprotease	peptide degredation	Gly, Leu
Aminopeptidase I	Zinc metalloprotease	glutathione degredation	Leu, Cys/Gly
Aminopeptidase Y	metalloprotease	unknown	Pro, Ala, Leu, Met, Phe, Tyr, Ser, Lys, Arg
Dipeptidylaminopeptidase B	serine dipeptidase	unknown	Xaa-Ala, Xaa-Pro

The industry has become more efficient and controlled with the utilization of exogeneous enzymes purchased from industrial suppliers. These supplier culture organisms that naturally provide high concentrations of a specific enzyme. The target enzymes are then isolated and purified for use in food manufacturing. These exogenous enzymes break down proteins and hydrolyze RNA. A typical process includes, cell rupturing, protease treatments to solubilize proteins, 5'-phosphodiesterase treatment to hydrolyze RNA into 5'-Nucleotides 5'-GMP, deaminase treatment to convert adenosine 5'-monophosphate (AMP) into 5'- IMP, and lastly, utilization of glutaminase to transform the amino acid, glutamine, into the flavor enhancing amino acid, L-glutamate (Chae et al., 2001). This process can exist with some or all the enzymes listed.

For processes that specifically target the hydrolysis of RNA with the 5'-phosphodiesterase enzyme, creating 5'-Nucleotides, the endogenous enzymes located in the

vacuole must be deactivated to prevent degradation to the RNA. This is typically achieved by exposing the yeast cream to temperatures above 90°C for approximately 20 minutes. These steps occur within a solution of yeast and water. At the culmination of the processing steps, a separation technique was applied to remove insoluble cell wall material from the water-soluble fraction which is the yeast extract. The cell wall fractions are a byproduct of this type of manufacturing. The typical composition of a yeast extract can be seen in Table 2.

Table 2. Major Components of Yeast Extract

Component	**% (Dry Solids Basis)**
Carbohydrates	28.67%
Fat	0.54%
Fiber	12.46%
Protein	58.98%

Yeast cell walls are approximately 15-30% of the dry weight of the cell and are composed mostly of mannoprotein, β-1,3-glucan, branched β-1,6-glucan, and chitin (Lipke & Ovalle, 1998). The typical composition of yeast cell walls is listed in Table 3. Mannoproteins are proteoglycans, meaning they are a combination of polysaccharides and proteins and consist of approximately 20% protein and 80% D-mannose (Moreno, 2012). Chitin is a β-1,4 polymer of N-acetylglucosamine which forms complexes with β-1,3-glucans (Lipke & Ovalle, 1998). This complex is a major portion of the inner yeast cell wall. β-glucans are polysaccharides made up of glucose units. Yeast β-glucans are located within the cell wall of the yeast and to efficiently isolate this material, disruption of the cell wall is suggested. The ruptured cell wall

allows for the intracellular components to be removed from the functional ingredient stream and increased surface area of the cell wall for further processing and isolation of the yeast β-glucans.

Yeast β-glucans have shown to offer extra health benefits due to the larger molecular size and variation in structure compared to other glucans available from barley, oat, and bacteria (Bzducha-Wróbel et al., 2015). This material has biological activities in the form of dietary fiber and functionality as an antioxidant. The addition of this ingredient into foods can provide additional healthy attributes as a prebiotic for enhanced gut health (Bacha et al., 2018). Prebiotics are sources of dietary fiber that promotes the growth and function of certain microflora while traveling through the digestion tract and can increase the health of the host (Damodaran & Parkin, 2017). Yeast β-glucan showed promising results in mice by elevating anti-inflammatory cytokines. Additionally, it had a higher antioxidant capacity compared to oat and mushroom β-glucans. The bioactivity of the β-glucan was also proportional to the concentration within the diets consumed (Haiyasut et al., 2018). The water binding capability of yeast glucans provides additional food functionality which was highlighted in yogurt manufacturing research. The addition of yeast β-glucan was introduced during fermentation. The significance of the added β-glucan from spent brewer's yeast created a faster gelation framework during the fermentation process. This framework lead to a faster pH drop and overall quicker fermentation. This could save significant processing time in industry and correlate to lower production costs. In addition to the gelation effect, the prebiotic nature of the added dietary fiber may also be promoting microbial growth and efficiency which is increasing the fermentation rate (Raikos et al., 2018). Added β-glucan to the formulation increases the nutritional value of the yogurt even without the fermentation benefits identified.

Component	Mean molecular mass (kDa)	% of wall mass
β-1,3-glucan	240	50
β-1,6-glucan	24	10
Mannoprotein	100–200	40
Chitin	25	1–3

Table 3. Major Components of Saccharomyces cerevisiae Cell Walls (Lipke & Ovalle, 1998)

The β-glucan within the cell wall, using the standard autolyzation processing methods, have been reported in many published research papers. The concentration of β-glucan within the cell walls of the baker's yeast after the use of exogeneous enzymes has not been explored based on literature review at the time of this publication. Understanding the effect of modern yeast extract processing methods and the remaining concentrations of β-glucans can provide insight on the value of this yeast extract's waste stream for industry. Additionally, there is potential for some β-glucan to become solubilized into the yeast extract fraction during heating steps. To account for potential heat induced β-glucan solubility, the yeast extracts will also be tested to account for any yield loss in the cell wall fraction and potentially identify an added nutritional benefit in the yeast extract itself.

Three techniques for exogenous enzyme processes will be utilized in this research. Technique 1 will consist of only exopeptidase and Technique 2 will consist of an endoprotease and exopeptidase. Both techniques were designed to mimic the activity the yeast endogenous enzymes within the cell vacuole function during autolyzation and usage rates of enzymes are based on the manufactures recommendation (Amano Enzyme USA). These enzyme treatments

are also expected to deliver a high degree of hydrolysis of yeast proteins to increase solubility into water. Technique 3 was designed to perform a more specified approach to the processing of the yeast extract with the goal of keeping the RNA intact and protein hydrolysis. Only an endo-protease enzyme was used in this system for the protein degradation. Additional enzymes were introduced for RNA hydrolysis to create additional flavor enhancement capabilities (5'-GMP + 5'-IMP). The enzyme usage rates for this technique were also based on the manufacturers recommendation (Amano Enzyme USA). Techniques 1 and 2 are expected to solubilize more protein from the yeast cell than Technique 3. This was based on the functionality of the enzymes and the higher usage rates. The high protein hydrolysis and solubilization can increase the overall concentration from the cell wall fractions produced from the yeast extraction process and deliver a higher overall β-glucan concentration.

In complicated food systems such as yeast and yeast extracts, the use of multiple analytical methods is required to identify effectiveness of processes and the determination of results. The solubilization of yeast components was based on looking at the solids yield, solids in compared to solids out approach. This value determined how much of the solids were removed from the yeast cell into the water fraction, which is the yeast extract.

The Kjeldahl method determines total nitrogen content which was used to determine protein content using a conversion factor that converts percent nitrogen to percent protein. Sulfuric acid along with a catalyst, $K_2SO_4/CuSO_4$, digests a sample converting nitrogen containing organic compounds into ammonium sulfate (Schuck et al., 2012).

$$\text{Organic Compounds} + H_2SO_4 \longrightarrow (NH_4)_2SO_4 + CO_2 + H_20$$

The addition of sodium hydroxide to the reaction mixture releases ammonia, then distilled and collected into hydrochloric acid. The titration of this mixture using sodium hydroxide results in identifying the total nitrogen.

$$(NH_4)_2SO_4 + 2NaOH \longrightarrow Na_2SO_4 + 2NH_3 + 2H_2O$$

Total nitrogen (TN) analysis was used to determine crude protein (CP) content using a standard conversion factor (CP=TN x 6.25). This information provides an understanding of how much protein was and was not solubilized by the enzymes to determine efficiency within individual fractions. This conversion factor is assuming that approximately 16% of protein is nitrogen and all nitrogen in the system originates from protein even if non-protein nitrogen exists which may overestimate the protein value in this system but this is an approved AOAC 984.13, Kjeldahl method (Gosukonda, 2020).

The purpose of the enzymatic treatments on the yeast cells is to solubilize the protein through degradation mechanisms. By cleaving the proteins and creating peptides and free amino acids, the characteristic of the protein's changes from hydrophobic to hydrophilic. This physical change permits the movement of these biological components into the water fraction which is the yeast extract. By allowing the proteins to be solubilized and pulled away from the insoluble cell wall fraction, the remaining β-glucans can become more concentrated. The Kjeldahl method was used to calculate the total nitrogen in the yeast, yeast extract, and cell walls. This data can be used to understand what further treatments can be applied to the cell walls to ensure all proteins are solubilized and removed.

Lastly, we identified the individual β-glucans in the system and quantified their concentrations. This required two types of analysis, total glucan determination and Fourier-

transform infrared spectroscopy (FTIR). The Megazyme Enzymatic Yeast β-Glucan Assay Procedure (K-EBHLG 01/20) was utilized to determine the total glucan of the yeast cell walls and water-soluble yeast extraction fractions. The various glucans within the fractions, β-1,3/1,6-glucan, β-1,3/1,4-glucan, and β-1,3-D-glucan are solubilized in 2 M potassium hydroxide (McCleary & Monaghan, 2002). The Glucazyme™ enzyme mixture was added to break down the β-glucan into D-Glucose. The GOPOD reagent has two steps:

1. The D-Glucose was oxidized by glucose oxidase to form hydrogen peroxide (H_2O_2) and D-gluconolactone.
2. The H_2O_2 reacts with 4-aminoantipyrine and p-hydroxybenzoic acid in the presence of peroxidase which produces a pink colored quinonimine dye (Fernando & Soysa, 2015). This can be measured with a spectrometer at 510 nm to determine concentration.

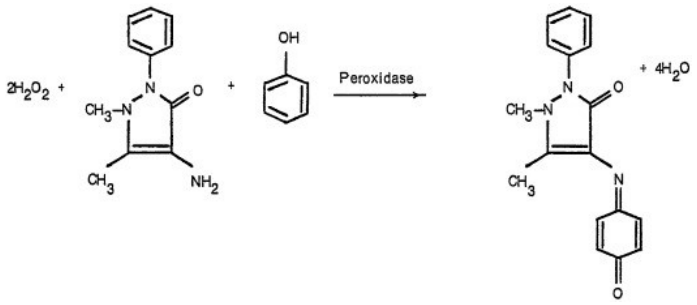

Figure 1. Mechanism for the reaction between hydrogen peroxide, 4-aminoantipyrine, p-hydroxybenzoic acid, and peroxidase (Fernando & Soysa, 2015).

A Thermo Scientific™ Nicolet iS 10 FTIR Spectrometer was used to determine glucan ratio in the dried samples of yeast extracts and cell walls. The instrument was also equipped with Attenuated Total Reflectance (ATR) technology and a diamond crystal platform. OMNIC™

Spectra™ software and TQ Analyst EZ software were used to capture the spectra and perform spectral analysis. A schematic of an FTIR analyzer can be seen in Figure 2.

This analysis included peak identification and area calculations of specified peaks. A baker's yeast glucan sample, G5011-25MG, was purchased for glucan peak identification (Sigma-Aldrich). Iso-propyl alcohol solution was used to clean FTIR sample.

Infrared (IR) spectroscopy is a technique of analyzing materials in both a quantitative and qualitative manner. The IR spectra can be divided into three regions (Rees, 2010):

- Far Infrared (400 – 10 cm^{-1})
- Mid-Infrared (4000 – 400 cm^{-1})
- Near Infrared (14285 – 4000 cm^{-1})

IR spectroscopy measures the absorbance of different frequencies of energy in the IR spectrum by molecules in solids, liquids, or gases (Rodriguez-Saona et al., 2017). The absorption can be quantified by detecting the amount of absorbed light energy. These frequencies correspond to the ground state and several excited states. A molecular vibration increase can be measured by exciting the bond by having it absorb light energy. The typical FTIR spectrometer includes an energy source, beamsplitter, sample cell, and a detector (Shukla & Iravani, 2018).

The total glucan analysis quantified the amount of glucan in a sample. The FTIR determined the ratio of different glucans in the samples. When the data was combined, a concentration of individual components was calculated. This approach is referenced in Chaiyasut et al. (2018).

The 5'-phosphodiesterase enzymatic hydrolysis of the yeast RNA efficiency was determined using a High Performance Liquid Chromatography (HPLC) methodology. The 5'-nucleotide composition was analyzed on a Thermo Scientific Dionex Ultimate 3000 UHPLC instrument system. The system consisted of a pump, autosampler, and a diode array detector (Figure 3). Thermo Scientific Dionex Chromeleon 7.2 software was utilized for running samples, data processing, and quantitative analysis.

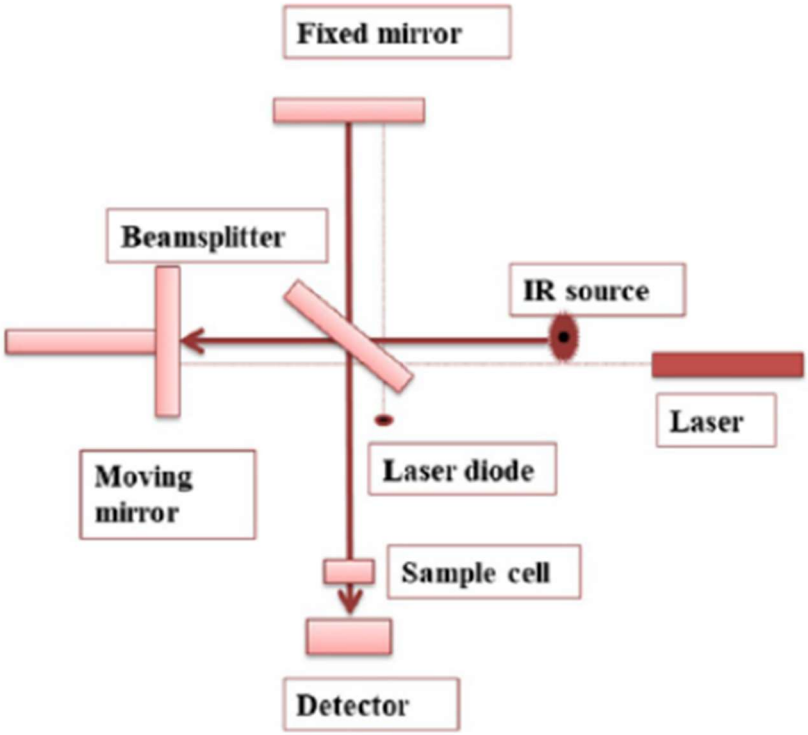

Figure 2. Schematic diagram of FTIR (Shulka & Iravani, 2018)

The use of HPLC to separate and determine species in biological materials is essential to this analysis. HPLC is a type of chromatography that employs a liquid mobile phase and a very finely divided stationary phase with high pressures to obtain satisfactory flow rates (Skoog, 2004). The ranges for this analysis are between 2000-3000 psi.

The analytical hierarchy for all samples can be seen in Figure 4.

