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Extracting (1,3/1,6)-β-Glucans from *Saccharomyces cerevisiae*: A Fungal Immunotherapeutic

By

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An Undergraduate Thesis Submitted in Partial Fulfillment
of the Requirements for the
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East Tennessee State University

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Dr. Rachel Greene, Reader  4-10-16

Dr. Doug Lowman, Reader  Date

Dr. Hilary Malatino, Reader  4/15/16
Abstract

The goal of this research was the development of a method to extract pure (1,3/1,6)-β-glucans from *Saccharomyces cerevisiae*. These β-glucans are of pharmaceutical importance because an animal’s immune system can recognize glucan molecules, and these molecules can act as immunomodulators, essentially turning on the immune system. The problem in the past has been that previously published methods produce β-glucans with low side chain lengths and few branching occurrences. This issue was tackled by a multivariable approach that reduced extraction steps, initial sample size, and concentrations of reagents used. This method has been shown to produce greater yields of β-glucans while maintaining high purity. Analyses such as $^1$H-NMR and GC-MS have been used to confirm the content of the extracted glucans. Ideally, this research will generate interest for further β-glucan studies and ultimately be utilized pharmacologically with immunocompromised individuals.
1. Introduction

1.0 Saccharomyces cerevisiae

*Saccharomyces cerevisiae* is a fungal species that is characterized as yeast. It has several practical applications in areas such as brewing beer or baking bread. Additionally, it is easy to obtain and relatively inexpensive, thus making it a good model for experimentation. It is unicellular and a eukaryotic species, and it shares other characteristics of fungal species such as reproduction by budding, and a complex cell wall.\(^1\)

1.1 Fungal cell wall

The fungal cell wall is generally intricate and is comprised many parts. Its composition in *S. cerevisiae* contains lipids, mannans, glucans, and chitin. The presence of chitin is one of the aspects of fungal cell walls that separates it from the cell walls of plants.\(^2\) A simplified schematic of the theoretical structure of a fungal cell wall is shown in Figure 1.1.0.

![Generalized structure of a fungal cell wall](image)

The fungal cell wall provides for the organism in several ways. Not only does it act as a secondary defense system, but the chitin helps to provide a stronger structural integrity to the fungus. But chitin is not the most noteworthy part of the fungal cell wall. The fungal cell wall contains polymers of glucose that are commonly
referred to as glucans. These glucans have specific linkages and conformations that are unique to fungal species.4

Glucose molecules are named according to the location of their alcohol group on the first carbon as shown in Figure 1.1.1. In the alpha conformation, the alcohol group is on the same side of the ring as the alcohol group on the second carbon, and in the beta form, they are in opposition. Glucans are essentially polymers of glucose that have a particular connectivity. In cereals and grains, the connectivity of these glucose molecules follows a (1,3/1,4) linkage, as shown in Figure 1.1.2.

While these are still classified as glucans, they are not the glucans that are found in fungal species. This is particularly important because there is a significant difference among the glucans regarding the way the immune system responds to each.

Fungal species feature what is known as a (1,3/1,6)-linkage seen in Figure 1.1.3. This (1,3/1,6)-linkage is important because it is recognized by the immune system as a sign of infection. In generalized terms, when a fungal infection is in
its early stages, the (1,3/1,6)-
linkage is the first marker the
immune system sees before it
starts to generate antibodies.
For the purposes of
immunotherapeutics, it is
fortunate that this particular
marker is not dangerous on its own. These glucans can be used to preemptively
start the immune system.

1.2 Various uses of fungi in food production and pharmaceuticals

Many fungi,
including \( S. \text{cerevisiae} \),
have been used for many
years in the production of
various types of food and
drink. \( S. \text{cerevisiae} \) has
often been called baker’s yeast because it is the leavening agent in bread. This
leavening occurs through a fermentation process that yeast perform naturally. It
is also this same process that allows for the production of many alcoholic
beverages such as beer. Essentially, yeast breaks down glucose molecules for
food and energy, and the byproducts are pyruvate and ethanol as shown in
Equation 1.2.0.
Since the discovery of penicillin in 1928, fungal species have been heavily used to produce various antibiotics. The type of resistance that is gained from the ingestion of certain fungal species is due to the antimicrobial adaptations that fungi have evolved throughout history. Unfortunately, many of these antibiotics were and continue to be overused by the general population. As a result, many pathogens have evolved resistance to these fungal-based drugs, such as methicillin resistant *Staphylococcus aureus* (MRSA).

