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Optimized Acid/Base Extraction and Structural Characterization of β-glucan from Saccharomyces Cerevisiae

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Optimized Acid/Base Extraction and Structural Characterization of β-glucan from *Saccharomyces Cerevisiae*

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by

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ABSTRACT

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β-glucan is a major component of the fungal cell wall consisting of (1→3)-β linked glucose polymers with (1→6)-β linked side chains. The published classical isolation procedure of β-glucan from *Saccharomyces cerevisiae* is expensive and time-consuming. Thus, the aim of this research was to develop an effective procedure for the extraction of glucans. We have developed a new method for glucan extraction that will be cost effective and will maintain the native structure of the glucan. The method that we developed is 80% faster and utilizes $^1/_3$ of the reagents compared to the published classical method. Further, the method developed increases the yield from 2.9 % to 10.3 %. Our new process has a branching frequency of 18.4 down from 197 and a side chain of 5.1 up from 2.5. The data indicate a more preserved native structure of isolated glucans.

DEDICATION

This research work is dedicated to God Almighty for his unconditional grace and mercies, my mother, Matilda Bekoe for her prayers and support, SSG Fred O. Bekoe for his support throughout my education, my sister and all my loved ones.

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LIST OF ABBREVIATIONS

NMR Nuclear magnetic resonance

TFA Trifluoroacetic acid

BF Branching frequency

SC side chain

DP Degree of polymerization

DMSO Dimethyl sulfoxide

DI Deionized

rpm Revolutions per minute

CWP Cell wall proteins

NRT Non-reducing terminal

GPI Glycosylphosphatidylinositol-modified

PPCO Polypropylene Copolymer

CHAPTER 1

INTRODUCTION

The yeast *Saccharomyces cerevisiae* is a model organism that is commonly referred to as baker's yeast. It is used as a leavening agent in the production of bread. It generates carbon dioxide which causes the bread's aerated structure as well as adds flavor to the bread.[1,2] "*Cerevisiae*" is a scientific name which was derived from an ancient terminology used to describe beer. It belongs to the fungus or mold family. *S. cerevisiae* is a single celled organism that contains a nucleus and other membrane organelles. Yeast undergoes rapid cell growth and division that produces daughter cells under different conditions in a process called budding. The size of the yeast varies and depends on the species of interest; its typical range is between 3-40 µm in diameter.[1-3]

When *S. cerevisiae* is exposed to numerous environmental conditions such as osmotic pressure, heat, and desiccation, its growth reduces and this affects its metabolic activity. In order to retain metabolic activity and improve bio-production under these environmental stress conditions, one must employ yeast which can grow under these environmental stress conditions.[3]

The Fungal Cell Wall

The fungal cell wall is a unique structure that maintains the shape and structure of the fungal cell and acts as the main point of contact between the cell and its environment.[4-7] The cell wall is a tough, flexible layer, but can sometimes be fairly rigid. It is found outside the cell membrane providing the cell with protection as well as serving as a filtering mechanism. The cell wall is primarily composed of four main components: glucan, chitin, mannan, and mannoprotein as shown in Figure 1.