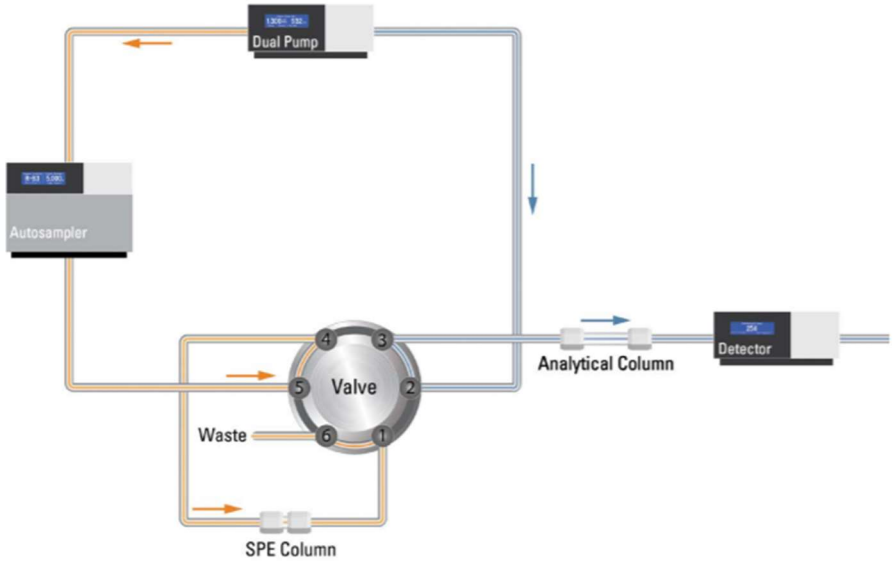

Figure 3: Dionex Ultimat 3000 HPLC Diagram

1.2 Objectives

The objective of this research was to identify how exogeneous enzyme treatments on *Saccharomyces cerevisiae* yeast cells impact the concentration of β-glucan, specifically, branched β-1,3/1,6-glucan, within the remaining cell wall post yeast extraction processes. Table 3 shows the major components of the cell wall which highlights the concentrations of the β-glucans. Research has shown the effectiveness of autolyzation processes which account for endogenous enzymes within the cell vacuole doing the work to solubilize the proteins both attached and non-attached to the cell walls. Current industrial process associated with yeast extraction manufacturing primarily use exogeneous enzymes isolated from other microorganisms

as the main pathway for protein solubilization and cell wall rupturing. To effectively identify how the β-glucan concentration varies using modern yeast extraction methodologies, evaluations to determine the overall nutritional impacts are necessary.

The two aims of this research are:

1. Determination of the total polysaccharide and β-1,3/1,6-glucan concentration of the yeast cell walls post processing of yeast extracts utilizing exogeneous enzyme processing techniques.
2. Identification and quantification of soluble β-glucan concentration existing in the yeast extract fraction.

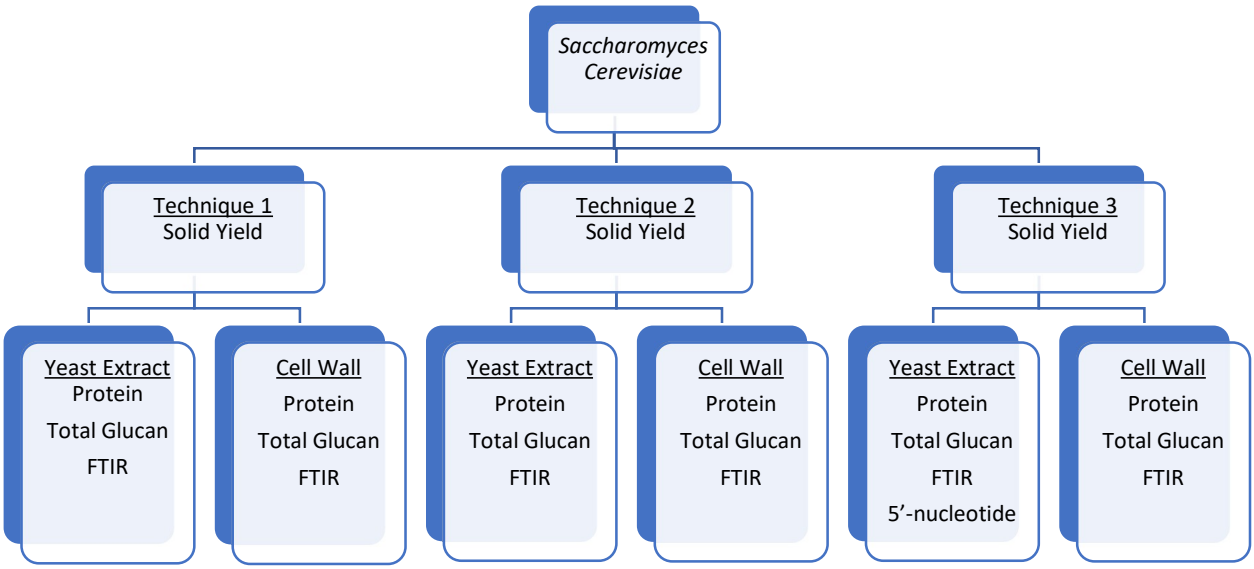

Figure 4. Analytical Hierarchy

Chapter 2. Experimental

2.1 Yeast Extraction Process

2.1.1 Materials

Saccharomyces cerevisiae yeast cream (Red Star, Product Code 84107), YL-T NAL (liquid endo-protease from *geobacillus stearothermophilus* – Amano, Japan), Umamizyme-K (Peptidase from *Aspergillus oryzae*-Amano, Japan), RP-1G-K (5'-phosphodiesterase from *Penicillium citrinum*-Amano, Japan), Deamizyme 50000G-K (Deaminase from *Aspergillus melleus*-Amano, Japan), sodium hydroxide (50%), temperature-controlled shaking water bath (VWR Shel Lab-1217), industrial size centrifuge (IEC Universal Model), Corning PC620D stirring hot plate, CEM Smart Trac 6 solids analyzer, deionized water (DI)

2.1.2 Peptidase Enzymatic Treatment-Technique 1

Baker's yeast cream was added to a 2000 mL glass beaker and percent solids was determined using a CEM Smart Trac 6 solids analyzer. The baker's yeast cream was then standardized to 10% solids using DI water and diluting to 1000g. The mixture was heated to 90^{o}C for 20 minutes to deactivate the yeast endogenous enzymes. A stirring hot plate was used. A magnetic stir bar was utilized to ensure proper mixture and heat transfer during this step. After heat treatment, the yeast cream was cooled to 50°C and pH was adjusted to 6.0 using sodium hydroxide (50% conc.). The heated and pH adjusted mixture was then separated into 6 x 150g aliquots in 300 mL glass jars with screw top lids. Formula listed in Table 4. The samples were placed into a temperature-controlled shaking water bath. Protein hydrolysis and cell lysis was achieved with the addition of Umamizyme at 50^{o}C and held at temp for 16 hours while shaker oscillation was set at 90 RPMs. Enzyme dosage was determined on a yeast solids basis.

The Umamizyme enzyme was diluted with DI water to achieve a 10% solution (w/w). After the enzymatic processing of the yeast cream, the samples were heated to 90°C for 10 minutes in the water bath to deactivate enzymes. Samples were centrifuged for 20 minutes at 3,000 rpm using an industrial size centrifuge. Water soluble fraction was decanted, and weight was recorded. Cell wall fraction was mixed with an additional 100g of DI water and centrifuged again for 20 minutes. Water soluble fraction was combined with first fraction and weight was recorded. The cell wall fraction was saved for further analysis.

Table 4. Formulation of Peptidase Yeast Extract Process

Sample#	Yeast Wt. (g)	%Solids	10% w/w Umamizyme Soln. (g)
1	150	11.97%	3.59
2	150	11.97%	3.59
3	150	11.97%	3.59
4	150	11.97%	3.59
5	150	11.97%	3.59
6	150	11.97%	3.59

2.1.3 Protease and Peptidase Enzymatic Treatment – Technique 2

Baker's yeast cream was added to a 2000 mL glass beaker and percent solids was determined using a CEM Smart Trac 6 solids analyzer. The baker's yeast cream was then standardized to 10% solids using DI water and diluting to 1000g. The mixture was heated to 90°C for 20 minutes to deactivate the yeast endogenous enzymes. A stirring hot plate was used. A magnetic stir bar was utilized to ensure proper mixture and heat transfer during this step. After heat treatment, the yeast cream was cooled to 50°C and pH was adjusted to 7.0 using sodium hydroxide (50% w/w conc.). The heated and pH adjusted mixture was then separated into 6 x 150g aliquots in 300 mL glass jars with screw top lids. Formula listed in Table 5. The

samples were placed into a temperature-controlled shaking water bath. Protein hydrolysis and cell lysis was achieved with the addition of YL-T at 50°C and held at temp for 5 hours while shaker oscillation was set at 90 RPMs. Enzyme dosage was determined on a yeast solids basis. The Umamizyme enzyme was diluted with DI water to achieve a 10% solution (w/w). After the enzymatic processing with YL-T, the pH of the samples was adjusted to 6.0 using sodium hydroxide (50% soln.). Protein and peptide hydrolysis are continued with the addition of Umamizyme at 50°C and held at temp for 16 hours while shaker was set at 90 RPMs. Enzyme dosage was determined on a yeast solids basis. The Umamizyme enzyme was diluted with DI water to achieve a 10% solution (w/w). After the enzymatic processing of the yeast cream, the samples were heated to 90°C for 10 minutes in the water bath to deactivate enzymes. Samples were centrifuged for 20 minutes at 3,000 rpm using an industrial size centrifuge. Water soluble fraction was decanted, and weight was recorded. Cell wall fraction was mixed with an additional 100g of DI water and centrifuged again for 20 minutes. Water soluble fraction was combined with first fraction and weight was recorded. The cell wall fraction was saved for further analysis.

Table 5. Formulation of Endoprotease and Exopeptidase Yeast Extract Process

Sample#	Yeast Wt. (g)	%Solids	10%YL-T w/w Soln. (g)	10% w/w Umamizyme Soln. (g)
1	**150**	**11.99%**	**1.80**	**1.80**
2	**150**	**11.99%**	**1.80**	**1.80**
3	**150**	**11.99%**	**1.80**	**1.80**
4	**150**	**11.99%**	**1.80**	**1.80**
5	**150**	**11.99%**	**1.80**	**1.80**
6	**150**	**11.99%**	**1.80**	**1.80**

2.1.4 5'-Nucleotide Enzymatic Process-Technique 3

Baker's yeast cream was added to a 2000 mL glass beaker and percent solids was determined using a CEM Smart Trac 6 solids analyzer. The baker's yeast cream was then standardized to 10% solids using DI water and diluting to 1000g. The mixture was heated to 90ºC for 20 minutes to deactivate the yeast endogenous enzymes. A stirring hot plate was used. A magnetic stir bar was utilized to ensure proper mixture and heat transfer during this step. After heat treatment, the yeast cream was cooled to 50°C and pH was adjusted to 7.0 using sodium hydroxide (50% conc.). The heated and pH adjusted mixture was then separated into 6 x 150g aliquots in 300 mL glass jars with screw top lids. Formula listed in Table 6. The samples were placed into a temperature-controlled shaking water bath. Protein hydrolysis and cell lysis was achieved with the addition of YL-T at 50ºC and held at temp for 5 hours while shaker oscillation was set at 90 RPMs. Enzyme dosage was determined on a yeast solids basis. The YL-T enzyme was diluted with DI water to achieve a 10% solution (w/w). The samples were then heated to 65°C and pH was adjusted to 5.0 using hydrochloric acid (6M). RP-1 was diluted with DI water to a 5% solution and added for Nuclease treatment and held at temperature for 16 hours while shaker oscillation was set at 90 RPMs. Upon completion of this hold time, the temperature was lowered to 50°C and the pH was adjusted to 6.0 using sodium hydroxide (50% conc.). Deaminase was diluted with DI water to a 1% dilution and was added and held for 5 hours with shaker oscillation was set at 90 RPMs. After the enzymatic processing of the yeast cream, the samples were heated to 90°C for 10 minutes in the water bath to deactivate enzymes. Samples were centrifuged for 20 minutes at 3,000 rpm using an industrial size centrifuge. Water soluble fraction was decanted, and weight was recorded. Cell wall fraction was mixed with an additional 100g of DI water and centrifuged again for 20 minutes. Water soluble fraction was

combined with first fraction and weight was recorded. The cell wall fraction was saved for further analysis.

Table 6. Formulation of 5'Nucleotide Yeast Extract Process

Sample#	Yeast Wt. (g)	%Solids	10% w/w YL-T Soln. (g)	5% w/w RP-1 Soln. (g)	1% w/w Deaminase Soln. (g)
1	150	11.50%	1.29	0.69	0.35
2	150	11.50%	1.29	0.69	0.35
3	150	11.50%	1.29	0.69	0.35
4	150	11.50%	1.29	0.69	0.35
5	150	11.50%	1.29	0.69	0.35
6	150	11.50%	1.29	0.69	0.35

2.2 TOTAL NITROGEN CONTENT

2.2.1 Materials

Foss Tecator Digester, Foss Tecator Digestion Block (heater) set at 420°C, 250 mL digestion tubes with rack, weigh boats, Whatman #4 70mm filter paper (cut in half), Kjeldahl tablets (FisherTab CT-37, 3.5 g K_2SO_4+0.4 g $CuSO_4$), concentrated sulfuric acid (95.0-98%), 50% sodium hydroxide, 500 mL Erlenmeyer flasks, 0.1 N hydrochloric acid, 0.1 N sodium hydroxide (NaOH), 0.1% methyl red indicator solution, analytical balance, and DI water.

2.2.2 Kjeldahl Method

2.2.3 Procedure

Samples were weighed onto filter paper and then placed into 250 mL digestion tubes. Two Kjeldahl tablets are added to each tube along with 15 mL of concentrated sulfuric acid. The digestion tubes were place onto the heating block (Figure 5) which was set to 420°C. Once the

temperature was attained, the digestion process was initiated and allowed to react for 60 minutes. Upon reaching required time requirements, the digestion tubes were to cool to room temperature prior to distillation.

Figure 5: FOSS Heating Block

The Foss auto-distillation unit (Figure 6) was turned on 30 minutes prior to use. Once the unit was warmed up, two blank tubes were run to ensure steam generator and pumps are primed for sample digestion tube. A 500 mL Erlenmeyer flask was filled with 50 mL 0.1 N HCl and 3 drops of the methyl red indicator solution. The flask was placed in the unit to collect the distillate from the sample. The sample tube that has completed the digestion process was removed from the rack and placed into the distillation unit. To each tube, 80 mL of DI water and 50 mL of 50% NaOH was added to the tubes. This was completed through an internal automated program. This should change the color of the sample to a black solution to signify the mixture has been effectively neutralized. The steam generator was activated by the prog, and steam was injected through the mixture for 4 minutes. During this time, the steam with the dissolve ammonia was condensed through a cold-water condenser and collected in the receiving flask

with the indicator. The flask was then titrated to a clear-yellow endpoint using 0.1 N HCl and the volume of titrant was recorded.

Figure 6: FOSS Automatic Distillation Unit

To calculate the total nitrogen content of each sample, the following formula was used:

$$TN = \frac{14.007 \times (V_a - V_b) \times \mathrm{N}}{w_s}$$

Figure 7. Total Nitrogen Calculation (Shuck et al., 2012)

Where TN is Total Nitrogen expressed as grams per kilograms of sample, 14.007 is the atomic mass of Nitrogen, V_a is the volume in mL of 0.1 N acid solution added to receiving flask, V_b is the volume in mL of 0.1 N Sodium Hydroxide added, N is the normality of the acid/base used for titration (0.1 N), and w_s is the weight of the sample in grams.