### 1.3 Historical background of β-glucan extraction

In 1941, Hassid, *et al.* detailed their experimental procedure for the extraction of (1,3/1,6)-β-glucans. Their method employed a series of extractions that used various acid, base, and ethanol solvents. While their method was successful in yielding pure samples of the desired β-glucans, the yields that were achieved were low. Additionally, the native structure of these glucans was severely degraded. The polymerization of glucans is one of the key, high levels degradation could interfere with the immune system’s ability to recognize (1,3/1,6)-β-glucans. The glucan’s native structure is generally defined by the lengths of its side chains and the frequency of branching that occurs. Both of these are dependent upon the 1,6-linkage shown above in Figure 1.1.2. Hassid’s method will be referred to as the classical method and is important to note because their attempts were some of the first that focused on extracting the β-glucans from *Saccharomyces cerevisiae*.

One attempt to improve upon the classical method has been to utilize the efforts of enzymes to extract the (1,3/1,6)-β-glucans. In particular, Shiavone, *et
al. have worked on perfecting this methodology. While their methods have produced results with high yields and purity, the enzymes that are required for their extraction process are not cost efficient. While enzymes are expensive, they are also extremely sensitive to their storage conditions. With the use of (1,3/1,6)-β-glucans as pharmaceutical grade immunotherapeutics, it would be difficult to produce the desired product at an economically feasible price.

Dr. Rachel Greene also worked to optimize the extraction methods that were available. Some of the methods employed involved the reduction of the number of extraction steps. Additionally, different acids were used such as hydrochloric acid, phosphoric acid, and formic acid. By attempting to optimize these conditions, Greene’s methods provided better resolution of the native structure of the (1,3/1,6)-β-glucans because there were novel structures observed. By maintaining the native polymerized structure, the understanding of how (1,3/1,6)-β-glucans interact with the innate immune system increased.

1.4 Calculation of β-glucans in sample

For this research, the samples were analyzed via nuclear magnetic resonance (NMR) spectroscopy, see Section 2.2. After data was collected, a few peaks were integrated with JEOL Delta v5.0.4 computer software. The integrations describe relative amounts of various types of glucan that are present in the isolated sample. Figure 1.4.0 shows which peaks were integrated. These peaks were selected based upon the hydrogen atoms that were present in these regions. From these integrations, ratios of the peak integrations were used to determine the side-chain frequency and branch lengths. The desired result is a
high average side chain length (ASCL) and a low average branching frequency (ABF). The black box below in Figure 1.4.0 encloses the anomeric hydrogen; this hydrogen is positioned on the first carbon of each glucose molecule, C-1. Because each glucose molecule has this hydrogen, its peak was normalized to 1000 to use as a reference. Next, the red box surrounds the doublet that is associated with the hydrogen on the first carbon when a (1,3) linkage is present. Lastly, the blue box displays the doublet for the protons that are attached to the C-6 with a (1,6) linkage.

Once the spectrum was integrated, the ratios were used to determine the branching frequency and side chain lengths as seen in Sample Calculation 1.4.0. To do this, the difference of the integrations of the red box and the blue box was found. Having normalized the tall doublet to 1000, the difference was divided into 1000. By using this ratio, the branching frequency was determined. The branching frequency refers to the

<table>
<thead>
<tr>
<th>Integration Ratios:</th>
</tr>
</thead>
<tbody>
<tr>
<td>(blue-red) = n</td>
</tr>
</tbody>
</table>
| \[
\frac{1000}{n} = \text{Branching Frequency} \\
\frac{\text{blue}}{n} = \text{Side Chain Frequency}
\] |

Sample Calculation 1.4.0 – Calculation of branching frequency and side chain frequency; colors correspond to Figure 1.4.0
number of glucose molecules in the (1,3) backbone that must be present before a (1,6) branching will occur. For the spectrum in Figure 1.4.0, the difference between the red box and blue box is approximately 45; therefore, 1000 would be divided by 45 to get a branching frequency of around 22.

Next, the side chain length was found by dividing the blue peaks’ integration by the difference, so for Figure 1.4.0 the side chain length would be 159 divided by 45, which reveals that the side chain length was around 3.5 for this sample. This method of analysis was used for every sample. The data were then recorded in a spreadsheet (Appendix 1.4.0.a) for easy comparison with the various methods.