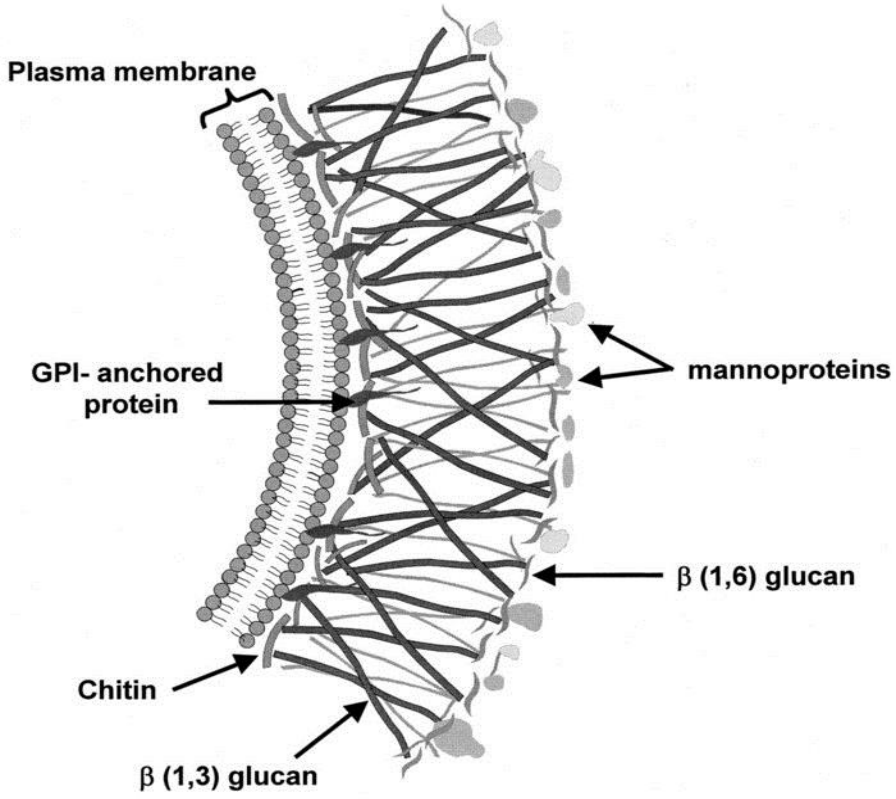

Figure 1. Detailed structure of the cell wall of yeast *Saccharomyces cerevisiae*.[7]

The fungal cell wall acts as a pressure vessel that protects the cell from over-expansion when water enters the cell. There are theories of heavy cross-linking between the cell wall components but the actual nature of this cross-linking has yet to be established.[8,9] The fungal cell wall also plays an essential role in various biological processes that determine the reproduction of cell-cell and cell-matrix interaction, with other physical activities.[10]

Saccharomyces cerevisiae is of great importance because of its wide range of applications. The cell wall of *S. cerevisiae* is composed of a complex cross-linked network of glucans and chitin. Chitin is a linear carbohydrate polymer of N-acetylglucosammine which is believed to be attached to a number of glucans. It is typically found at the budding sites of the cell and represents about 1-2% of the total mass of the cell.[10,11] Even though it forms a relative small composition of the total cell wall mass, it is important for the viability of the cell. Chitin has the ability to form complexes with the glucan. These complexes normally form a crystalline structure, which acts as a foundation upon which the rest of the cell wall is built.[10,12]

Of the major cell wall components in *Saccharomyces cerevisiae,* glucan represents about 50% of the cell wall mass.[13] Both the chitin and the glucan confer a very high mechanical resistivity to the cell wall. Table 1 gives the percentage range of the various components of the cell wall of *S. cerevisiae.* Beta-glucans can also be linked to cell wall proteins (CWP) via covalent bonding. CWP accounts for about 35-40% of the cell wall and it is characterized by high mannose *N*- and/or *O*-glycosylated proteins. The complex structure of the fungal cell wall makes it an important target for antifungal therapy.[10,14]

Table 1. The cell wall components of *Saccharomyces cerevisiae.*[13]

Component	Cell wall mass (%, dry weight)
Glucans	50-55
Lipids	1-3
Mannoprotein complex	35-40
Chitin	1-2

General Applications of Fungi

Fungi have been used extensively in the production of food for decades. In recent times, the wide application of fungi has been exploited in the pharmaceutical, flavoring, and chemical industries. Due to their importance, detailed research has been carried out on the physiological activity of these fungi. *S. cerevisiae* is the most studied fungal species due to its wide range of applications in the industrial world, particularly in the food and pharmaceutical industries.[15]

Microbial proteases that are derived from different type fungal sources have been proven to be useful in meat tenderization due to its substrate specificity.[15,16] *Aspergillus Oryzae* is the most common fungal protease that has been employed commercially for the production of various foodstuffs like soy sauce and butter.[16] *A. oryzae* has been proven to be a safe and reliable source of proteases. A typical *A. oryzae* protease example, aspartic protease, has maximal activity between the pH ranges of 2.5-6.0, which makes it effective in the application of food tenderizing because table meat cuts have a typical range of pH 5.4-5.8.[10] Another useful fungi in

the food production industry is *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, it is used in the production of yogurt as a bio-preservative agent.[17]