Once the TN was determined for the sample, the value was multiplied by 6.25 which was a generic conversion factor used to calculate %Crude Protein (CP) for %Total Nitrogen (TN).

$$CP = TN \times 6.25$$

2.3 Total Glucan Analysis

2.3.1 Materials

Glucazyme™(exo-1,3-β-glucanase, endo-1,3-β-glucanase, β-glucosidase and chitinase suspension), GOPOD Reagent Buffer (p-hydroxybenzoic acid and sodium azid), GOPOD Reagent Enzymes (Glucose Oxidase plus Peroxidase and 4-aminoantipyrine), D-Glucose Standard Solution (1.5 g/mL), control fungal β-glucan (43%), round bottom glass test tubes (16x125 mm), test tube caps, variable volume pipettors w/disposable tips, Mettler Toledo analytical balance, Beckman Coulter DU520 UV-VIS Spectrophotometer, vortex mixer, temperature-controlled shaking water bath (VWR Shel Lab-1217), Thermo Sorvall ST8 benchtop centrifuge, 15 mL conical centrifuge tubes, glacial acetic acid, NaOH (50%), potassium hydroxide (KOH) (45%), DI water

2.3.2 Reagents

2.3.2.1 GOPOD Reagent

GOPOD Reagent Buffer (50 mL) was added to a 1 L volumetric flask and diluted with DI water. After mixing, the diluted GOPOD Reagent Buffer was poured into a 2000 mL glass beaker. The GOPOD reagent enzymes were then added to the beaker and mixed.

2.3.2.2 Sodium Acetate Buffer (200 mM, pH 5.0)

Glacial acetic acid (11.6 mL) was added to 900 mL of DI water in a 1000 mL glass beaker. The pH was adjusted to pH 5.0 using 4 M NaOH. The buffer was then poured into a 1 L volumetric flask and diluted to mark with DI water.

2.3.2.3 Sodium Acetate Buffer (1.2 M, pH 3.8)

Glacial acetic acid (68.6 mL) was added to 800 mL of DI water in a 1000 mL glass beaker. The pH was adjusted to pH 3.8 using 4 M NaOH. The buffer was then poured into a 1 L volumetric flask and diluted to mark with DI water.

2.3.2.4 Potassium hydroxide (2 M)

Potassium hydroxide-45% (171.3 mL) was added to 800mL of DI water in a 1000 mL glass beaker. Contents of beaker were then poured into a 1 L volumetric flask and filled to mark with DI water.

2.3.2.5 Sodium hydroxide (4 M)

Sodium hydroxide-50% (20.9 mL) was added to 80 mL of DI water in a 100 mL glass beaker. Contents of beaker were then poured into a 100 mL volumetric flask and filled to mark with DI water.

2.3.3 Procedure

The yeast extract, yeast cell wall, and standards were dried in a vacuum oven set at 70°C for 16 hours. Approximately 20 mg of each dried sample were weighed into a 16x100 mm culture tubes. The 2 M potassium hydroxide (0.4 mL) was added to each tube and mixed for 30 minutes in an ice water bath. After the hold time was completed and the samples were still in the ice bath, 1.6 mL of the 1.2 M sodium acetate buffer was added to each sample tube and mixed using the vortex mixer. 40 uL of the Glucazyme^{TM} was then added and tubes were capped. The sample tubes stayed in the ice water bath for an additional two minutes while mixing. The tubes were then transferred to a temperature controlled heated water bath set at 40°C and incubated for 16 hours.

When the incubation period was completed, 10 mL of DI water was added to each tube. The contents were mixed using a vortex mixer were transferred to a 15 mL conical centrifuge tube. The tube was then placed into a benchtop centrifuge and centrifuged for 10 minutes at 3000 rpm. 0.1 mL aliquots of each centrifuge tube are transferred to a new 16x100 mm glass test tube. 4 mL of GOPOD reagent was added to each test tube including a reagent blank and a D-glucose standard. The reagent blank consisted of 0.1 mL of sodium acetate buffer (200 mM, pH 5.0) plus 4.0 mL GOPOD reagent. The D-glucose standard consists of 0.1 mL D-glucose standard (1.5 mg/mL) plus 4.0 mL GOPOD reagent. The tubes were placed into a temperature controlled heated water bath and incubated at 40°C for 20 minutes.

During incubation, the spectrophotometer was turned on and allowed to warm up for 30 minutes. The wavelength was set to 510 nm. The reagent blank sample was placed into a plastic cuvette and then placed into the instrument. The absorbance reading was zeroed. Absorbance was measured for each sample and the value was recorded. The same cuvette was used for all absorbance measurements and rinsed three times with DI water in between samples.

Using the following formula, the %w/w of total β-Glucan can be calculated (McCleary & Monaghan, 2002):

$$\beta\text{-Glucan (\%w/w)} = \Delta E \times F \times 12.04/0.1 \times 100/W \times 1/1000 \times 162/180$$

Where ΔE = absorbance read against reagent blank; F = conversion from absorbance to µg = 150 µg of D-glucose standard divided by absorbance of 150 µg reaction with GOPOD; 12.04/0.1 = volume correction (0.1 mL taken from 12.04 mL); 100/W = factor to present β-glucan as a percentage of sample weight; 1/1000 = conversion from µg to mg; 162/180 = factor to convert from free D-glucose to anhydro-D-glucose as occurs in β-glucan.

2.4 Fourier Transform Infrared Spectroscopy (FTIR) Glucan Ratio Analysis

2.4.1 Procedure

The samples needed to be dried prior to analysis on the FTIR to increase concentration of targeted β-Glucans. The yeast extract, yeast cell wall, and standards were dried in a vacuum oven set at 70°C for 16 hours.

The crystal was cleaned using iso-propyl alcohol and a Kimwipe. Then, an IR background scan was completed prior to analysis to identify environmental spectra. A small amount of dry sample and standard powders were placed on the crystal platform one at a time. A mechanical press compacted the sample over the platform to prep the sample for analysis. The ratio of glucan in the samples was analyzed the FTIR spectrometer using a spectral range of 4000 – 400 cm^{-1} at a 4 cm^{-1} resolution. The instrument performed 32 scans for each sample. The spectral data was processed for baseline correction and normalization using the OMNIC™ Spectra™ software. The TQ Analyst EZ software was used to identify the peaks and calculate their area with peak resolve analysis.

2.5 5’-Nucleotide Analysis Using HPLC

2.5.1 Materials

Cytidine 5'-monophosphate disodium salt (CMP), guanosine 5'-monophosphate disodium salt (GMP), uridine 5'-monophosphate disodium salt (UMP), 5’-inosine monophosphate disodium salt (IMP), 0.1N HCl, 50% NaOH. variable volume pipettors w/disposable tips, Mettler Toledo analytical balance, 15 mL conical centrifuge tubes, 0.45-micron syringe filter, ammonium phosphate (HPLC Grade), phosphoric acid (HPLC Grade, 85%).

2.5.2 Reagents

2.5.2.1 Stock Solution of 5'-Nucleotide salts (100 ppm)

Four separate stock solutions were created for each 5'-Nucleotide salt (5'-CMP, 5'-GMP, 5'-IMP, 5'-UMP). 100 mg of the Nucleotide salt was added to a 1000 mL volumetric flask and diluted with DI water to mark. The flasks were mixed well by inverting until the salt was fully dissolved.

2.5.2.2 Preparation of 5'-Nucleotide Standard

4 mL of each 5'-Nucleotide stock solution was pipetted to a common 250mL volumetric flask. Then 25 mL of 0.1N HCl was added to the flask. Flask was filled with DI water to mark and mixed well. A 0.45-micron syringe filter was used to fill HPLC vial and then placed into the HPCL auto sampler.

2.5.2.3 Ammonium Phosphate Buffer (125 mM, pH 3.00)

28.75g of HPLC grade ammonium phosphate monobasic (Fluka) was weighed into a 2000 mL volumetric flask and filled to mark with DI water. The solution was mixed well until all salt crystals were fully dissolved. The solution was then transferred to a 2000 mL glass beaker and pH was adjusted to 3.00 using HPLC Grade phosphoric acid (85%). The pH adjusted buffer was then poured through a 0.45-micron filter with the aid of a vacuum filter system.

2.5.3 Procedure

2.5.3.1 Sample preparation

Approximately 100 mL of each 5'-Nucleotide yeast extract sample was poured into a 100 mL glass beaker. 50% NaOH was used to adjust the pH of each sample to 8.50. The pH adjusted sample was poured into a 15 mL centrifuge tube and centrifuged for 10 minutes at 4000 RPMs. After the sample was centrifuge, 4.0 mL was pipetted into a 250 mL volumetric flask. 25 mL of 0.1 N HCl was added to the flask and then filled to mark with DI water, mixed well. A 0.45-micron syringe filter was used to fill HPLC vial and then placed into the HPLC auto sampler.

2.5.3.2 Analysis

Standards and samples were analyzed under the following conditions: stationary phase, Syncronis Amino 5µ 250 × 4.6 mm (Fisher Scientific). Flow rate: 1.0 mL/min. Oven temperature: 25°C. UV detector: detection wavelength 254 nm for 5'-UMP, 5'-GMP and 5'-AMP, and 5'-CMP (Hua & Huang, 2010). Injection volume: 100 µL. Run time: 20 min. Mobile phase: 125 mM ammonium phosphate buffer pH 3.0.

Each individual 5'-Nucleotide was identified in the standard and the peaks were assigned with their corresponding identifier (CMP, UMP, IMP, GMP). The calculations were carried out by comparing the individual peak areas from the standard and samples.

2.6 Statistical Analysis

The results obtained from analysis were expressed as the mean ± standard deviation of the six replicates within the sample set. Statistical analysis was completed using Microsoft Excel for Office 365. Data was analyzed using ANOVA single factor calculations to determine significant difference between data sets. If significance was identified, $p < 0.5$, *post-hoc* analysis was used to pair two data sets together at a time and performing a t-Test: Two-Sample Assuming

Equal Variance (Thumm, 2000). Grubbs Outlier Test was applied to the sample sets for each technique to identify any outlier data using Minitab.

Chapter 3. Results

3.1 Soluble Solids Yield Based on Enzyme Treatment

The yeast extraction process was performed with the goal to solubilize the insoluble yeast components specifically proteins. The efficiency of this process was identified by calculating the solids captured within the yeast extract fraction and compare it to the total solids that went into the system from the yeast prior to enzyme treatments. This calculation identified the solids yield for each extraction process. When the solids analysis was performed, the weight of yeast cream was annotated and removed from the yield calculation. Yield analysis data is presented in Tables 7-9.

Table 7. Solids Yield Analysis Data-Technique 1

Sample#	%Solids of Supernatant	Supernatant Total Wt. (g)	Total Solids (Supernatant Wt x Supernatant Solids% (g)	%Yield
1	6.75%	217.00	14.65	82.76%
2	6.86%	216.82	14.87	84.08%
3	6.75%	215.58	14.55	82.39%
4	6.77%	216.46	14.65	82.72%
5	6.91%	216.80	14.98	84.74%
6	5.84%	212.12	12.39	69.95%[1]

Notes: [1] %Yield value determined outlier (Figure 7). Removed from future calculations.

Table 8. Solids Analysis Data-Technique 2

Sample#	%Solids of Supernatant	Supernatant Total Wt. (g)	Total Solids (Supernatant Wt. x Supernatant %Solids) (g)	%Yield
1	6.25%	214.68	13.42	75.54%
2	6.46%	215.26	13.91	78.45%
3	6.55%	215.38	14.11	79.59%
4	6.50%	215.32	14.00	78.98%
5	6.29%	213.42	13.42	75.59%
6	6.51%	211.06	13.74	77.60%

Table 9. Solids Analysis Data-Technique 3

Sample#	%Solids of Supernatant	Supernatant Total Wt. (g)	Total Solids (Supernatant Wt x Supernatant Solids% (g)	%Yield
1	3.21%	215.1	6.90	39.00%
2	3.42%	212.2	7.26	40.98%
3	3.54%	215.46	7.63	42.90%
4	3.60%	214.96	7.74	43.63%
5	3.56%	213.82	7.61	42.93%
6	3.49%	219.1	7.65	43.10%

An outlier test was performed on the solid yield analysis results for all sample sets using Grubbs outlier test. This analysis was calculated in Minitab to determine if any of the values were considered an outlier. One value was identified, the solid yield in sample #6 of Technique 1 and was removed for further statistical analysis. The graphical representation can be seen in Figure 8.

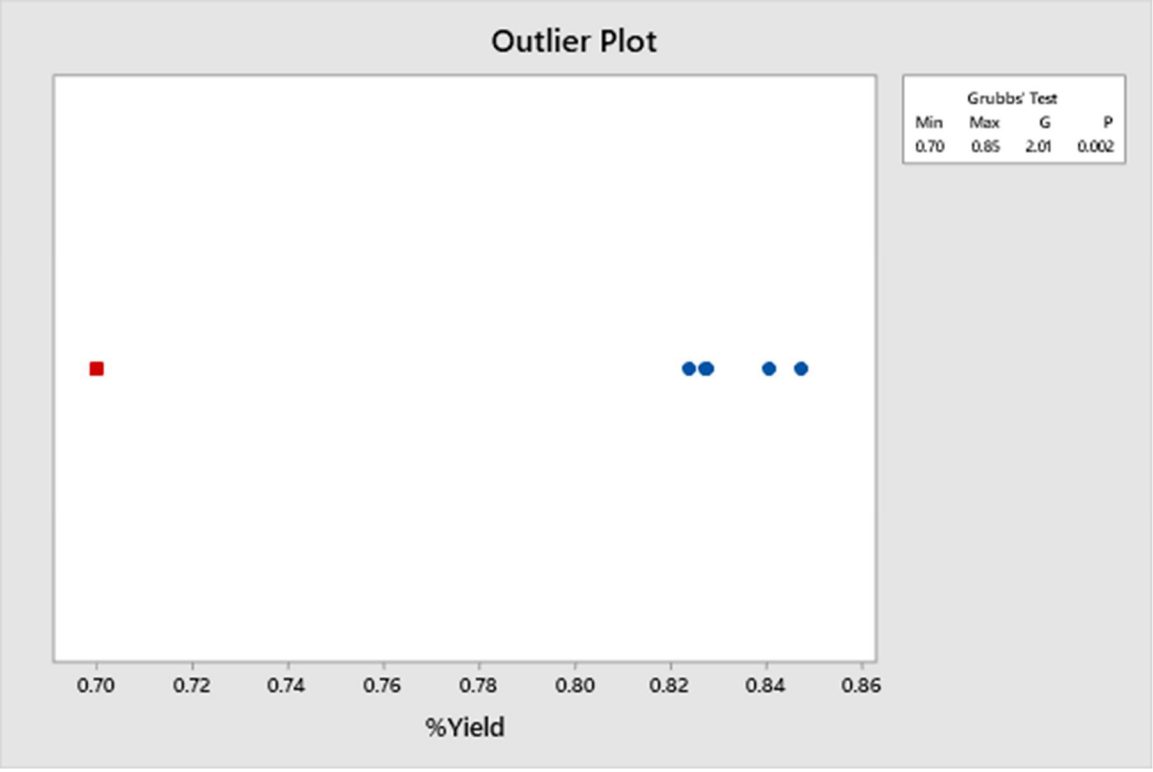

Figure 8. Outlier Plot for Technique 1 Yield Analysis

The highest solids yield achieved was produced in the 2% Umamizyme with an 83.34 ± 1.02% average. The 1% YL-T + 1% Umamizyme treatment resulted in a solid yield of 77.62 ± 1.73%, followed by the 5'-Nucleotide process which only captured 42.09 ± 1.76% of the initial solids that were added to the system. These values are depicted in Figure 9. The use of the exopeptidase within these systems shows the significant increase total soluble solids achieved compared to the system that used only the endo-protease (YL-T NAL). Statistical analysis of the yield data determined there was a significant difference between the yields produced in the 5'-Nucleotide yeast extracts compared to the other two. This same analysis also determined that there was no significance between the Umamizyme only extract process and the YL-T + Umamizyme process.