1.5 Research aims

The ultimate goal of this research was to optimize the extraction process for (1,3/1,6)-β-glucans. The ultimate extraction process would yield pure (1,3/1,6)-β-glucans while maintaining their native structure. In order to achieve this, some experimental variables were tested. Chapter 2 describes these variables which included the number of extractions with boiling ethanol, the scalability of the method, and determining the optimal acid concentration to be used. The structure was then investigated with the NMR to determine if more of the native structure was retained.
2. Materials and Methods

2.0 Sources for solvents and materials used

*S. cerevisiae* was obtained from Red Star Yeast & Products and kept at 4°C. Sodium hydroxide (NaOH) pellets from Fischer Scientific were dissolved to the desired concentration with deionized (DI) water at 18 MΩ. Specific concentrations desired of o-phosphoric acid (H₃PO₄) were prepared from an 85% stock solution from Fischer Scientific. For the final boiling step, 200 proof ethanol from Pharmco-AAPER was used. A Sorvall Legend RT+ with 250 mL Nalgene PPCO bottles; later, 50 mL Thermo Scientific were used for centrifugation. For mass measurements, an Adventurer Pro AV114 was used. A Thermo Scientific Super Nuova Multiplace Spinning Hotplate was used for boiling and stirring solutions and suspensions. A Thermo Scientific Orion Star A211 pH meter was used for neutralization steps. A VirTis BenchTop Pro Freeze Dryer was used for lyophilization of the samples. For $^1$H NMR, the final product was dissolved in 1 mL of deuterated dimethyl sulfoxide (DMSO-d₆) from Sigma-Aldrich. Trifluoroacetic acid from Sigma-Aldrich was also used. For the analysis with nuclear magnetic resonance, a JEOL 400 MHz NMR was used. Delta v5.0.4 software was used for integration and comparison.

2.1 Three times boiling with 1N NaOH, 1N H₃PO₄, and absolute ethanol

In order to extract the glucans from the yeast samples, a method developed by Dr. Rachel Greene was employed. Greene’s method was shaped after what is referred to as the classical method that was developed by Hassid *et al*. Section 2.1 will cover Greene’s method which will be referred to as the
reduced method. A brief flowchart of the steps of the method is shown in Figure 2.1.0.

![Flowchart of general steps involved in reduced method of glucan extraction](image)

The samples were dry *Saccharomyces cerevisiae*, commonly known as baker’s yeast. For each set of extractions, samples were completed in sets of four trials simultaneously. Before the samples were weighed, a 1 N solution of sodium hydroxide, NaOH, and a 1 N solution phosphoric acid, H₃PO₄, were prepared. Approximately 1 L of both the acid and base solutions were prepared for each extraction.

In Greene’s reduced method, approximately 4 g of *S. cerevisiae* was weighed and deposited into 500 mL Erlenmeyer flasks. Each of the exact weights and were recorded and assigned a unique ID. Each of the yeast samples were boiled, with stirring, for fifteen minutes in 200 mL of the base. The suspensions were then removed from the hotplate and allowed to cool.
After cooling, the suspensions were transferred into labeled 200 mL centrifuge vessels. Using a top loading balance, the vessels were weight balanced for centrifugation. The samples were then centrifuged at 5000 rpm for five minutes. After centrifugation, the supernatant was discarded. The pellet was then washed, to remove any remaining NaOH residue, in approximately 200 mL of DI. The sample was then centrifuged again. The suspensions were then weight balanced again and centrifuged at 5000 rpm for five minutes. Afterwards, the supernatant was discarded again. The pellet was again resuspended in 200 mL of DI.

Next, the pH of the suspensions were adjusted to between 6.9 and 7.1. This was accomplished with dilute concentrations of NaOH and HCl (0.1 to 1 M). When the desired pH was achieved, the suspensions were weight balanced with DI and centrifuged, again discarding the supernatant and suspending the pellet in DI. Lastly, the vessels were balanced and put through the centrifuge one more time at 5000 rpm for five minutes. Again, the supernatant was discarded.

However, unlike the previous washings, this time the pellet was suspended in the H$_3$PO$_4$ prepared earlier. Using a graduated cylinder, 200 mL of the acid was used to suspend the pellet, and then each of the suspensions were poured into clean, 500 mL Erlenmeyer flasks with a clean stirring bar. The 200 mL volume of acid was used for rinsing the centrifuge vessels to ensure quantitative transfer. Once resuspended, the solutions were boiled for fifteen minutes at 200 rpm and then allowed to cool.
The suspensions were then weight balanced in centrifuge vessels with DI and the top loading balance. The vessels were then centrifuged at 5000 rpm for five minutes. The supernatant was again discarded. After one centrifugation cycle, the pellet was suspended in 200 mL of absolute ethanol in the same flasks in which the acid boiling took place. The Erlenmeyer flasks were then covered with cellophane and left in the hood overnight.

The ethanol suspension samples were then boiled for fifteen minutes at 200 rpm. Once the fifteen minutes had elapsed, the samples were allowed to cool. Next the suspensions were transferred to centrifuge vessels, balanced, and centrifuged. Unlike the previous steps, the ethanol suspension made it more difficult for the sample to pellet out of the suspension, so the samples were centrifuged at 7000 rpm for five minutes. The supernatant was discarded. The pellet was then suspended in approximately 200 mL DI, balanced, and centrifuged at 5000 rpm. Then, in the same manner as before, the suspension was neutralized to a pH between 6.9-7.1. It was then weight balanced and centrifuged at 5000 rpm for five minutes.