The application of fungi is not only limited to the food industries, plant *endophytic* fungi has a potent and novel natural bioactive activity, that has wide applications in the agricultural and medicinal industries.[18] Over the decades, many important bioactive compounds, such as antimicrobials, insecticidal, and anticancer agents, have been synthesized from *endophytic* fungi. Taxol (Paclitaxel), a highly potent anticancer drug, was classically isolated from the pacific yew tree *Taxus spp.* However, the production of Taxol from *Taxus spp*. was very low due to insufficient yew trees. With the increase in demand for Taxol, there was a need to find new ways of producing it apart from the usual source of *Taxus spp.* The bioactive compound Taxol was later discovered in *Taxomyces andreanae.*[18] Fungi *Siderophores* which is one of the widely used fungi in medicine have been utilized in the treatment of iron overload conditions such as b-thalassaemia.[19]

Cell Wall Glucan

In general, β-glucan is a natural polymer of D-glucose which is produced by many different types of organisms. It is found in the cell walls of fungi, plants, and algae. The glucose monomers are linked together by β-glycosidic bonds.[20-22] □-glucan is the major constituent of the cell wall of cereal grains, fungi and yeast.[23,24] The glucans resulting from the various sources have varied structures which determines the physical properties of the β-glucan, such as viscosity and solubility. Several β-glucans differ in glycosidic linkage position.[25,26]

β-glucan from *Saccharomyces cerevisiae*

β-Glucan from *Saccharomyces cerevisiae* consist of (1→3)-β-D-glucan backbone and (1→6)-β-D-glucan sidechains that forms a single structure as shown in Figure 2.[23,27] The (1→3)-β-D-glucan is responsible for the strength of the cell wall; it forms a triple helix 3-dimensional structure which has a spring-like mechanical properties. It is believed that the (1→6)-β-D-glucan links the (1→3)-β-D-glucan to the chitin and mannoprotein, but the actual linkages have not been determined.[27] The fungal cell wall's mechanical strength is largely due to (1→3)-β-glucan chains.[28-30] (1→3)-β-glucan chains belong to the hollow helix family; its shape is similar to a flexible wire spring that can exist in different forms. This unique property of β-glucan helps to explain the elasticity of the fungal cell wall.[28,31] Krainer and co-workers used ^{13}C-NMR on living cells to confirm that a portion of (1→3)-β-glucan have a helical structure.[28,32] When cells are in stationary phase, (1→3)-β-glucan molecules have been found to made up of about 1500 monomers of glucose unit. In their mature state, (1→3)-β-glucan are branched and contain (1→6)-β-glucan linked glucose unit. The degree of branching of (1→3)-β-glucan may also depend on environmental conditions such as growth phase. In general, (1→6)-β-glucan is a highly branched water-soluble polymer which consists of about 130 monomer units of glucose. It is believed that the (1→6)-glucan's function is to connect Glycosylphosphatidylinositol-modified (GPI) dependent proteins to the (1→3)-β-glucan network system. In the case of cell wall stress, (1→6)-β-glucan can function as acceptor site for chitin.[28]