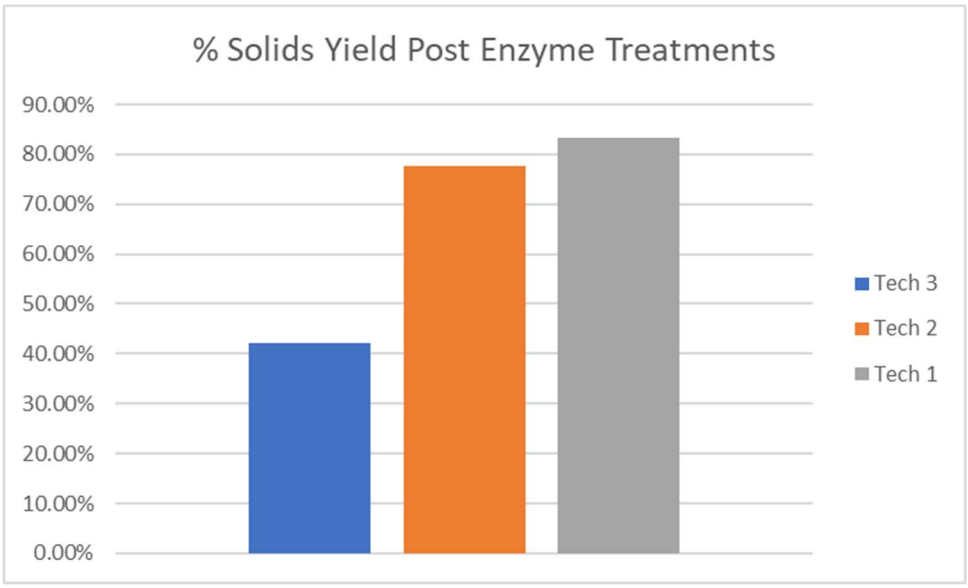

Figure 9. Solids Yield Results

The one-way ANOVA statistical analysis determined that there was significant difference within the data set of the solid yields results. Using a two-sample T-Test to compare each data solid yield data set to each other it was calculated that there was significant difference, $p<0.05$.

3.2 Determination of Crude Protein in the Yeast Extracts and Cell Wall Fractions

The protein values were standardized by calculating the protein on a dry solid basis (DSB). This value was obtained by dividing the crude protein value by the solid concentration of the samples. The total nitrogen and crude protein values for the cell wall fractions post process are listed in Tables 10-12. The yeast extract values are listed in Tables 13-15.

Table 10. Protein Analysis Data-Technique 1 Cell Walls

Sample #	Sample Wt. (g)	Titer (mL) 0.1N NaOH	%TN	%Crude Protein (TNx6.25)	%Solids	%Protein (DSB)
1	0.770	46.60	0.62	3.87	11.67%	33.13
2	0.754	47.00	0.56	3.48	11.32%	30.78
3	0.786	46.60	0.61	3.79	11.46%	33.05
4	0.783	46.70	0.59	3.69	11.42%	32.32
5	0.752	46.10	0.73	4.54	12.04%	37.72
6	0.745	47.30	0.51	3.17	11.40%	27.84

Table 11. Protein Analysis Data-Technique 2 Cell Walls

Sample #	Sample Wt. (g)	Titer (mL) 0.1N NaOH	%TN	%Crude Protein (TNx6.25)	%Solids	%Protein (DSB)
1	1.723	41.45	0.70	4.35	15.49%	28.05
2	1.648	43.50	0.55	3.45	15.02%	22.99
3	1.616	43.60	0.55	3.47	13.75%	25.22
4	1.634	43.75	0.54	3.35	14.07%	23.80
5	1.672	43.85	0.52	3.22	13.33%	24.16
6	1.678	43.50	0.54	3.39	13.58%	24.98

Table 12. Protein Analysis Data-Technique 3 Cell Walls

Sample #	Sample Wt. (g)	Titer (mL) 0.1N NaOH	%TN	%Crude Protein (TNx6.25)	%Solids	%Protein (DSB)
1	1.397	29.80	2.03	12.66	27.70%	45.71

2	1.522	28.95	1.94	12.11	27.51%	44.02
3	1.795	25.70	1.90	11.85	27.62%	42.92
4	1.64	28.00	1.88	11.75	27.52%	42.68
5	1.285	31.65	2.00	12.50	28.07%	44.55
6	1.516	28.20	2.01	12.59	27.98%	45.00

Table 13. Protein Analysis Data-Technique 1 Yeast Extract

Sample #	Sample Wt. (g)	Titer (mL) 0.1N NaOH	%TN	%Crude Protein (TNx6.25)	%Solids	%Protein (DSB)
1	2.038	38.85	0.77	4.79	6.75%	70.97
2	2.029	38.75	0.78	4.85	6.86%	70.77
3	2.038	38.05	0.82	5.13	6.75%	76.06
4	2.052	39.10	0.74	4.65	6.77%	68.70
5	2.051	40.10	0.68	4.23	6.81%	62.06
6	2.043	40.80	0.63	3.94	5.84%	67.52

Table 14. Protein Analysis Data-Technique 2 Yeast Extract

Sample #	Sample Wt. (g)	Titer (mL) 0.1N NaOH	%TN	%Crude Protein (TNx6.25)	%Solids	%Protein (DSB)
1	2.028	40.75	0.64	3.99	6.25%	63.90
2	2.026	40.50	0.66	4.11	6.46%	63.56
3	2.024	40.60	0.65	4.07	6.55%	62.09
4	2.051	40.50	0.65	4.06	6.50%	62.40
5	2.06	40.40	0.65	4.08	6.29%	64.87
6	2.031	40.35	0.67	4.16	6.51%	63.91

Table 15. Protein Analysis Data-Technique 3 Yeast Extract

Sample #	Sample Wt. (g)	Titer (mL) 0.1N NaOH	%TN	%Crude Protein (TNx6.25)	%Solids	%Protein (DSB)
1	2.027	44.35	0.39	2.44	3.21%	76.03[1]

2	2.023	44.70	0.37	2.29	3.42%	67.08
3	2.048	44.45	0.38	2.37	3.54%	67.03
4	2.037	44.50	0.38	2.36	3.60%	65.67
5	2.024	44.60	0.37	2.34	3.56%	65.62
6	2.044	44.75	0.36	2.25	3.49%	64.44

Notes: [1] *%Protein value determined outlier (Figure 8). Removed from future calculations.*

An outlier test was performed on the protein analysis results for all sample sets using Grubbs outlier test. This analysis was calculated in Minitab to determine if any of the values were considered an outlier. One value was identified, the protein value in sample #1 of Technique 3 and was removed for further statistical analysis. The graphical representation can be seen in Figure 10.

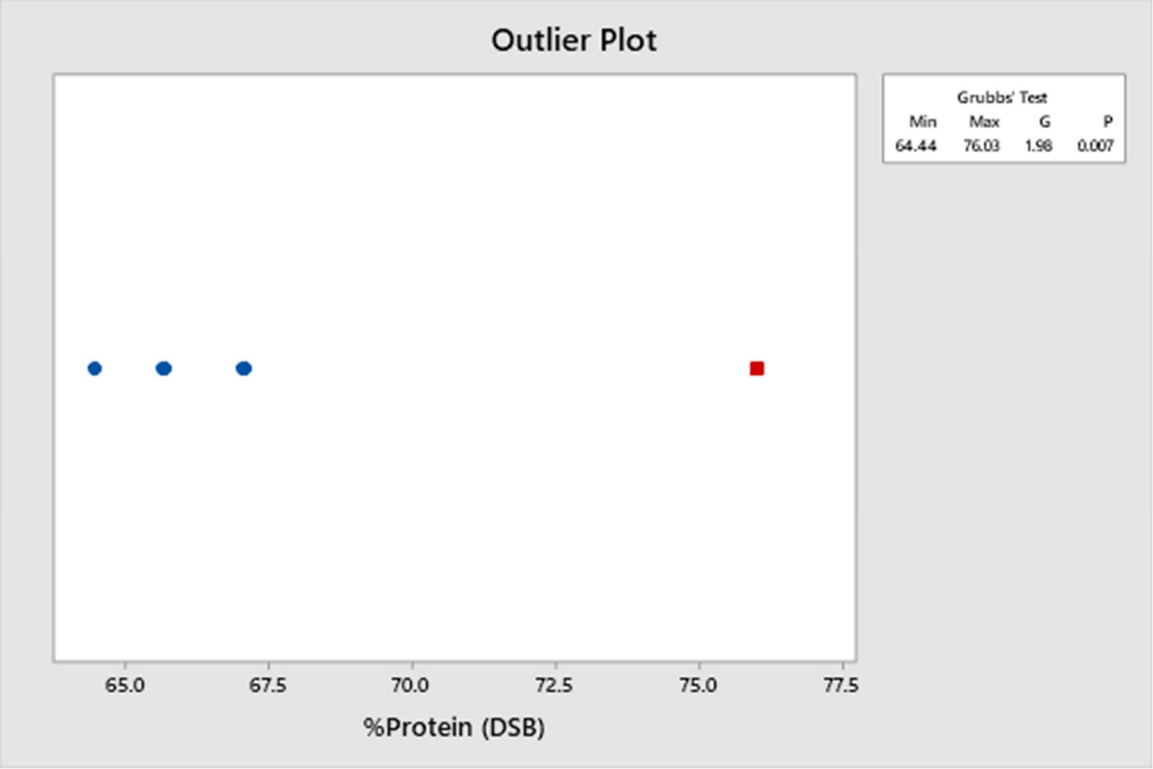

Figure 10. Outlier Plot of Protein Analysis Data for Technique 3 Yeast Extract

The one-way ANOVA statistical analysis determined that there was significant difference within the data set of the protein values for the protein within the cell walls. Using a two-sample T-Test to compare each protein data set to each other it was calculated that there was significant difference, $p<0.05$, between all groups. Applying the same statistical measurements significant difference was only calculated for the yeast extract from Technique 1, Technique 2 and 3 were not determined to be significantly different.

In addition to the cell walls and yeast extract protein analysis, the yeast cream used as the starting material for the processes was also tested. The initial protein values for the yeast was listed in Table 16.

Table 16. Yeast Crude Protein Results

Sample #	Sample Wt. (g)	Titer (mL) 0.1N NaOH	%TN	%Crude Protein (TNx6.25)	%Solids	%Protein (DSB)
Yeast 030221CBS1	0.769	40.89	1.66	10.37	17.99	57.66
Yeast 030121CBS2	0.784	39.70	1.84	11.50	17.76	64.77

3.3 Total Glucan Analysis of Yeast Cell Walls and Yeast Extracts

The total glucan analysis of samples was performed using the Megazyme Enzymatic Yeast β-Glucan Assay. This analysis determined total glucan content but did not differentiate between the types of glucan within the system. The spectrophotometer was used to obtain absorbance values in the visible light spectrum at a wavelength of 510nm. The absorbance values of the samples were compared to the known absorbance values of 150 µg of a D-glucose standard after it was reacted with the GOPD reagent. This standard was measured in quadruplicate and the average was used for "F" in the total glucan analysis calculation which

was referenced in the procedure explained earlier. The absorbance values of the D-glucose standard and their average are listed in Table 17. Using this calculated value, the total glucose can be determined within the individual samples. A 43% Yeast β-glucan Control was provided in the assay kit.

Table 17. Absorbance Measurements of D-Glucose Standard at 510nm

Rep. 1	Rep. 2	Rep. 3	Rep. 4	Average Abs
1.239	1.228	1.238	1.240	1.236

The outlier statistical analysis tool was used on all the data sets and determined that there were no outliers within the results for total glucan. The control sample was measured in duplicate which is based on the manufacturer's recommendation for the assay kit (Table 18). The moisture level of the control was 5.1%, when this value was applied to the calculated β-Glucan concentration it results in 43.01 g/100g calculated total glucan on a dry solid basis.

The enzymes used in the individual processing methods were tested to determine potential glucose contributions to the total glucan analysis. The four enzymes tested were also measured in duplicate (Table 19). This methodology was not applied to the different sample sets since there are six iterations of the same processing method. Tables 20-25 represent the data associated with the total glucan analysis for all the sample sets of the dried material. Drying the material was necessary to ensure concentration was high enough for analysis.

Table 18. Yeast β-Glucan Control

	Wt. (mg)	Rep 1 Abs.	Rep 2 Abs.	β-Glucan (g/100g) Rep. 1	β-Glucan (g/100g) Rep. 2	Average
Yeast β- Glucan Control (43%)	21.5	0.664	0.671	40.61	41.03	40.82

Table 19. Total Glucan Analysis of Enzymes

Enzyme	Wt. (mg)	Rep 1 Abs.	Rep 2 Abs.	β-Glucan (g/100g) Rep. 1	β-Glucan (g/100g) Rep. 2	Average
YL-T NAL	31.1	0.012	0.013	0.51	0.55	0.53
Umamizyme	24.2	0.457	0.448	24.83	24.34	24.58
Deaminase	22.1	0.041	0.042	2.44	2.50	2.47
RP-1	25.6	0.080	0.074	4.11	3.80	3.95

Table 20. β-Glucan in Technique 1 Cell Wall Samples

	Sample#	Wt. (mg)	Abs.	β-Glucan (g/100g)
Technique 1: Cell Wall	1	23.5	0.458	25.62
	2	26.1	0.489	24.63
	3	22.8	0.422	24.34
	4	24	0.445	24.38
	5	23.4	0.431	24.22
	6	21.9	0.423	25.40
			Average	24.76
			Standard Deviation	0.60

Table 21. β-Glucan in Technique 2 Cell Wall Samples

	Sample#	Wt. (mg)	Abs.	β-Glucan (g/100g)
Technique 2: Cell Wall	1	22.1	0.391	23.26
	2	20.6	0.402	25.66
	3	23.3	0.492	27.76
	4	20.7	0.436	27.69
	5	23.1	0.422	24.02
	6	24.8	0.456	24.18
			Average	25.43
			Standard Deviation	1.94

Table 22. β-Glucan in Technique 3 Cell Wall Samples

	Sample#	Wt. (mg)	Abs.	β-Glucan (g/100g)
Technique 3: Cell Wall	1	26.6	0.31	15.32
	2	23.8	0.294	16.24
	3	21.5	0.282	17.25
	4	24.8	0.324	17.18
	5	23.3	0.285	16.08
	6	26.6	0.356	17.60
			Average	16.61
		Standard Deviation		0.87

Table 23. β-Glucan in Technique 1 Extract Samples

	Sample#	Wt. (mg)	Abs.	β-Glucan (g/100g)
Technique 1: Extract	1	21.4	0.059	3.62
	2	20.6	0.056	3.57
	3	21.5	0.052	3.18
	4	24.3	0.058	3.14
	5	21.2	0.056	3.47
	6	22.7	0.057	3.30
			Average	3.38
		Standard Deviation		0.21

Table 24. β-Glucan in Technique 2 Extract Samples

	Sample#	Wt. (mg)	Abs.	β-Glucan (g/100g)
Technique 2: Extract	1	24	0.06	3.29
	2	22.3	0.06	3.54
	3	21.2	0.05	3.10
	4	23.9	0.062	3.41
	5	21.2	0.054	3.35
	6	24.8	0.065	3.45
			Average	3.36
		Standard Deviation		0.15

Table 25. β-Glucan in Technique 3 Extract Samples

	Sample#	Wt. (mg)	Abs.	β-Glucan (g/100g)
Technique 3: Extract	1	21.7	0.023	1.39
	2	20.6	0.024	1.53
	3	24	0.027	1.48
	4	24	0.023	1.26
	5	20.1	0.021	1.37
	6	22.6	0.024	1.40
			Average	1.41
			Standard Deviation	0.09

Statistical analysis using a Two-Sample T-Test assuming equal variance, compared the data sets within each cell wall or extract analysis results for the total glucan concentrations. Techniques 1 and 2 showed no significant differences when both sample set results were compared to each other. The Technique 3 glucan concentrations in the cell wall and extract fractions were identified to be significantly different compared to Techniques 1 and 2.