The pellet was then suspended in 200 mL of ethanol, boiled for fifteen minutes, centrifuged, the supernatant was discarded, and it was suspended in an additional 200 mL of ethanol. Then the same procedures for boiling and centrifugation were followed. Next, the pellet was suspended in 200 mL DI. Then the suspension was boiled in Erlenmeyer flasks merely to ensure the excess ethanol was evaporated, and subsequently allowed to cool. Just as before, the suspension was then centrifuged at 5000 rpm for five minutes. The pellet was
suspended again in DI, and centrifuged at 5000 rpm for a final time. Lastly, the pellet was suspended in less than 15 mL of DI and frozen on an angle of approximately 15° in a labeled conical vial with a lid. The angle created more surface area, which facilitated lyophilization.

### 2.2 NMR Analysis

The samples were analyzed with 400 MHz $^1$H NMR. Initially, a dry weight of the entire sample was taken in order to assess percent yield by mass. To get the samples into solution for NMR, approximately 18 to 22 mg of the dried samples were placed into small vials. To these vials was added 1 mL of deuterated dimethyl sulfoxide (DMSO-d$_6$) and a few drops of trifluoroacetic acid (TFA), which moved the water peak downfield and out of the way of the peaks of interest to ensure their resolution was optimal. Previous research has shown that glucans are soluble in DMSO.$^{10}$ Evidence of solubility was supported by a clear solution; impurities such as residual proteins or lipids created increased pigment in the solution.

The $^1$H NMR analysis was done according to the protocol designed by Dr. Lowman.$^{11}$ This was the procedure followed for all NMR collection on the glucan samples. The NMR original protocol required the following specifications:
• Number of Scans: 16 (originally 64)
• Acquisition Time: 3.2768 seconds
• Interpulse Delay: 15 seconds (includes Acquisition Time + Relaxation Delay)
• Relaxation Delay: 15 seconds
• Data Points: 32,768
• Sweep Width (X_Sweep): 25 ppm
• X_Offset: 5.0 ppm
• Using 90° pulses
• Probe Temperature: 80°C (353 K)
• Total 1D \( ^1 \text{H} \) collection time:
• Chemical shift reference set to 2.50 ppm

Once spectral data had been collected, it was analyzed. First, the software Delta v5.0.4 was used to correct the baseline. The software’s “Baseline Correction (Interactive)” option was used, and various anchor points were placed on the spectrum to adjust the baseline. Next, to ensure that the reference DMSO-d\(_6\) peak was at exactly 2.50 ppm, the software was used to shift the reference peak to a desired ppm.\(^{12}\) The peaks of interest mentioned in Chapter 1 were integrated and values were recorded.

**2.3 Alternative Method I: One ethanol boiling step**

Sections 2.3-2.5 discuss alternate methods employed to the reduced method in order to further optimize and reduce the extraction steps required. Classical methods employed three times boiling steps for even the acid and base, but Greene’s reduced method had showed that the additional boiling steps were unnecessary.\(^{13}\) Therefore, it was reasonable that the ethanol boiling steps could be reduced to a single boiling event with ethanol. All of the steps from Dr. Greene’s original reduced method, Section 2.1, were followed up to the point of the first ethanol addition. With this new, altered method, the pellet was then suspended in ethanol and left covered overnight.
The next day, the procedure picked up with boiling the ethanol suspension for fifteen minutes. The suspension was then centrifuged at 7000 rpm for five minutes. The pellet was then suspended in DI. Next, the suspension was boiled in DI to remove excess ethanol, then the suspension was centrifuged at 5000 rpm for five minutes in DI two more times, discarding the supernatant. Lastly, the pellet was suspended in less than 15 mL of water and frozen on a slant at a 15º angle for lyophilization.

2.4 Alternate Method II: Reducing overall volumes and materials

This method employed all of the steps from the reduced method (2.1) as they were modified for Section 2.3, but it added an additional variation to Greene’s reduced protocol. In this method, all volumes and quantities were reduced to one-fourth of their original measurements, i.e. a 1 g yeast sample in a 125 mL Erlenmeyer flask with 50 mL of solvent for each flask, etc. Again, the same lyophilization steps and $^1$H NMR techniques were used to analyze the samples.