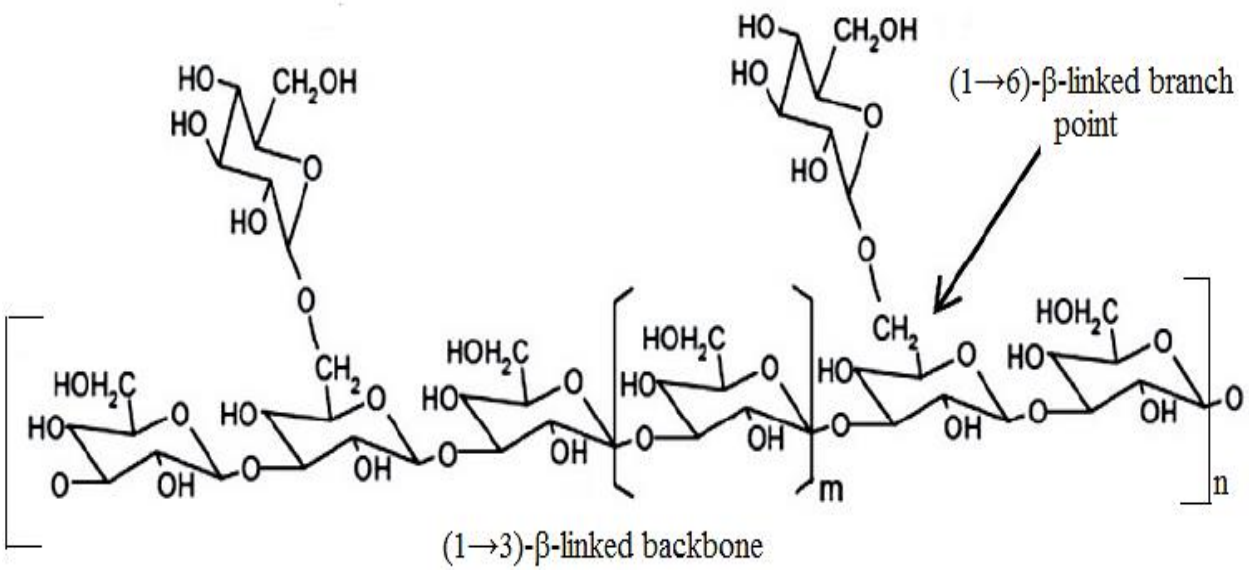

Figure 2. Chemical structure of β-glucan from *Saccharomyces cerevisiae*.[9]

Applications of β-glucan

There is a wide range of applications for β-glucans in food production such as a fat substitute, a thickening agent, and an emulsifier.[9,33] β-glucans have been used in the food industry in the production of frozen desserts, sauces, salad dressings, soft dough, and cake filling. β-glucans has the ability to retain water and has an oil-binding property allowing it to be used in the production of sausages and other meat products.[34]

β-glucan has been shown to be an immunostimulant for humans. It has been used as a therapeutic for people with immunosuppressed diseases to help improve their immune system.[35-37] It has been proven experimentally that β-glucans protect mammals from various kinds of infections and increase immune system cell's cytotoxicity against cancer.[25] β-glucans have also been proven to be involved in a process related to the reduction in postprandial blood glucose

and cholesterol levels.[38,39] β-glucan has been recently included on the list of products that contributes to the reduction of cholesterol in blood by FDA (United States Federal Drug Administration) and EFSA (European Food Safety Authority), and in so doing, has a recommended daily intake of 3 g to attain this positive effect.[38-40]

Classical Method for Extracting β-glucan from Cell Wall of Fungi

The classical method for extracting glucan from a fungal cell wall was developed in 1941. This classical method requires sequential base, acid, and ethanol extractions of the yeast from the fungal cell wall for several days.[41] This method is not only time consuming, but it also requires large volume of acid (HCl), base (NaOH) and ethanol for the extraction which is not environmentally friendly. While the classical method is known to effectively isolate the glucan from all other components of the fungal cell wall, it degrades the glucan to a high extent by reducing the polymeric chains. The final product obtained therefore does not reflect the actual nature and the native structure of the glucan in the fungal cell wall.[41,42]

The actual native structure of the beta-glucan is not known. However, the literature supports the assumption that the lower branching frequency and higher side chain length, the closer the beta-glucan is to the native structure. Lowman and coworkers used the classical extraction method to study the structure of beta glucans in *S. cerevisiae*. By working on a standard *S. cerevisiae,* they reported a branching frequency and side chain length of 198.8 and 0.37 respectively. Their NMR spectra proved that the classical method has been able to isolate the glucan from all other components of the fungal cell wall. However, due to the higher value of branching frequency and smaller value of side chain, the glucan isolated had most of its

polymeric chains reduced. Due to the degradative nature of the classical method of extraction, it was imperative for a new method for the extraction of glucan from the fungal cell wall to be developed in order for the structure of the glucan to be determined.[41]