3.4 FTIR Glucan Ratio Analysis Data

Full spectrums of each sample and a yeast β-glucan standard were scanned by the Thermo Scientific™ Nicolet iS 10 FTIR. The spectral range was set to 4000 – 400 cm^{-1} at a 4 cm^{-1} resolution and 32 scans were completed per sample. These scans were then processed using the OMNIC™ Spectra™ software. The data was then used within the TQ Analyst EZ software to identify the peaks and calculate their area with peak resolve analysis. Each technique's sample set were scanned individually and then the individual spectra were combined using the spectral averaging tool within the OMNIC software. The spectral range for these computations was adjusted to 1175-925 cm^{-1} to focus specifically the polysaccharide region associated with different types of β-glucans (β-1,6-glucans; β-1,3-glucans; β-1,4-glucans) (Haiyasut et al., 2018).

The approximate wave number associations are listed in Table 26 and Figure 11 depicts the full spectra scan of the Technique 2 cell wall samples.

Table 26. FTIR wave number glucan associations (Haiyasut et al., 2018)

Wave number (cm^{-1})	Assigned As
~985	β-1,6-glucan
~1038	β-1,4-glucan
~1111	β-1,3-glucan
~1161	β-1,3-glucan

The assigned wave numbers were then used to identify the related peaks in the FTIR spectra obtained for the samples. Peak resolve and normalization processes within the software were applied to the averaged spectrum to create a clear distinct peak area which would otherwise be hidden. This peak area was then used to determine glucan ratios for each sample set.