2.5 Alternate Method III: Titrating the acid concentration

Method III investigated the concentration of $\text{H}_3\text{PO}_4$ required to successfully isolate $\beta$-glucan from $S. \text{cerevisiae}$. In order to test this, all steps of Sections 2.1-2.4 were used, but the acid concentration of sample sets was reduced by a factor each time, i.e. 1 N $\text{H}_3\text{PO}_4$, then 0.1 N $\text{H}_3\text{PO}_4$, then 0.01 N $\text{H}_3\text{PO}_4$, and finally 0.001 N $\text{H}_3\text{PO}_4$. The same $^1$H NMR techniques were applied to these samples.
2.6 **Tabulated summary of methods utilized**

The method conditions for each variation are tabulated in Figure 2.6.0.

The frequency of boiling steps with the base and the acid are omitted from this table because they were unchanged at one extraction step each throughout all trials.

<table>
<thead>
<tr>
<th>Section</th>
<th>Method ID</th>
<th>Initial Sample Size</th>
<th>Base Conc.</th>
<th>Acid Conc.</th>
<th>Ethanol Boiling Freq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Reduced</td>
<td>4 g</td>
<td>1 N</td>
<td>1 N</td>
<td>3 times</td>
</tr>
<tr>
<td>2.2</td>
<td>Alternate 1</td>
<td>4 g</td>
<td>1 N</td>
<td>1 N</td>
<td>1 time</td>
</tr>
<tr>
<td>2.3</td>
<td>Alternate 2</td>
<td>1 g</td>
<td>1 N</td>
<td>1 N</td>
<td>1 time</td>
</tr>
<tr>
<td>2.4</td>
<td>Alternate 3</td>
<td>1 g</td>
<td>0.1 N</td>
<td>0.1 N</td>
<td>1 time</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.01 N</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.001 N</td>
<td></td>
</tr>
</tbody>
</table>

*Table 2.6.0: Tabulated summary of methods used*
3. Results and Discussion

3.0 Recap of research questions

Through this research, there was a considerable volume of data collected on the extraction processes of β-glucans. The ultimate goal was to obtain β-glucans that most accurately represent the native structure of β-glucans. Achieving this goal could provide the an effective therapeutic. Because this research contained so many different variables in terms of numbers of extractions, types of solutions used, and size of the samples, it was possible to determine a more efficient extraction process for β-glucans from S. cerevisiae. The questions that were addressed were:

1. Could the frequency of extractions with boiling ethanol be reduced to one? (Section 2.3)
2. Could the method be scaled down while maintaining previous results? (Section 2.4)
3. Could the acid concentration be reduced to provide further optimization of the extraction steps involving boiling acid? (Section 2.5)

3.1 Reduction of frequency of extractions with boiling ethanol

First, one of the most major improvements from the classical method was to reduce the number of extractions with boiling ethanol. It was previously demonstrated that β-glucans could be successfully extracted through the employment of three extractions with acid, base, and ethanol.6 Section 2.3 shows the steps that were taken to reduce the number of extractions with boiling
ethanol. The ethanol helped to remove lipids that remained in the sample because lipids are soluble in alcohols.\textsuperscript{14}

Through the work of Asare, it was discovered that one extraction with the chosen acid and base was sufficient to remove the undesired parts of the fungal cell wall.\textsuperscript{13} Because of this result, the constant frequency of one extraction step with acid and base have been omitted from all tables. Table 3.1.0 shows the average data obtained for the reductions of ethanol extractions from 3 times to 1 time. As stated in Section 1.4, the desired result is a high average side chain length (ASCL) and a low average branching frequency (ABF). Section 1.4 details the derivation of the ABF and ASCL.\textsuperscript{15}

![Figure 3.1.0 – NMR Spectra showing variation in frequencies of extractions with boiling ethanol. The top spectrum (JE1-8-D) represents a sample where only 1 extraction step with boiling ethanol was employed. The bottom spectrum (JE1-11-B) is a sample where three extraction steps with boiling ethanol were used.]

<table>
<thead>
<tr>
<th>Sample IDs</th>
<th>Initial Sample Size</th>
<th>Ethanol</th>
<th>Mean % Yield</th>
<th>ABF</th>
<th>ASCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>JE1-8-(A-D)</td>
<td>4 g</td>
<td>1 time</td>
<td>5.45%</td>
<td>21.2</td>
<td>3.54</td>
</tr>
<tr>
<td>JE1-11-(A-D)</td>
<td>4 g</td>
<td>3 times</td>
<td>4.67%</td>
<td>21.2</td>
<td>3.48</td>
</tr>
</tbody>
</table>

Table 3.1.0: Results of samples JE1-8-(A-D) and JE1-11-(A-D) are shown. The variable of this sample set is the frequency of ethanol boiling step shown in red.