Other Developed Methods for β-glucan Extraction from Fungal Cell Wall

Freimund and coworkers developed a method that produced a less degraded β-glucan that is free from other components of the fungal cell wall of *S. cerevisiae*.[43] In their method, yeast cell wall was treated with hot water and enzymatic Savinase. This method was efficient in isolating the glucan from other components of the fungal cell wall; it also enabled the isolation of mannoprotein, which is a physiologically active compound of big interest. They used NMR spectroscopy to confirm the structure of the isolated glucan.[43] However, this method is time consuming, and most importantly, it involves enzymatic Savinase, which is very expensive, and hence does not make the method cost-friendly. Other methods developed by Lui and coworkers and also by Javmen and coworkers involved the autolysis of the fungal cell wall, and further treatment with protease.[25,44] These methods also involve the use of enzymes that makes the extraction process expensive.

Optimized Extraction Method

To overcome the most important barrier in glucan extraction, that is, the cost involved when using protease enzyme used other documented methods, it is imperative to develop a new method for the extraction of β-glucan from the fungal cell wall. In order for this new method to be acceptable, it has to overcome the challenges of the earlier developed methods, that is, it has

to be very simple, less time consuming, and, most importantly, must be cost-effective. This new method should be able to isolate the glucan from all other components of the fungal cell wall and must also enable further analysis and characterization of the glucan extract. A new method, which involves the treatment of the yeast sample with a mild acid and base extraction was developed by Dr. Greene. My project was primarily to determine the optimum extraction method where the glucan extracted has the most native structure while also producing a pure product that is free of traces of other components of the cell wall. The treatment of the yeast sample with hot acid and base dissolved the proteins and other polysaccharides in the yeast, and after centrifuging the solution, the solid remaining was the glucan.[39] In order to remove all residual lipids, the solid glucan was treated with ethanol. Proton NMR analysis was used to confirm the structure and whether the glucan isolated was free of other components of the cell wall.

Calculating the Average Branching Frequency and Average Side Chain Length

In order to determine the extent of degradation of the isolated glucan, the branching frequency and side chain length were calculated. The BF gives information about the branching of the 1,6-β-glucan side chain unit from the 1,3-β-glucan main backbone unit. This calculated number gives information of the number of repeat units of 1,3-β-glucan between two 1,6-β-glucan branching units. A low BF means the average distance between two branching 1,6-β-glucan unit is less, which means there is more branching unit of the 1,6-β-glucan. Reduced BF means increased branching units, which means less degradation has been done to the glucan extract. The side chain length, gives the average length of the 1,6-β-glucan side chain. A higher SC means less degradation to the glucan structure. A schematic showing how the glucan forms the 1,3-β-glucan with 1,6-β-glucan side chains is given in Figure 3.[20]

To determine the average branching frequency and the side chain length, the integrated areas of the resonances assigned to H_1 of side chain non reducing terminal (H_1 SC NRT), H_1 of side chain (H_1 SC), and H_6 of side chain (H_6 SC) glycosidic bond as shown in an example NMR in Figure 4 were compared to H_1 of the (1→3)-β-linked repeating units in the polymer backbone. The BF and SC were calculated from the equations below. Equation 1 gives information of the peak area for one glucose unit. Equation 2 gives information of the length of the side chain based upon the number of glucose repeat units. Equation 3 gives the branching frequency; it is a ratio of side chains to repeat glucose units. The assigned protons in Figure 4 refer to the glucosyl repeat units in the polymer (1→3)-β-linked backbone. The expanded region of the spectra from 4.32 to 3.96 ppm represent the multiple repeating points in the resonances for the anomeric proton, H_1 SC, and one of the methylene protons of the H_6 SC, of the (1→6)-β-linkage of the side chain respectively.[20]