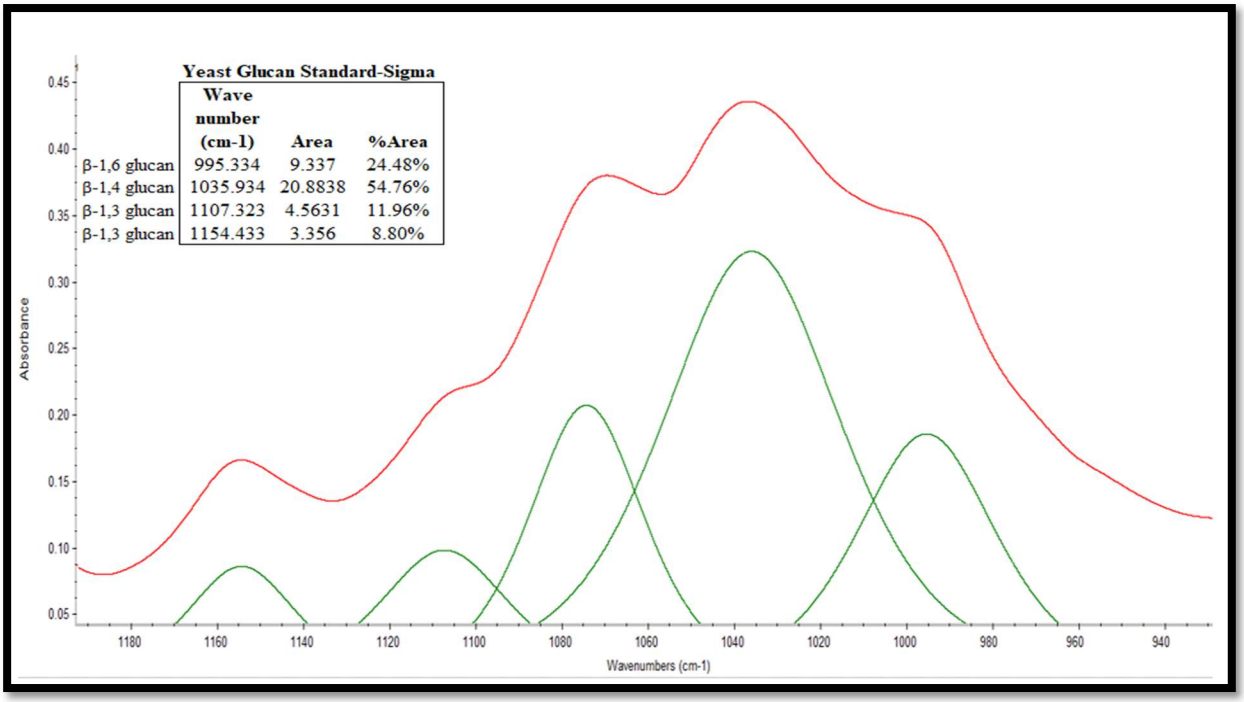

Figure 11. FTIR Spectra of yeast β-glucan standard

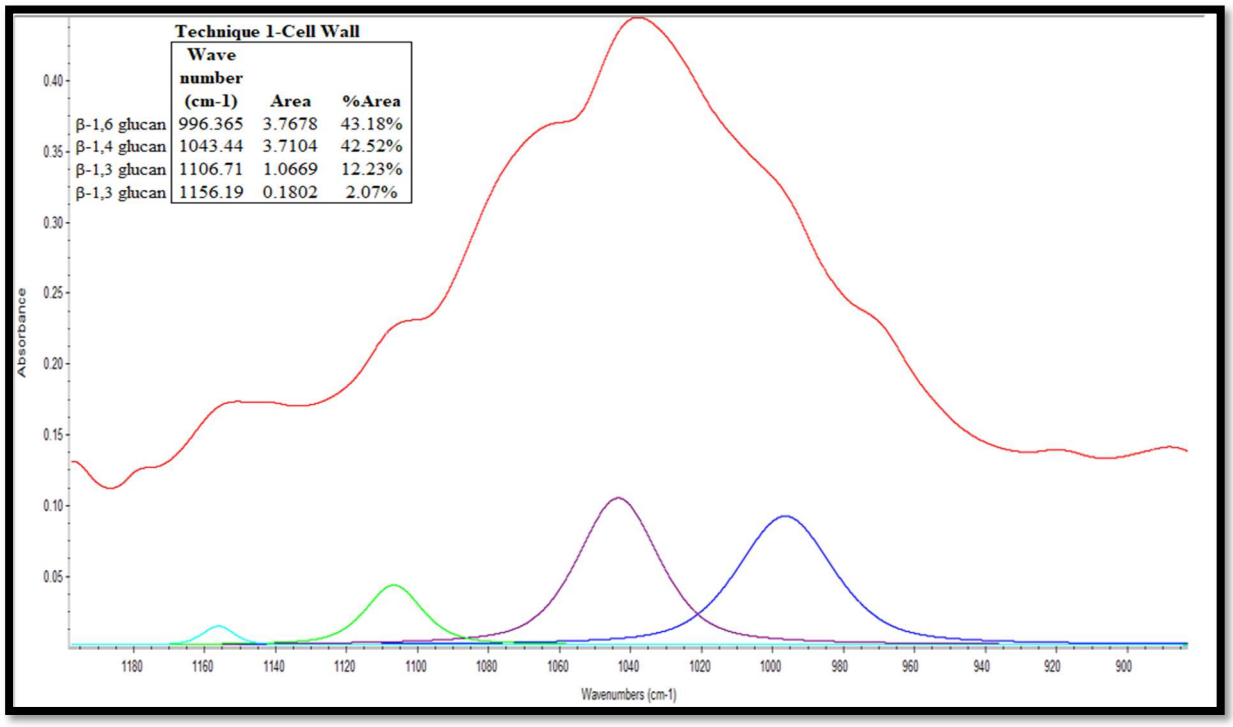

Figure 12. FTIR Spectra of technique 1-cell wall samples

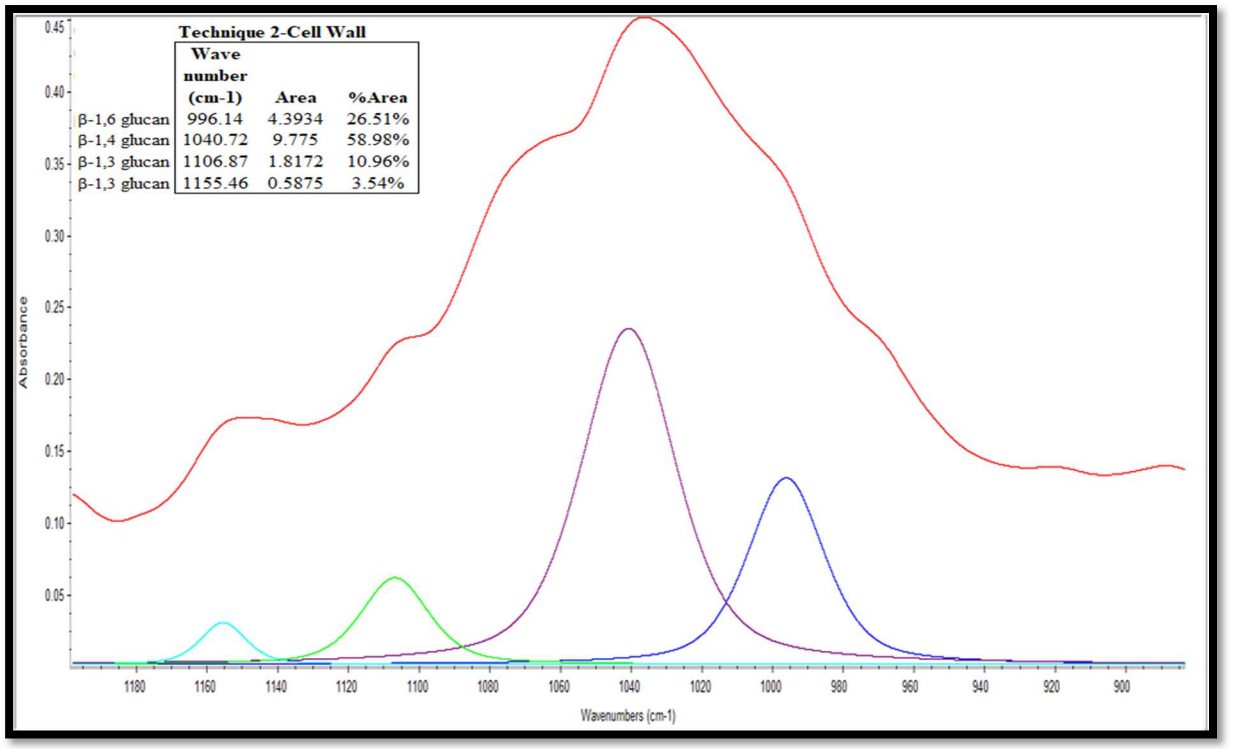

Figure 13. FTIR Spectra of technique 2-cell wall samples

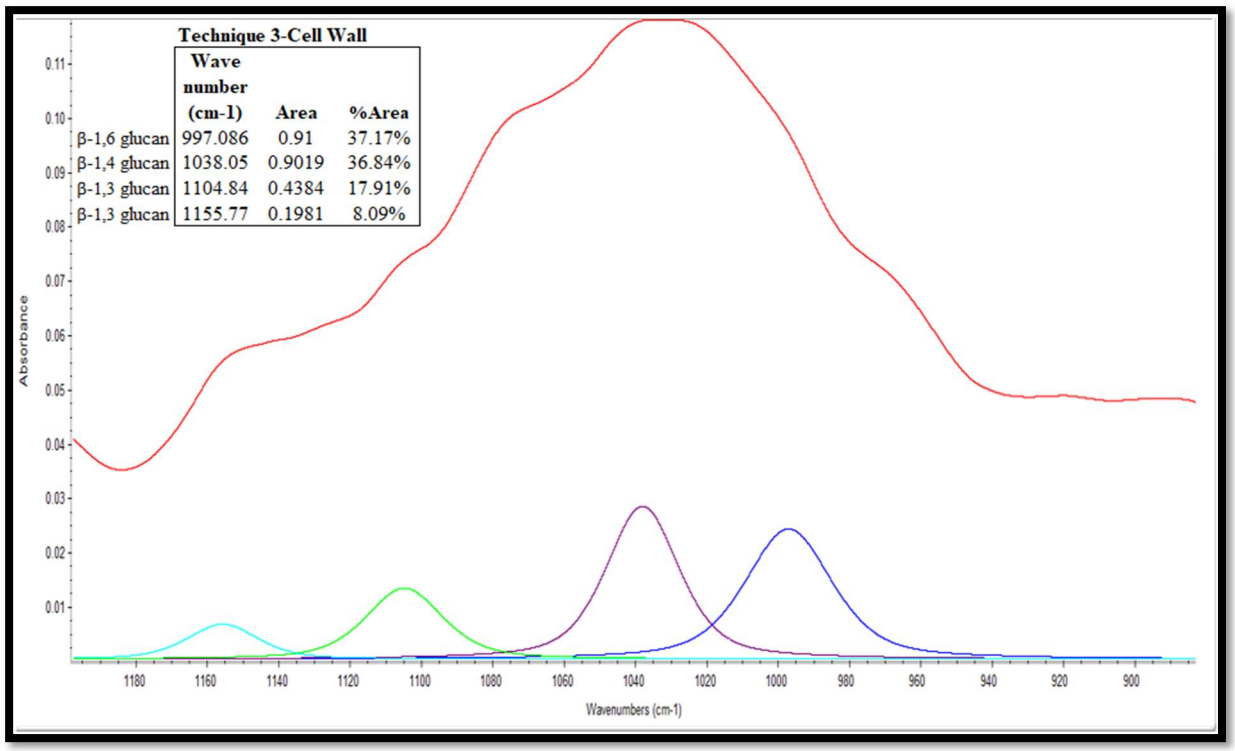

Figure 14. FTIR Spectra of technique 3-cell wall samples

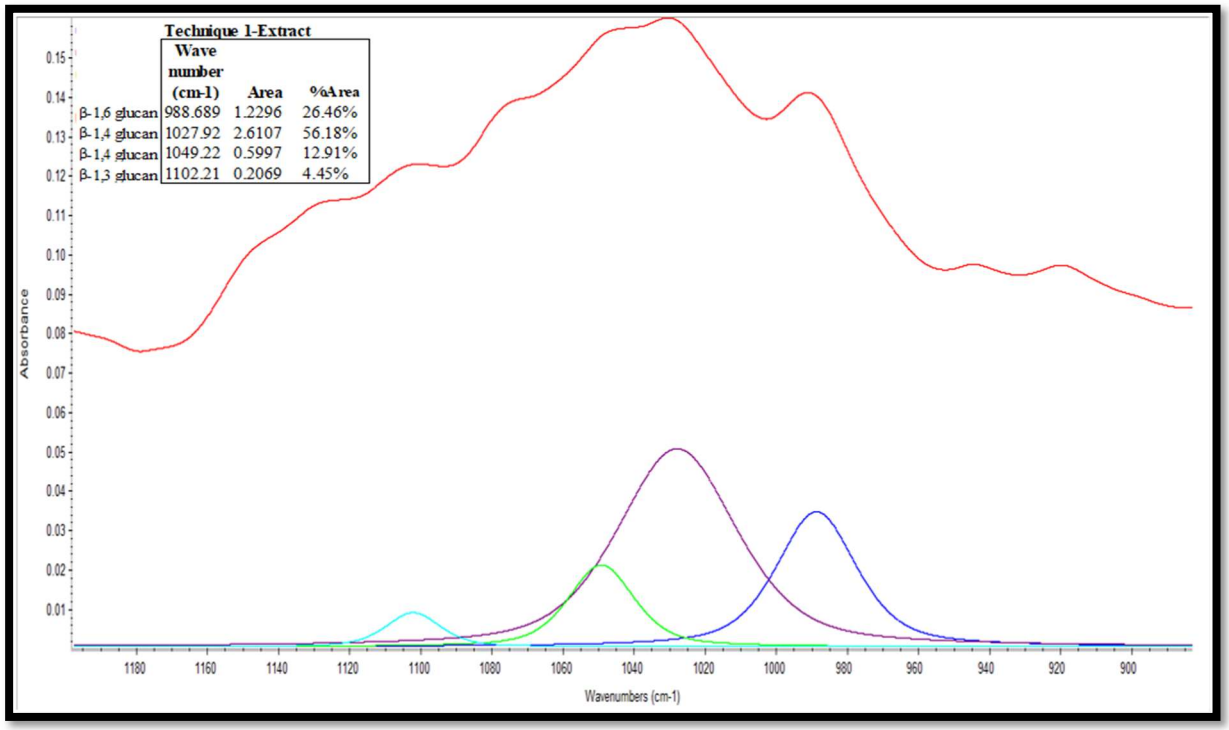

Figure 15. FTIR Spectra of technique 1-extract samples

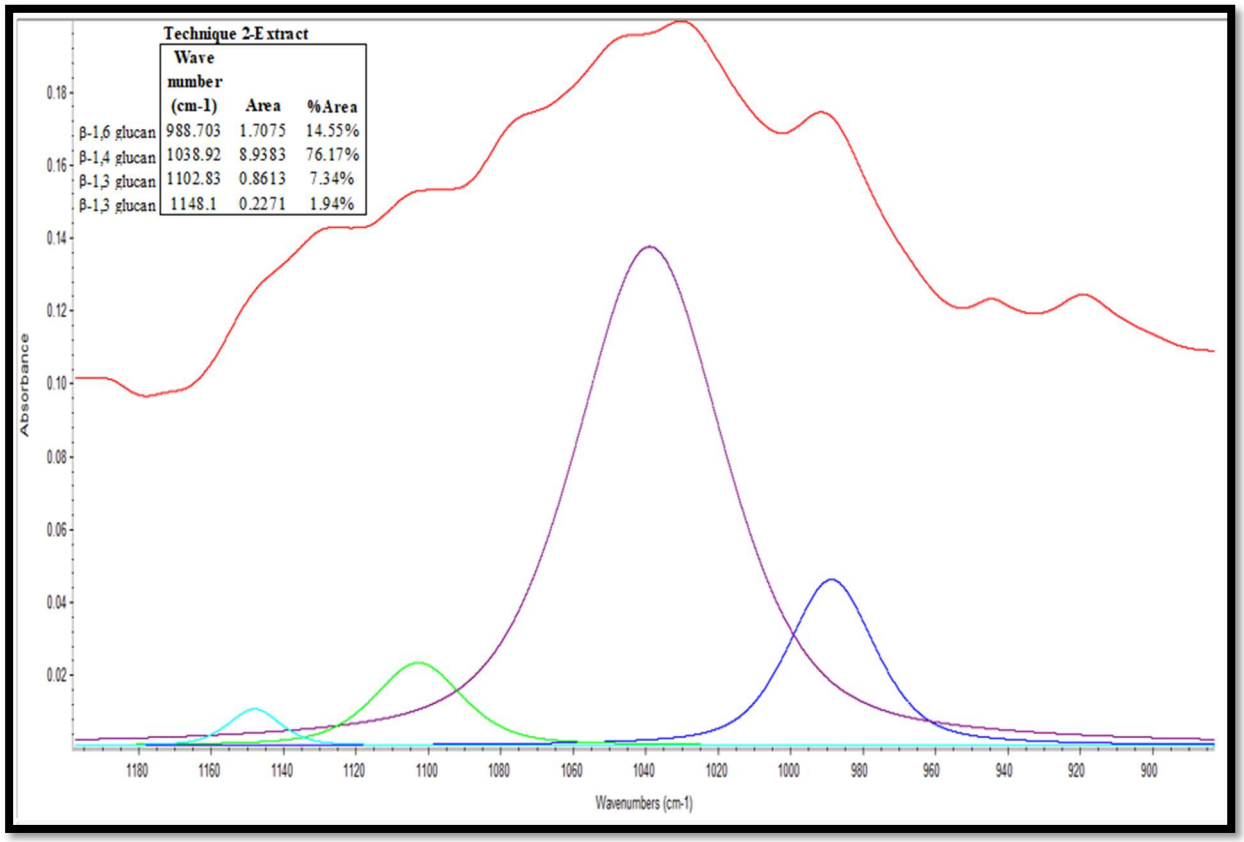

Figure 16. FTIR Spectra of technique 2-extract samples

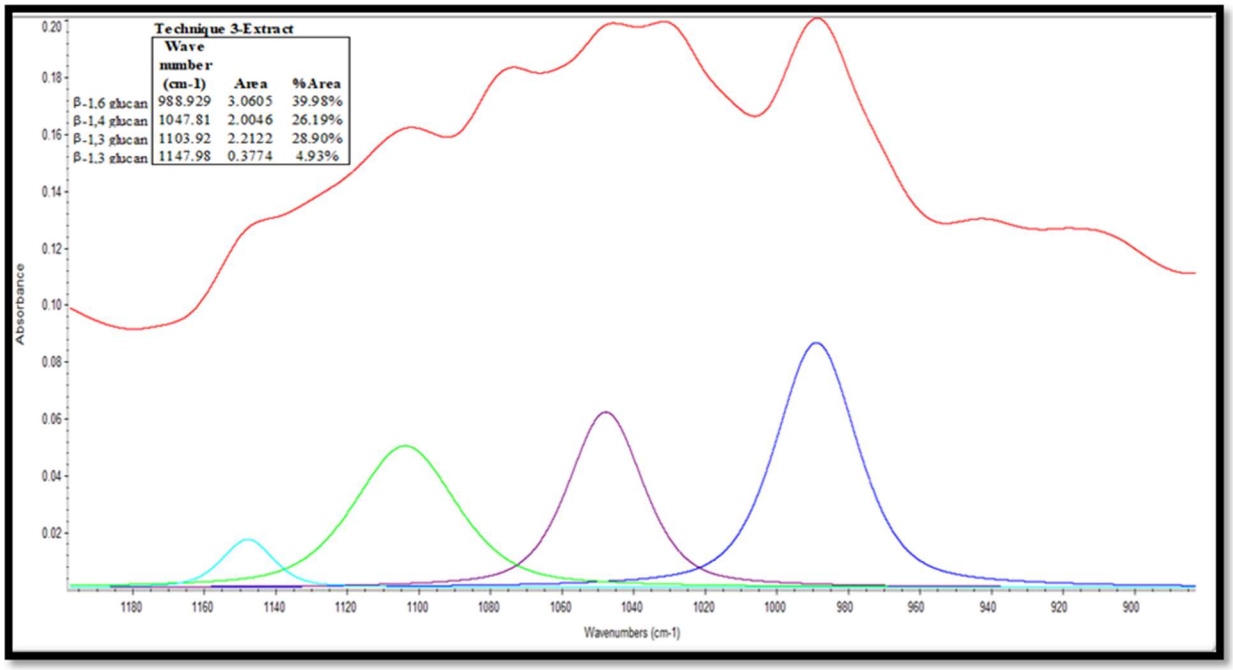

Figure 17. FTIR Spectra of technique 3-extract samples

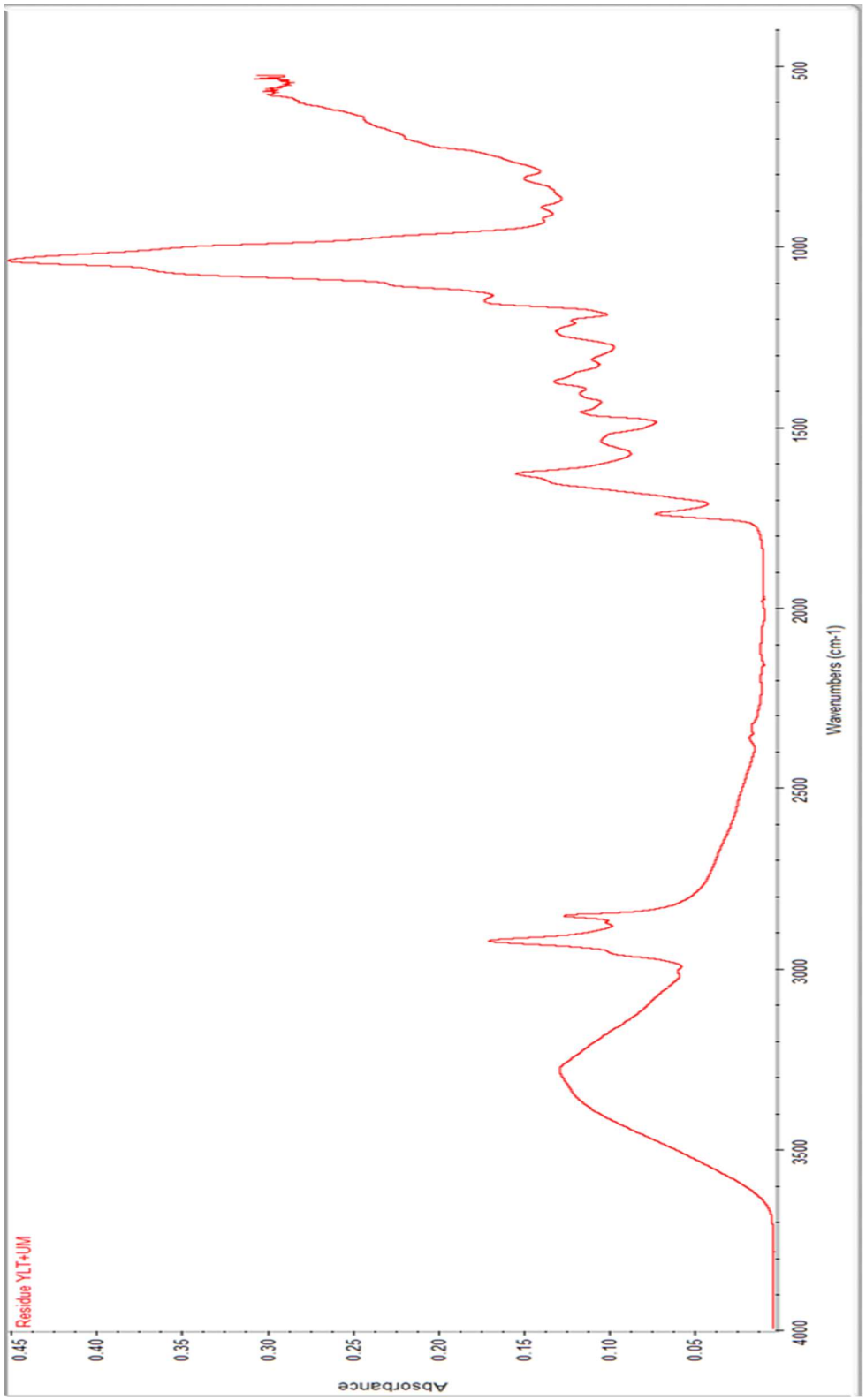

Figure 18. FTIR full spectral scan for Technique 2 cell wall samples.

3.5 HPLC Analysis of 5'-Nucleotides in Yeast Extract

The HPLC analysis of the yeast extract fractions identified and quantified the concentrations of four components: 5'-GMP, 5'-UMP, 5'-CMP, and 5'-IMP. An external standard was used at a known concentration of the 5'-Nucleotides to quantify the concentration of the materials in the yeast extract.

The standard and samples were analyzed in duplicate. The calculated peak areas for each the two injections of the standard are averaged (Table 27). Using the peak area ratio for each peak, known standard concentration, and dilutions provided in the procedure, the percent composition can be calculated (Table 28). The solids concentration for each sample was used to convert the values to a dry solid basis (Table 9). Chromatograms are shown for a stand run and a sample run to show peak clarity and retention time similarities. The retention time is depicted next to the name of the peak and uses minutes as the unit of measurement (Figures 19 and 20).

Table 27. Peak area of individual 5'-nucleotides. Unit s= mAU/min

	STD	1	2	3	4	5	6
5'-CMP	2.027	7.512	8.068	8.182	8.093	7.832	7.668
5'-UMP	3.112	15.227	16.547	16.716	16.673	16.142	15.656
5'-GMP	4.668	25.418	27.652	28.068	27.916	26.886	26.414
5'-IMP	5.688	35.842	38.732	39.108	38.822	37.174	35.828

Table 28. 5'-Nucleotide concentrations in Technique 3 yeast extract (DSB).

Sample#	1	2	3	4	5	6
5'-CMP	1.15%	1.16%	1.14%	1.11%	1.09%	1.08%
5'-UMP	1.52%	1.55%	1.52%	1.49%	1.46%	1.44%
5'-GMP	1.70%	1.73%	1.70%	1.66%	1.62%	1.62%
5'-IMP	1.96%	1.99%	1.94%	1.90%	1.84%	1.80%
Total	6.34%	6.44%	6.30%	6.15%	6.00%	5.95%