The results of these data support the reduction of the ethanol boiling step to 1 time. Peaks resulting from residual lipids in the sample would appear around
the 5.0 ppm region. There are two very small peaks in the extraction with only one boiling step with ethanol at 5.0 ppm and 5.2 ppm in sample JE1-8-D in Figure 3.1.0. This peak is small enough to be accounted for by instrumental noise. The spectra in Figure 3.1.0 indicate that there is very little difference between the two extraction methods.

3.2 Reduction of initial sample size of *S. cerevisiae*

The next step was to determine if the structure of the isolated glucan would vary if the sample size and extraction volumes were reduced. This was a crucial step in learning whether or not the extraction processes would be able to be scaled up or down while yielding the same high quality glucan extract. Because it was anticipated that the end product of this research could possibly lead to the pharmaceutical use of β-glucans, it was necessary to determine if these methods could be scaled effectively. By demonstrating the method could be scaled between 1 g samples and 4 g samples, there was support for adjusting the scale further for pharmaceutical purposes. Reducing the scale helped to make method development much more cost efficient due to fewer supplies being required.

Figure 3.2.0 shows that the 1 g sample (JE1-13-B) and the 4 g sample (JE1-11-B) did not have significant variations. There was not an observable increase in impurities between 2.9 to 4.9 ppm or a degradation of peak height or sharpness. Therefore, reducing the extraction method had little impact on the extracted glucan structure. This supported that scaling the method between 4 g samples and 1 g samples had little effect on the extraction product. Because the
starting materials and reagent volumes were reduced to a quarter of their original size, the 1 g method was utilized for further extractions. Table 3.2.0 displays the results of the scale reduction.

Table 3.2.0 – Results of samples JE1-13-(B-D) and JE1-11-(A-D) are shown. The variable of this sample set is the initial sample size. Note: Sample JE1-13-A was omitted from the averages because some of the sample was lost due to laboratory error. As a result, no spectral data were able to be obtained.

Table 3.2.0 displays the average results of four replicates of each set of the smaller scale extractions. It was observed that the reduction in the extraction scale from 4 g samples to 1 g samples not only made the process more time efficient for method development, but it also sustained results similar to the larger scale, typical extractions that had been performed previously. These results support that the initial batch size does not have a significant impact on the β-glucan extracted. In addition to having used a quarter of the initial yeast sample, the amounts of extraction solvents were also reduced; this was helpful because it
reduced the amount of time required for boiling and cooling of the suspensions, and it was more cost effective and better for the environment. Through several different trials, there was good evidence that the method was capable of handling different sized samples.

Figure 3.2.1 shows that varying both the initial sample size and the frequency of extractions with boiling ethanol does not significantly alter the results. Table 3.2.1 shows the average results of the combined variables of extraction frequency with ethanol and initial sample size on the glucan product. This is interesting because it couples two of the variations on the reduced method while maintaining comparable glucan products. This tandem result helps to support both variations of the extraction method. The percent yield in JE1-11-(A-D) does seem to be slightly lower than JE1-15-(A-D). When there are more extraction steps, \textit{i.e.} JE1-11, there is going to be more sample lost because quantitatively transferring the glucan suspension is unachievable.

![Figure 3.2.1 – NMR Spectra showing both variation in initial sample size and frequency of extractions with boiling ethanol. The top spectrum (JE1-15-D) shows an initial sample size of 1 g and only one extraction with boiling ethanol. The bottom spectrum (JE1-11-B) shows an initial sample size of 4 g and three extractions with boiling ethanol.](image-url)
<table>
<thead>
<tr>
<th>Sample IDs</th>
<th>Initial Sample Size</th>
<th>Ethanol</th>
<th>Mean % Yield</th>
<th>ABF</th>
<th>ASCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>JE1-15-(A-D)</td>
<td>1 g</td>
<td>1 time</td>
<td>6.06%</td>
<td>22.1</td>
<td>3.50</td>
</tr>
<tr>
<td>JE1-11-(A-D)</td>
<td>4 g</td>
<td>3 times</td>
<td>4.67%</td>
<td>21.2</td>
<td>3.48</td>
</tr>
</tbody>
</table>

Table 3.2.1 – Data for sample sets JE1-15-(A-D) and JE1-11-(A-D). These sample sets compare varying initial sample size and frequency of extractions with boiling ethanol.

Similar to Figure 3.2.0, the spectra demonstrate that there is very little difference between the various conditions. This provides better support for reducing both the initial sample size and frequency of extractions with boiling ethanol.