$$\text{SC } H_6 - \text{SC } H_1 = X \quad \ldots\ldots (1)$$

$$\frac{\text{SC } H_6}{X} = \text{SC} \quad \ldots\ldots (2)$$

$$\frac{\text{BB } H_1}{X} = \text{BF} \quad \ldots\ldots (3)$$

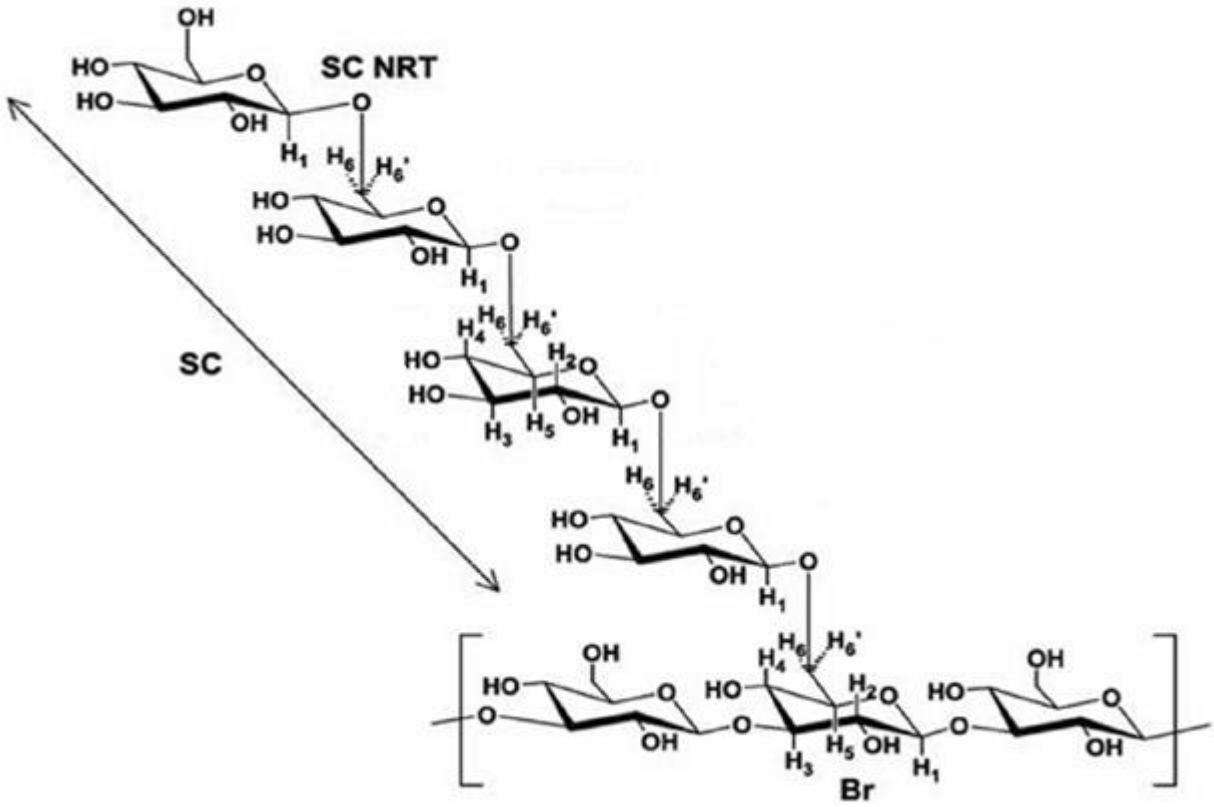

Figure 3. A schematic diagram of the structure of poly-(1→6)-β-D-glucan side chain which contains n repeat units and is attached to a (1→3)-β-linked backbone chain.[20]