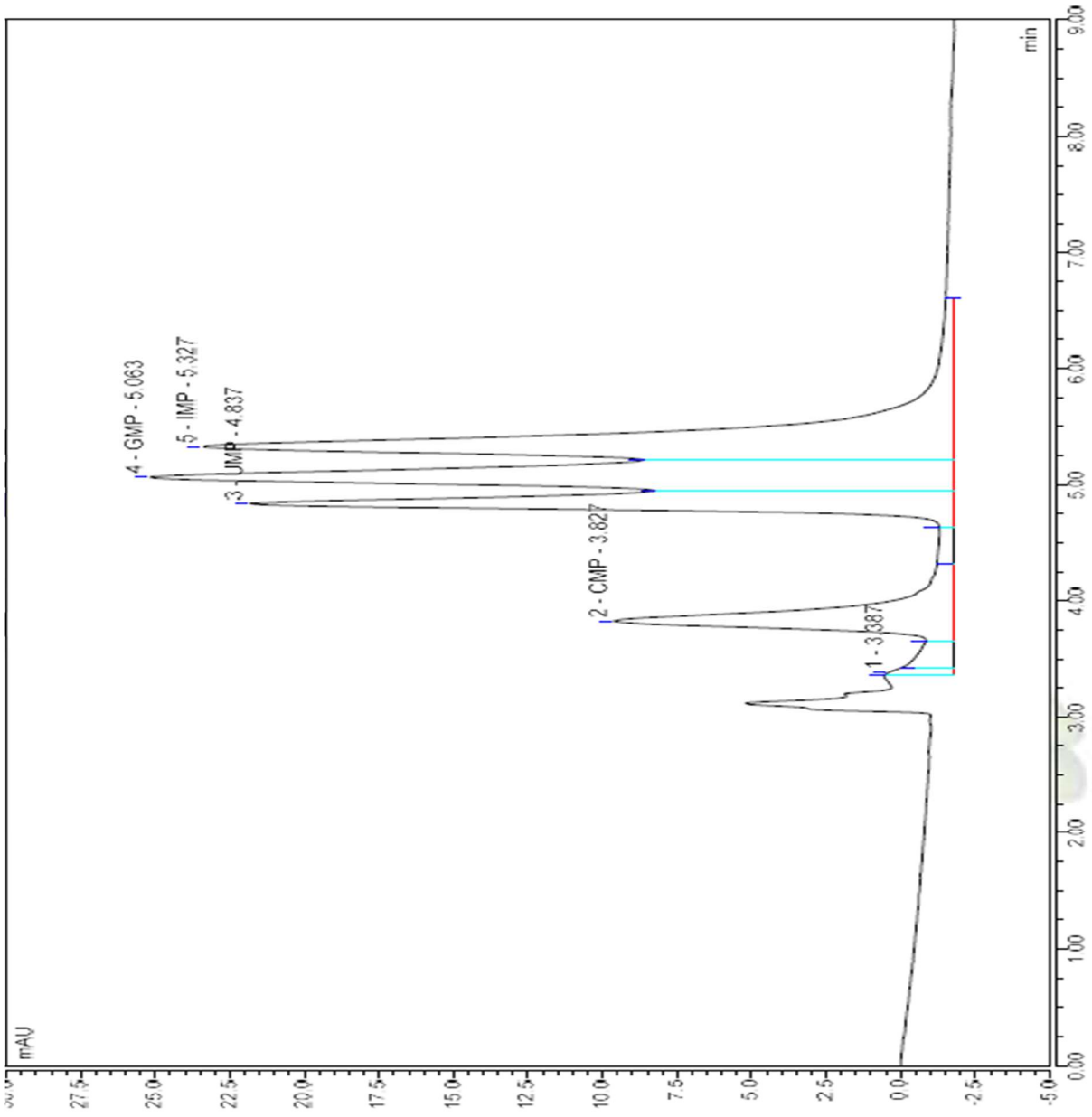

Figure 19. HPLC chromatogram of 5'-nucleotide standard

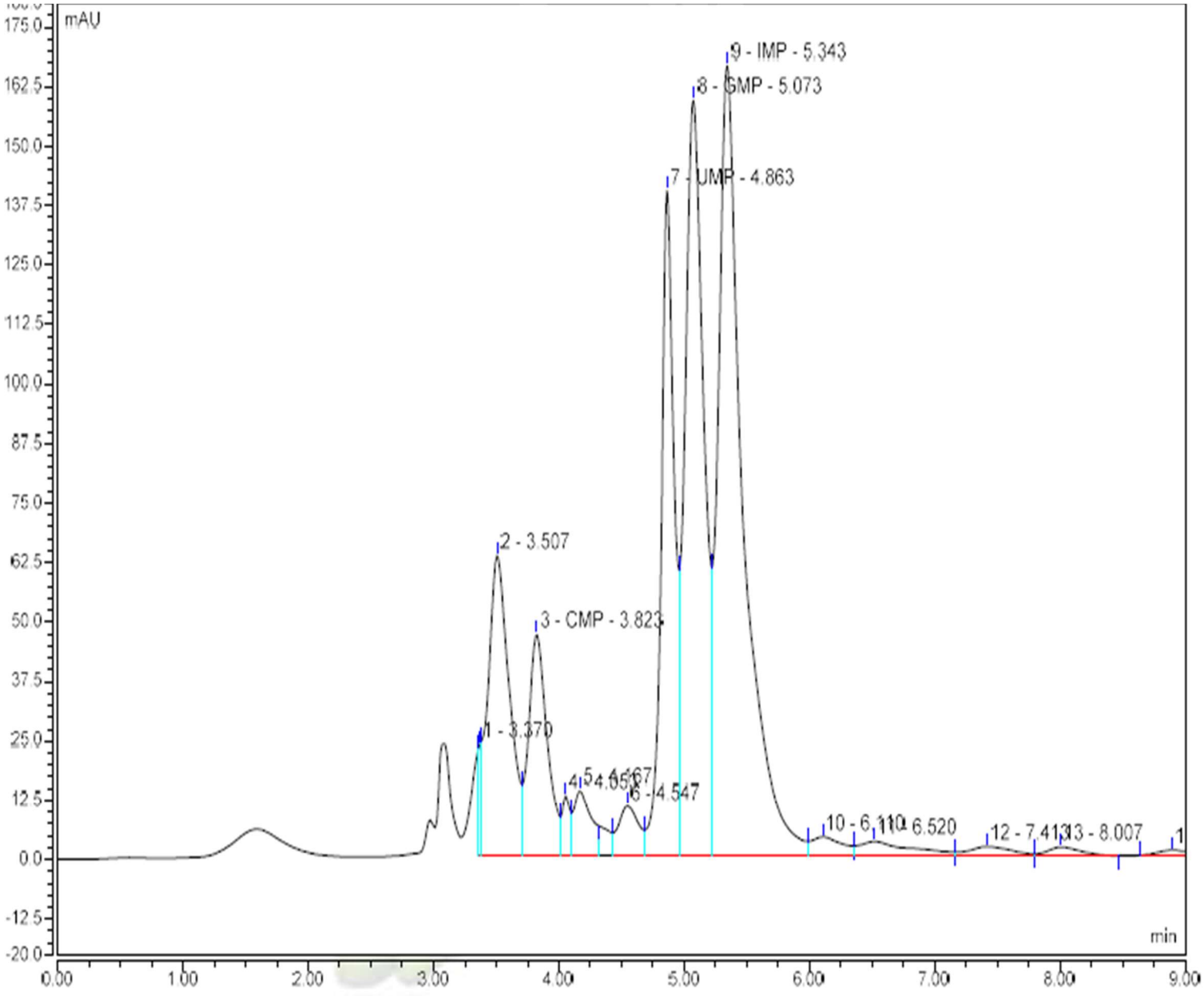

Figure 20. HPLC chromatogram of Technique 3 extract sample

Chapter 4. Discussion

4.1 Soluble Solids Yield Post Enzymatic Treatment of Yeast

A majority of the solubilization of the yeast components such as proteins was supported by the direct addition of the exogenous enzymes in each of the three process. Technique 1 used Umamizyme (Peptidase), Technique 2 used YL-T NAL (Endo-Protease) and Umamizyme (Peptidase), and Technique 3 used YL-T NAL (Endo-Protease), RP-1 (5'-phoshodiesterase), and a deaminase. The thermal treatment of the yeast (90°C for 20 minutes) had a primary role to deactivate the yeast endogenous enzymes within the vacuole but there is likely a small contribution during the heat step to perform minor lysing of the yeast cells. This value was not reported but was supported by related yeast research involving thermal treatments of yeast (Bzducha-Wróbel et al., 2014). The lysing of the cell wall inherently allows the internal yeast cell components to leach out into the water medium and provides more exposed cell wall surface area. The 20-minute time at temperature likely induced a low concentration of lysed yeast cells. Understanding how longer times at temperatures above 90°C may affect the efficiency of the enzymatic process could have an added benefit if the enzymes do not need to focus on the lysis and only on the protein degradation.

Reported values of solubilized material using a purely autolyzed yeast extract vary and are typically between 50-60% (Bzducha-Wróbel et al. 2014; Champagne et al., 1999; Cook, 1958). This reported value does exceed the yield result from the 5'-nucleotide process but was far less than what was achieved from the use of only exogenous enzymes introduced into those systems. Natural variability exists within a biological organism including their concentration and activity levels of endogenous enzymes. The inconsistency in enzyme concentration and

activity inherently causes variable results in solid yield and protein degradation. Additionally, the optimal conditions for the endogenous enzymes in the yeast vary as well. Optimal pH of the primary enzymes active in yeast autolysis (Proteinase A, Proteinase B, Carboxypeptidase Y, Carboxypeptidase S) range from 4-7 and optimal temperatures range from 40-60°C (Table 1). Identifying a pH and temperature to ensure optimal activity for all enzymes during autolysis is not achievable. The best option is to select a mean value that allows for an average efficiency across the range.

The use of exogenous enzymes provides a solution to this approach. When a single enzyme is being added into a system, the control over pH, temperature, and concentration are absolutes. The process can be designed to ensure maximum efficiency within a process step. This conclusion was fully supported by the soluble solid yields achieved in Techniques 1 and 2 with yields of 83.34% and 77.62% respectively, also shown in Figure 21.

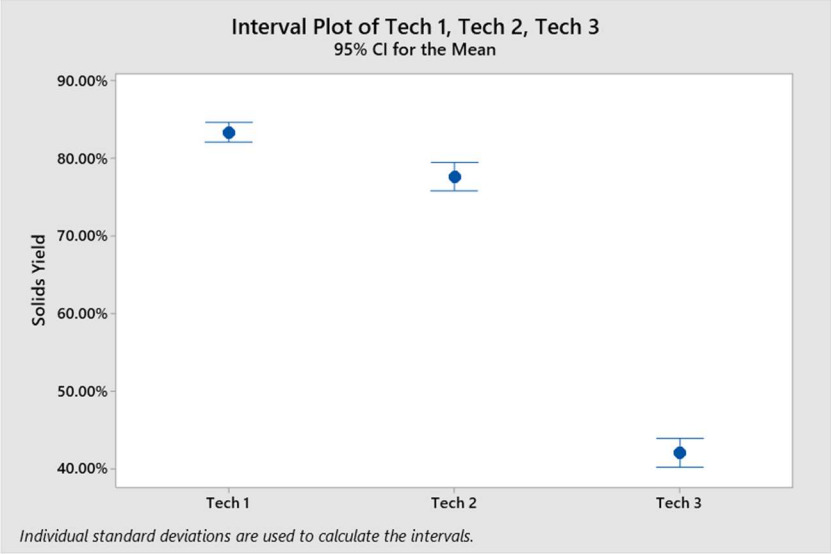

Figure 21. %Solids Yield in Yeast Extract Techniques

The efficiency identified supports the movement into using these enzymes in an industrial setting. The use of exoproteases in conjunction with endoproteases has been identified to increase the degree of hydrolysis of proteins and produce a more acceptable taste in their corresponding yeast extracts (Chae et al., 2001). This corresponds to the enzyme formulation identified in Technique 2.

There was no significant difference calculated for Technique 1 and 2 allowing for the soluble solids yield goals to be tied into a specified flavor profile driven by the release of free amino acids, such as glutamate, to be the deciding factor on enzyme treatment selection.

4.2 Protein Analysis Results of Cell Wall and Extracts Associated with Different Processing Techniques

The protein results can be reviewed as two different sets. The cell wall fraction and the yeast extract fraction. These sets were separated from the yeast cream post enzymatic process by centrifuging and decanting the water-soluble portion to isolate the individual fractions. The determination of crude protein content of these fractions along with the starting yeast material protein value provides additional insight into the extent of breakdown and release of yeast cellular components.

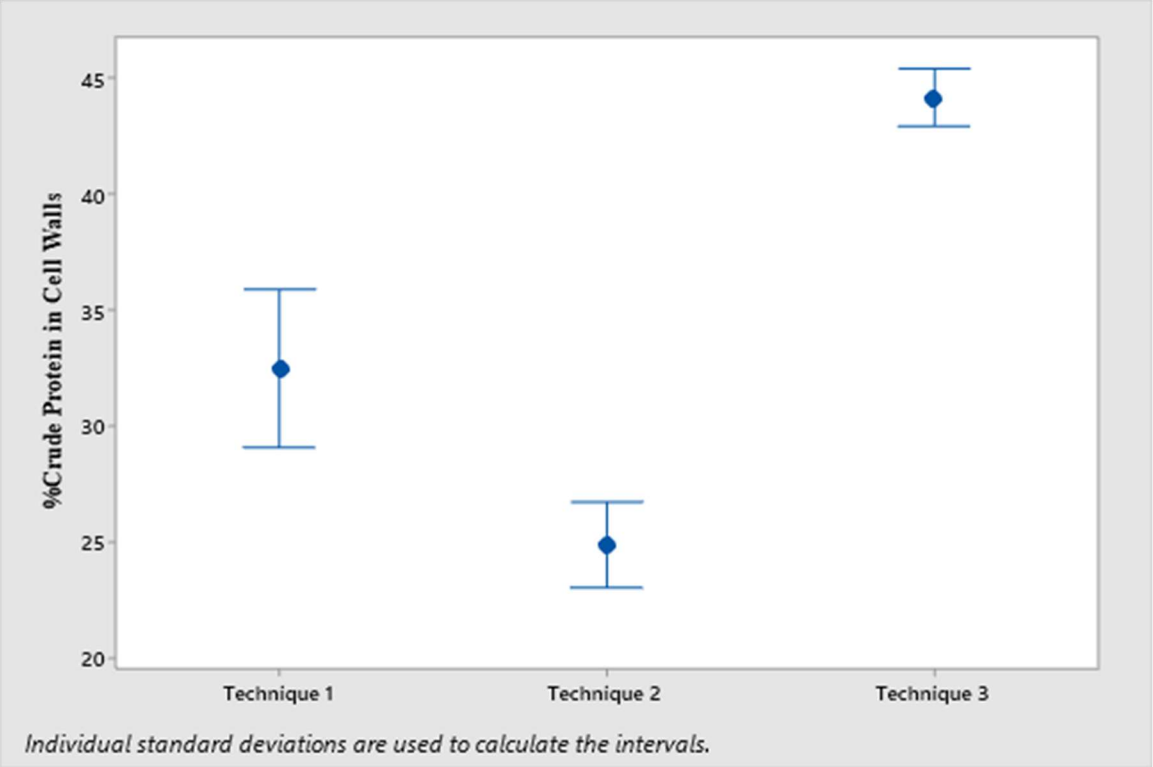

Figure 22. Interval Plot of %Protein in Cell Wall Samples. Calculated on a dry solid basis (DSB)

The enzymatic treatments had variable impacts to the protein content remaining in the cell wall as shown in Figure 22. The dot on the graph represents the mean value for the samples and the lines represent the 95% confidence interval of the means. Technique 3 reported the highest protein load remaining in the cell wall fractions. This was a result of the limited functionality of only an endo-protease used in the processing method. This type of enzyme acts best on intact proteins by limitedly hydrolyzing to create lower molecular weight compounds such as peptides and cleaving the non-terminal amino acids within the molecule (Contesini et al., 2018). Technique 3 resulted in the lowest soluble solids yield and highest amount of residual protein remaining on the cell walls which is a direct relationship to the enzyme functionality in the system and lack of capability to hydrolyze the yeast proteins to a point of solubilization in comparison to the enzyme treatments in the other techniques.

Technique 2 reported the lowest protein concentration remaining in the cell wall fraction. The key difference within this enzyme treatment was the coupled effect of using the endoprotease along with an exopeptidase. The exopeptidase breaks down peptide bonds between amino acids and the end of the polypeptide chain (Contesini et al., 2018). The use of these two types of enzymes in a yeast extract system exhibit the ability to perform a higher degree of hydrolysis and degradation removing more proteins from the cell walls.

The use of only an exopeptidase, as was the case in Technique 1, reduced the protein load in the cell walls more than the treatment with just an endo-protease, Technique 3. This demonstrated that the solubilization of the yeast proteins was impacted by the reduction in molecular size when the terminal end of the protein chain was reduced rather than the non-terminal ends.