### 3.3 Acid titration

The final area of investigation was to determine the optimal acid concentration for these extractions. The optimal concentration for the base in the extraction steps using boiling sodium hydroxide had already been determined through Asare’s work to be 0.1 N sodium hydroxide. In order to determine the optimal acid concentration, the concentration of the phosphoric acid was reduced by a factor of 10 for each successive extraction set starting from 1 N H$_3$PO$_4$ and working down to 10$^{-3}$ N H$_3$PO$_4$. While there was very little difference between the 1 N, 10$^{-1}$ N, and 10$^{-2}$ N, the integration data from the NMR spectra indicated that the products were becoming more impure. The spectra in Figure 3.3.0 highlight the impurities that arise from reducing the acid concentration primarily in the 3.4 and 3.6 ppm regions marked with the red rectangles. Therefore, the optimal concentration for acid had to be determined based on goals of purity and maintaining native structure. The data for these spectra are tabulated in Table 3.3.0.
Figure 3.3.0 – NMR Spectra showing the difference in varying acid concentration for the extractions with areas of potentially increasing impurity in red boxes. The top spectrum (JE1-22-A) has 0.1 N for its acid concentration. The middle spectrum (JE1-25-A) has 0.01 N for its acid concentration. The bottom spectrum (JE1-28-A) has 0.001 N for its acid concentration.

Table 3.3.0 – Results of acid titration. Note: percent yields are by weight, so the presence of impurities, likely in JE1-28-(A-D), would appear to be an increase in percent yield. The coloration of the final product for JE1-28-(A-D) also lacked the characteristic off-white coloration, but instead looked light brown.

<table>
<thead>
<tr>
<th>Sample IDs</th>
<th>Acid Conc.</th>
<th>Initial Sample Size</th>
<th>Ethanol</th>
<th>Mean % Yield</th>
<th>ABF</th>
<th>ASCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>JE1-22-(A-D)</td>
<td>0.1 N</td>
<td>1 g</td>
<td>1 time</td>
<td>7.69%</td>
<td>17.6</td>
<td>4.70</td>
</tr>
<tr>
<td>JE1-25-(A-D)</td>
<td>0.01 N</td>
<td>1 g</td>
<td>1 time</td>
<td>9.34%</td>
<td>18.0</td>
<td>5.44</td>
</tr>
<tr>
<td>JE1-28-(A-D)</td>
<td>0.001 N</td>
<td>1 g</td>
<td>1 time</td>
<td>12.25%</td>
<td>14.6</td>
<td>4.76</td>
</tr>
</tbody>
</table>

Because these impurities are difficult to detect with a varying acid concentration, it is difficult to determine which acid concentration is optimal. It is suspected that some of the impurities that appear are the results of another monosaccharide unit called mannans. Mannans possess acid-labile portions, so it would make sense that they would be left behind under lower acidic conditions.\textsuperscript{16} Mannans are not necessarily bad for one’s health, but because the purpose of this extraction is to obtain pure, isolated $\beta$-glucans, this effect is
undesired. The change in ABF and ASCL is generally helpful in determining the optimal conditions, but these results do not show a significant difference. It appears that the higher concentration of phosphoric acid would be preferred in terms of purity. Another extraction with conditions of 0.1 N NaOH and 1 N H₃PO₄ should be performed to compare to sample JE1-22-A (Figure 3.3.0).
4. Conclusions and Future Work

4.0 Conclusions

The results of this research project showed great promise towards the optimization of the extraction of (1,3/1,6)-β-glucans. This research investigated extraction optimization by reducing the number of steps required and testing various acid concentration. By learning more about ways to enhance the extraction process for (1,3/1,6)-β-glucans, the potential for this process to be used by modern pharmaceutical companies increased greatly. This was because the results showed great favor with regard to reduced cost and extraction time.

The first part investigated whether or not the number of extraction steps with boiling ethanol affected the purity and sample size of the resulting β-glucan product. Compared to the traditional method, reducing the number of steps with boiling ethanol did not show a significant change in the purity of the final product. Additionally, this reduction resulted in higher percent yields by mass as compared to the starting masses. In some instances, the percent recovery of material was almost doubled when the extraction steps with boiling ethanol were reduced. This result is very important to be able to market this method in the future, and will be the basis of some potential ideas for future work (see Section 4.1).

The next area investigated was the reduction of the sample size in order to assess whether or not the extraction methods were scalable. The results showed the initial sample mass of S. cerevisiae did not have a significant effect on the final sample’s composition or size. This also makes the extraction process
more favorable for industrial purposes. Further, these results implied that the method can be scaled and still provide desired results without sacrificing any of the desired product’s quality.

Finally, the optimization of the acid concentration revealed interesting and important information about its role in the extraction process. By reducing the acid concentration in many trials, it was demonstrated that while the acid plays a very small role in the removal of undesired fungal cell wall components, it is still essential. There is research that shows there are acid labile portions of fungal cell wall structures, such as mannans, which are left behind by gentler extraction methods. Without being able to remove these acid labile portions, the final product composition contained impurities. It is likely these impurities were difficult to detect because of the similarity of composition between fungal glucans and mannans. By reducing the acid concentration significantly, small impurities began to arise which revealed the essential role of the extraction step that utilizes acid.