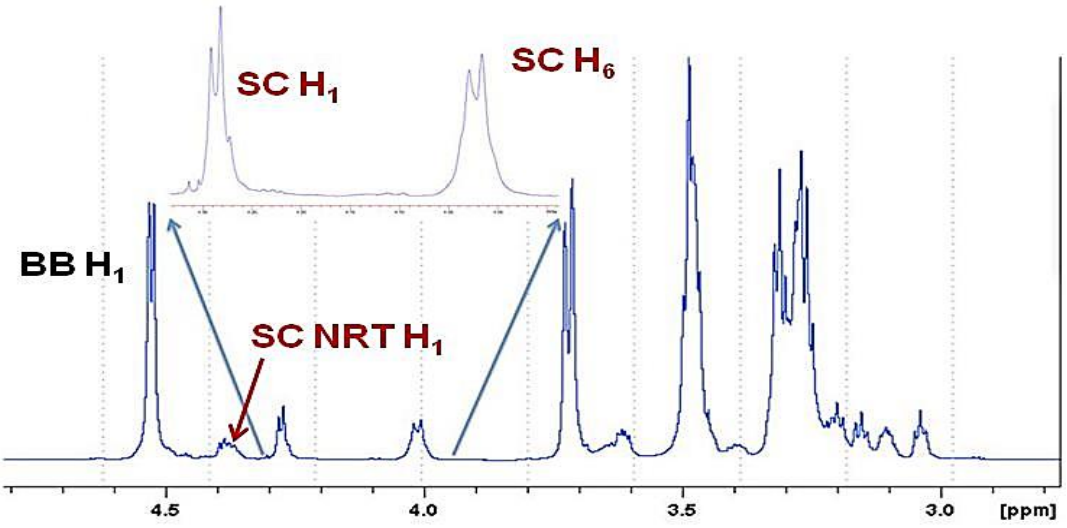

Figure 4. Proton NMR spectrum of (1→3,1→6)-β-glucan extracted from *C. glabrata*.[20]

It was reported by Tada and co-workers that the integration of the resonance assigned to H_1 SC and H_6 SC had a ratio of 1:1 for *Grifola frondosa*, which means the *Grifolan frondosa* had a single (1→6)-β-linked glucosyl repeat unit in the side chain of the backbone.[37] Interestingly, Lowman and co-workers found that the integrated areas assigned to H_1 SC and H_6 SC of the glycosidic linkages in the (1→6)-β-linked side chain have different ratios in glucan extracted from *Candida glabrata.* They determined that the integrated area of the H_6 SC (4.02 ppm) was larger than the integrated area of the H_1 SC (4.27 ppm); the integration of the two areas gave a ratio of 0.787:1 for H_1 SC and H_6 SC respectively. The difference in areas of H_1 SC and H_6 SC resonance gives the integral area assigned to the anomeric H_1 proton in the SC NRT. Based on their 0.787:1 ratio, they found out that in *C. glabrata,* the side chain contains about 4.7 (1→6)-β-linked repeating units. Comparing the ratios of areas assigned to H_1 of SC NRT and H_1 of the (1→3)-β-linked repeating units in the backbone of the polymer, they found out that the side chain is attached to the (1→3)-β-linked polymer backbone on an average of every 21 repeating units.[20]

In using different extraction conditions to isolate glucan from *Saccharomyces cerevisiae*, it was found that the average side chain length contains 2.5-6.2 (1→6)-β-linked repeating units *depending* on the extraction conditions. Comparing the ratio of the area assigned to H_1 of SC NRT and ^1H of the (1→3)-β-linked repeating units in the backbone of the polymer, it was also determined that the side chain is attached to the (1→3)-β-linked polymer backbone on an average of every 18.6-196.5 repeating units depending on the extraction conditions.

Research Aims

1. The primary aim of this research was to develop a milder and cost effective method for the extraction of β-glucan from *Saccharomyces cerevisiae*; this method should result in a more native glucan structure which has not been greatly degraded.

2. Another aim of this research was to use NMR spectroscopy to study and characterize the structure of β-glucan produced from *Saccharomyces cerevisiae* using the optimized extraction method.

CHAPTER 2

MATERIALS AND METHODS

Saccharomyces Cerevisiae

The yeast used in this research was a commercially available *Saccharomyces cerevisiae*. It was a dry sample purchased from Red Star Yeast and Products Corporation in Wisconsin, USA. It was stored in a refrigerator at a temperature of 4^0 C to maintain the dormancy of the yeast.