The review of protein data from the yeast extract fractions exhibit different trends as seen in Figure 23. The expected relationship between the protein values of the cell walls of the yeast would be inverse for the yeast extracts fractions. In other words, if the protein content is high in the cell walls, it should be low in the yeast extract fraction. This relationship did not hold up based on the protein data. The lowest protein concentration in the cell wall fraction was identified as Technique 2 with a mean value of 24.87% but yeast extract protein value resulted in 63.45% which was less than the yeast extract protein value from Technique 1, 69.34%. The expected correlation of low protein content in the cell wall fraction and high protein concentration in the extract fraction did not hold true for Technique 2 samples. Was the use of both functional enzymes, endoprotease and exopeptidase, helping the solubility of more than just proteins that diluted the concentration of the protein within the extract? A more intensive nutritional panel could be used to identify any subtle differences created by the enzymatic

treatments. Additionally, the use of the generic multiplying factor (6.25) to determine crude protein may attribute to overestimation of total protein values in the system.

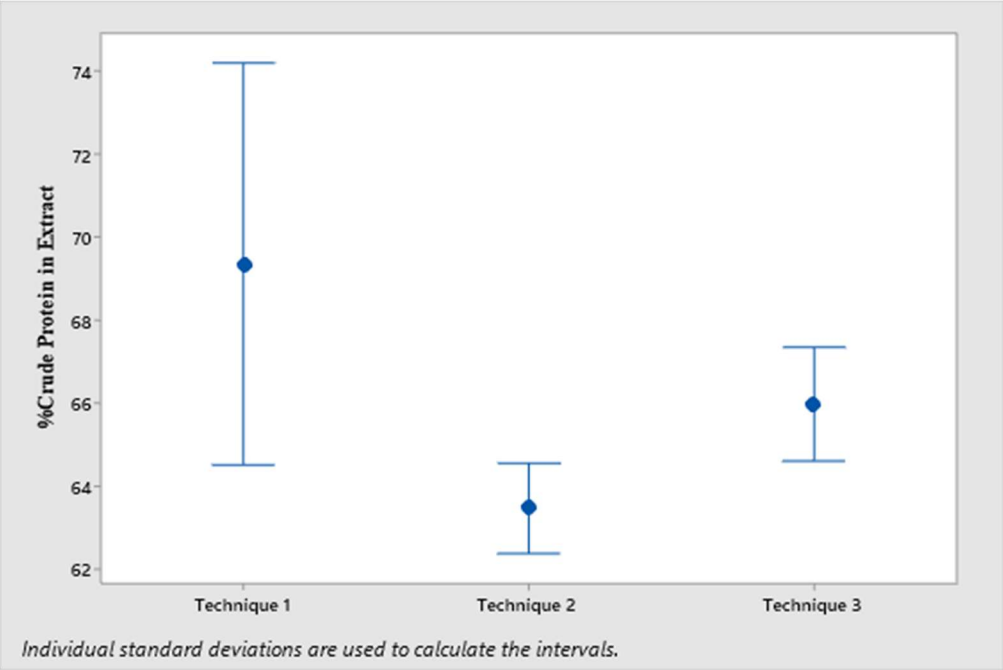

Figure 23. Interval Plot of Protein in Yeast Extracts. Reported on a dry solid basis (DSB)

4.3 Total Glucan Analysis Coupled with FTIR Analysis for Glucan Ratio Determination to Calculate Specific Concentrations of β-1,3/1,6-Glucans

The results of Techniques 1 and 2 showed very similar results for the total glucan concentration with averages of 24.76% and 25.43% respectively. Technique 3 had a lower value which was expected due to the higher protein content remaining in the cell wall material for that process. The total glucan analysis of the extract fractions did identify glucan material present that was solubilized during the various processing techniques. Hot water treatments were identified to potentially solubilize some glucans and all three techniques were exposed to hot water treatments during the yeast endogenous enzyme deactivations (Bzducha-Wróbel et al.,

2014). Although this processing step was applied equally to all three Techniques, there were concentration differences. Techniques 1 and 2 had similar calculated values but they had an approximate concentration that was two times higher than Technique 3. This data trends that the pathway to glucan solubilization is not only impacted by hot water treatments of the yeast cells but was also influenced by exogenous enzymes with exopeptidase activities. The enzymes that were used in the yeast process were confirmed to contain glucose activity during the analysis of total glucan. The most significant value was identified within the 2% DSB Umamizyme enzyme resulting in 24.83g/100g β-glucan. This result is likely due to a carrier within the powdered enzyme. Carriers are used in powdered enzymes to help during the drying process and lower the concentration of the enzyme to make it easier for formulations in large manufacturing settings due to typical low usage rates. The carrier used in this format might be a glucose base carbohydrate like dextrin. Even with this high of a concentration, the enzyme usage within the formulations never exceeded 1%. This impact would be negligible regarding impact on β-glucan analysis and calculations.

Coupling the total glucan analysis with FTIR spectral analysis, we can begin to understand the specific concentrations of individual β-glucans in the samples. The β-1,3 glucan concentrations in the cell wall samples from Techniques 1,2, and 3 were very comparable and varied very little across all processing conditions resulting in 3.99%, 4.03%, and 4.68% respectively. The β-1,6-glucan concentration in the cell walls was the highest in Technique 1 at 12.06% followed by Technique 2 and 3 with 7.35% and 6.68% respectively (Figure 24). Bzducha-Wróbel et al. (2014) reported combined values of β-1,3 and β-1,6-glucan using different disruption methods on yeast cell wall preparations which included autolysis. Based on their findings, the reported value of β-1,3/β-1,6-glucan was 12.5% @ pH 5 and 12.9% @ pH 7.

The combined concentration of β-1,3/β-1,6-glucan in the cell wall material produced from Technique 1 resulted in 16.06% showing the efficiency of the exopeptidase treatment to isolate β-glucan components through effective solubilization of non-glucan components in the yeast biomass. The use of a single enzyme during the processing step achieved a 24% increase in β-1,3/β-1,6-glucan within the cell wall material. The other two techniques achieved β-1,3/β-1,6-glucan levels at 11.38% (Technique 2) and 11.36% (Technique 1).

Based on the FTIR spectral analysis, a large concentration of β-1,4-glucan was identified within the cell wall samples. The methodology conducted for the FTIR analysis was referenced from Chaiyasut et al. (2018). Their identified absorption ranges for specific β-glucans was used as identification markers, listed in Table 26. This cited research also had the goal to identify the ratio of β-glucans within yeast and reported a much lower concentration of β-1,4-glucan than identified in this research. It was also reported that yeast contain a high content of β-1,3-glucan followed by of β-1,6-glucan which is consistent with other cited materials. There were some key differences regarding the sample prep between their values and the values reported in this research.

1. The extraction process in Chaiyasut et al. (2018) prepared their samples by extraction of only β-glucan using a sodium hydroxide incubation followed by an acetic acid incubation.
2. Yeast strain variance could also be a factor. The strain of yeast can have variable biological differences in the cell wall architecture. Chaiyasut et al. used *Saccharomyces cerevisiae* HII31, these results were based on *Saccharomyces cerevisiae* L106.

β-1,4-glucan functions within the yeast cell as a link with chitin and other β-glucan components (Samaan, 2017). The FTIR spectra clearly defined and quantified the largest peak in the identified spectral wavelength as β-1,4-glucan according to Chaiyasut et al. (2018). Based on the analytical results, the cell wall material produced through enzymatic process contains mainly β-1,4-glucan and β-1,6-glucan. Although the β-1,4-glucan values do not align with cited research, the combined values of β-1,3/β-1,6-glucan do show consistency with cited research, Bzducha-Wróbel et al. (2014).

The yeast extract samples had a detectable level of β-glucan concentrations (Figure 25). Like the cell wall samples, the major components were identified as β-1,4-glucan and β-1,6-glucan. Although β-glucans were identified within the yeast extract samples, the concentration values were low for any impactful nutrition claims since typical yeast extracts are used within food systems at less than 2%. The FDA has the Daily Recommended Value as 28g per day of dietary fiber. The value attributed to a food formulation is negligible.

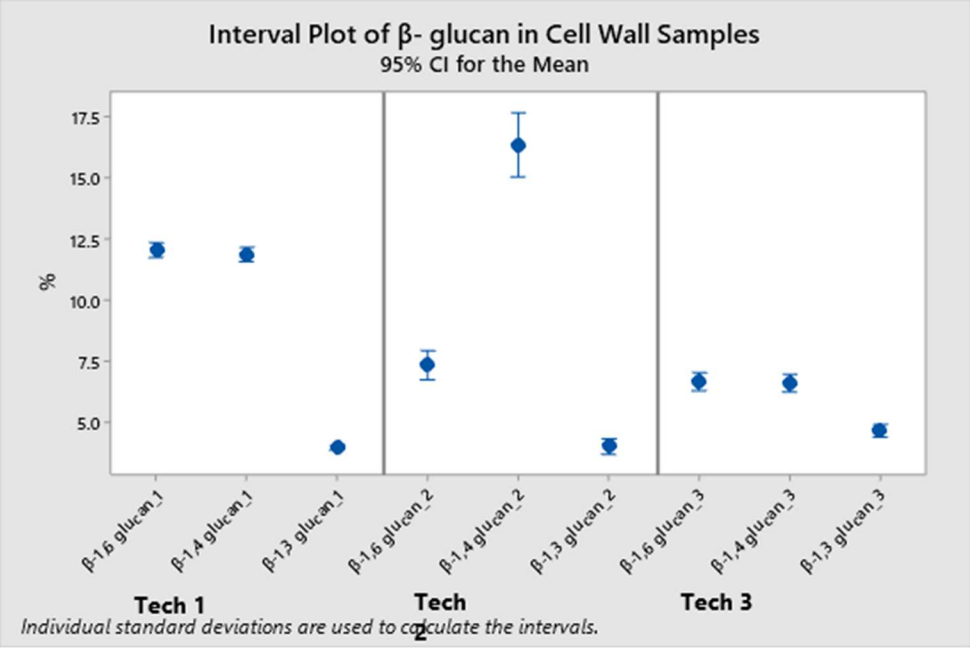

Figure 24. β-Glucan concentration by type in cell wall samples. % on a dry solid basis.

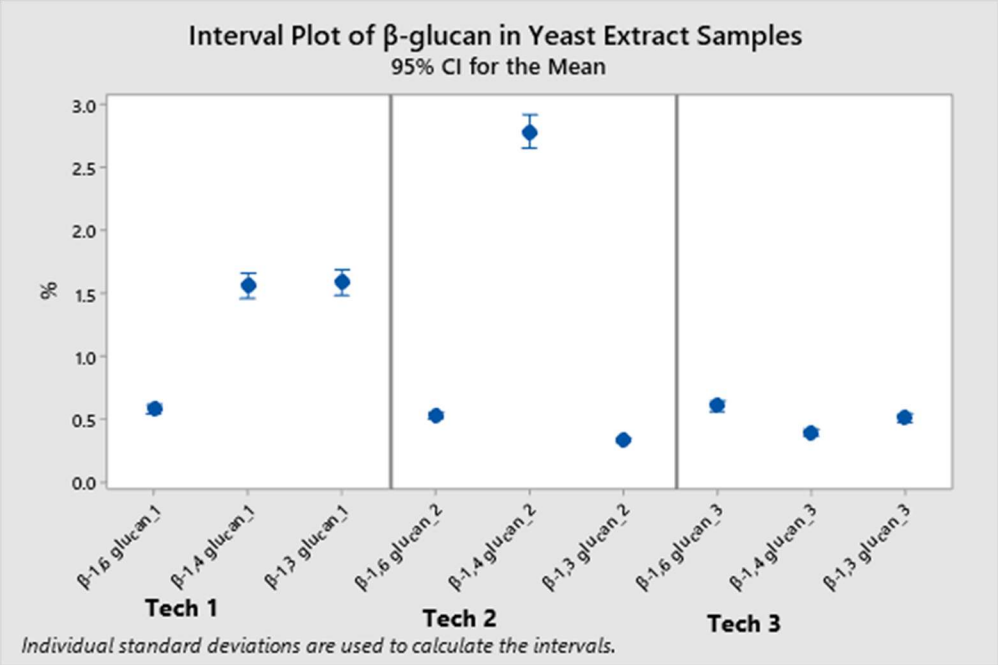

Figure 25. β-Glucan concentration by type in yeast extract samples. % on a dry solid basis.

Chapter 5: Conclusion

The use of exogenous enzymes to degrade and solubilize yeast proteins in this study resulted in the highest values when an exopeptidase was introduced to the system. The ability for this enzyme type to cleave proteins at their terminal end and create smaller fragments is believed to aid in the removal of yeast biomass from the cell wall materials. These results were supported with the use of 2% DSB Umamizyme in Technique 1 by obtaining the highest solids yield (83.34%) and the highest β-1,3/1,6-glucan concentration in the cell wall fraction post process (16.06% DSB). Additionally, this cell wall material contained an average of 32.47% crude protein and by developing additional processes, mechanical and/or enzymatic, the potential to increase the overall concentration of β-1,3/1,6-glucan by eliminating this protein is possible.

The reported values in this study for soluble solids yield for Techniques 1 and 2 (83.4% and 77.62%) supports the efficiency of the exogenous enzyme usage in the production of yeast extracts. These values were higher than any other reported values for autolyzed yeast extracts cited in this research. The efficiency of this type of processing is attributed to improved control of optimized processing environment (pH and temperature), along with standardization (concentration and activity level).

The yeast extracts produced from all three techniques did show evidence of containing β-glucan components. This evidence does support claims that β-glucan can be solubilized during heat treatments of yeast cells in water. Although, there was detectable levels, the levels were very low and did not present an opportunity to significantly increase the nutritional snapshot of the yeast extract as an ingredient in a food system.

The research supports that additional treatments of the cell walls will be necessary to provide a material that provides a meaningful concentration of β-1,3/1,6-glucan for improved health claims. The evaluation of different enzyme treatments on the yeast cells offers an opportunity to further concentrate the β-glucan materials to improve the functionality as an enhance dietary fiber. The use of chitinase may enhance the disruption of the cell wall architecture which would allow for more surface area and increase protein degradation for solubility. Identifying and quantifying the remaining mannoproteins can determine if the use of mannanase type enzymes could reduce the concentration of mannoproteins from the cell wall and increase the overall concentration of β-glucans.

The ability to solubilize β-glucans in a basic solution (2M KOH) identified in the Megazyme procedure for glucan analysis may be an opportunity for purification. The neutralization of the solubilized material would be required leading to the production of some type of salt. This would need to be evaluated for flavor and nutritional impacts based on the base and acid used in the processing method.

Chapter 6: Future Work

Since the main purpose of these yeast extracts is to enhance flavor and provide umami impacts, understanding the free amino acid profiles would be important to ensure the degree of hydrolysis of the proteins impacted and solubilized in these processes are also releasing free amino acids, specifically glutamic acid. Determination of the free amino acid content of the yeast extracts was not in scope for this research but could be used for further determinations of different pathways of protein degradation in support of releasing more protein from the cell wall.

Analysis on the yeast extract and cell wall materials to determine other distinguishing characteristics would provide insight into changes occurring during the different processing methods. Mannoprotein concentrations by analyzing D-mannose remaining on the cell wall and if any is solubilizing into the yeast extract can help improve preparation methods. Additionally, if the mannoprotein concentration is known, evaluating the efficiency of different enzyme treatments to solubilize this material can be tested. Protein concentration variability identified in the results would benefit by introducing multiple methods of analysis to ensure values identified are repeatable.

Lastly, the specificity of the glucan standard utilized for the FTIR methodology can be expanded into the individual β-glucan components identified (β-1,3-glucan, β-1,6-glucan, and β-1,4-glucan). By identifying the spectra associated with the individual components, the identification of the wavenumber can be verified based on reported values and enhance the results through robust verification processes. The use of an isolation technique on the β-glucan components prior to FTIR analysis can provide additional accuracy in the ratios identified.

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