By considering each of these conditions independently, it was much easier to determine the optimal conditions of the extraction process. Perhaps the most beneficial result from this research is that it set the stage for future research to be conducted on the extraction of (1,3/1,6)-β-glucans. Many of the efforts that were made aim to make the extraction process more favorable for industrial purposes. Luckily that also means that the process becomes easier for researchers to conduct further optimizations. There is still much work that can be done to improve this extraction process. Fortunately, working so intimately with this
extraction process revealed several avenues where this research could be improved.

There are still areas where this extraction scheme could be improved, but based off of this research, the best method for the extraction of (1,3/1,6)-β-glucans was determined. First, reducing the extractions with boiling ethanol to only one time provides not only similar results, but also allows for the total extraction to be completed more quickly. Second, it should be noted that the size of the initial sample does not yield any significant effects on the final product’s purity; therefore, when improving this method, smaller batches will allow for more progress while maintaining cost efficiency. Lastly, the optimal concentration for the solvents is not known precisely; however, based off of observed trends in this research, it is believed that the optimal solution concentrations with the solvents NaOH and H₃PO₄ would be 0.1 N and 1 N, respectively.

4.1 Future work

One of the first areas that needs to be addressed is the interpretation of the results of the research. While there is good reason to believe that the results that were obtained are precise and consistent, it is an entirely different task to assess their accuracy. One potential way to do this would be to perform these experiments with other forms of yeast. Another closely related yeast, such as S. paradoxus or S. mikatae, would be a good place to start. S. paradoxus has many similar structural features to that of S. cerevisiae. S. paradoxus is also the closest known family member to S. cerevisiae, but has not been domesticated by
humans. Applying the extraction process that was optimized for *S. cerevisiae* would reveal information about the accuracy of the results that were being obtained. By being able to compare the yields and composition of the products obtained from each species, greater understanding of the functionality of this method could be gathered. This could reveal new information about ways in which this method could be further optimized.

Additionally, there are some initial results from this research that indicate there may be some potential for a different acid to be used for the extraction process. In other trials, formic acid showed a similar result to that of phosphoric acid, but these results have not been fully investigated. There have been studies that have shown that formic acid is a more environmentally friendly solvent. Furthermore, it also has a lower cost and does not leave behind phosphates.

By utilizing an acid that showed similar promise, there is potential for greater overall yield and possibly better removal of the acid labile portions of the fungal cell wall. It would also be worthwhile to investigate the use of an alternative to sodium hydroxide because most prior research has only adjusted the acid concentration. However, the sodium hydroxide appears to be efficient. Further, sodium hydroxide provides a good solvent due to its shelf life and ease of use. Additionally, it is the strongest base relative to other similar bases such as potassium hydroxide or lithium hydroxide. Other bases not that do not include contain elements from Group 1 of the periodic table (e.g. ammonia, ethylamine) neither exhibit the same strength in terms of basicity nor are they easy to store in a laboratory.
In an attempt to further reduce the time required for this extraction process, additional steps could be taken. For instance, each time there is a step where an extraction is occurring by the use of boiling acid, base, or ethanol, the time specified in the methods is set at 15 minutes. There is a possibility that for some of these steps, if not all, the amount of time spent boiling could be reduced two-or-three-fold. This would significantly reduce the amount of time and energy required to extract the β-glucans. Likewise, the centrifugation steps could be altered in their duration. This could even reveal insights into the potentials of utilizing differential centrifugation to expedite some steps in the process.

Lastly, it would be beneficial to investigate more in-depth analyses using other analytical methods. By utilizing GC-MS, there would be further confirmation of the true contents of the final products that were obtained. GC-MS was utilized for a few samples in this experimental work, but utilization of this technique would be excellent for providing even greater insight into the native structure of the β-glucans. By utilizing methods similar to Arthur’s, periodic checkpoints could be performed to ensure the glucan’s native structure is being maintained. Through Arthur’s work, the fractionation patterns supported that the extraction processes were successfully isolating the β-glucans. With further research, similar analytical methods to GC-MS should be used to further confirm the isolation of β-glucans in the extracted product.
5.0 References


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21. Arthur, C. Linkage Analysis and Compositional Studies of β-Glucan from Saccharomyces cerevisiae and Compositional Studies of Mannan from
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Note: Sample JE1-13-A: First 1 g sample: The wrong size centrifuge flasks likely responsible for some sample loss. Sample A had too low of a yield for NMR. Average % Yield for this set calculated only from samples B-D.
7.2 Spectra for all samples

Data sets are labeled at top left (upright) of each spectrum for all samples.