Materials

All the chemicals that were used were bought from Sigma Aldrich, they were standard laboratory chemicals designed for research and development purposes. The base used (NaOH) was a laboratory reagent grade with the following specification; vapor density >1 (vs air), vapor pressure < 18 mmHg (20 °C), assay ≥ 98 %, form: pellets (anhydrous), impurities ≤ 1.0 % sodium carbonate, mp: 318 °C (lit). Two different acids were used. The HCl was a laboratory reagent grade with the following specifications; vapor pressure: 3.23 psi (21.1 °C), assay: 36.5-38.0 % (ACS specification), impurities ≤ 1 ppm free chlorine and ≤5 ppm extractable organic substances, color: Alpha: ≤ 10, free from suspended matter or sediment, bp >100 °C (lit.), density: 1.2 g/mL at 25 °C (lit.), anion traces: bromide (Br^-): ≤ 0.005%, sulfate (SO_4^{2-}): ≤1 ppm, sulfite (SO_3^{2-}): ≤1 ppm, cation traces: As: ≤0.01 ppm, Fe: ≤0.2 ppm, NH_4^+: ≤3 ppm, heavy metals (as Pb): ≤1 ppm. The phosphoric acid used was of ACS grade with the following specifications: vapor pressure: 2.2 mmHg (20^0), concentration: 85 wt. % in H_2O, bp: 158^0C (lit.), density: 1.685 g/mL at 25 °C (lit.). The ethanol used was of ACS reagent grade with an assay ≥ 99.5 % and a boiling point of 78.3 °C. Deuterated dimethyl sulfoxide (d_6-DMSO) with the

following specifications was used; isotopic purity: 99.96 atom % D, density: 1.190 g/mL at 25 °C (lit.), with a boiling point of 189 °C (lit.). The trifluoroacetic acid was of *R*eagentPlus grade, 99 % assay, and a boiling point and density of 72.4 °C and 1.489 g/mL at 20 °C (lit.), respectively. The extraction was performed in a 500 mL and a 250 mL Erlenmeyer flask. A 250 mL and a 50 mL polypropylene centrifuge bottles were used for centrifuging the solutions. All water used during this project was an (18 MΩ) deionized water.

A 400 MHz NMR was used for this project; it was set at the following parameters; Number of Scans: 16, Acquisition Time: 3.2768 seconds, Interpulse Delay: 15 seconds, Relaxation Time: 15 seconds, Data Points: 32,768, Sweep Width (X_Sweep): 25 ppm, X_Offset: 5.0 ppm, Pulses Angle: 90°, Probe Temperature: 80 °C. The chemical shift reference was set to 2.50 ppm, using the residual proton resonance of d_6-DMSO.

A Sorvall Legend RT+ centrifuge was used for this project. It had a capacity of 8 x 50 mL or 4 x 250 mL rotor, with a 230 V capacity. It had a speed limit of up to 9000 rpm. A four plate Super-Nuova Multi-place Stirrer and Stirring Hot plates with integral controls was used for all extractions. All weighings were completed with a METTLER TOLEDO's™ Basic Weighing balance. A Thermo Scientific Orion Star A111 pH Benchtop Meter was used for all pH steps.

Glucan Extraction

Classical Extraction Procedure

The classical extraction procedure was repeated during this research for direct comparison to the optimized method. Approximately 4 g of dry *S. Cerevisiae* was extracted with a 200 mL of 1 N NaOH for the base extraction procedure. The NaOH and all other solutions were prepared with deionized water (DI H_2O). The solution was then centrifuged at 5000 rpm and the supernatant was removed. The precipitate was then resuspended in another 200 mL of 1N NaOH. The yeast was extracted three times with boiling base solution. The residue after the base extraction was adjusted to a pH~7. After the pH, the residue was extracted three times with a boiling HCl solution. After the acid extraction step, the yeast was then extracted with boiling ethanol for three times. Between the first and third ethanol extractions the residue was left overnight in the presence of the ethanol. After the ethanol extraction, the resulting residue was resuspended in DI water and was then adjusted to a pH of ~7. After the residue was adjusted to a pH~7, it then boiled and washed three times in DI water. The residue was then dissolved in approximately 15 mL of DI water in a conical tube in the freezer. The solution was lyophilized and analyzed with the NMR. The number of extractions for each solution was reduced to one in the optimized extraction procedure.

Optimized Extraction Procedure

Sodium hydroxide solution was prepared for the base extraction. The concentration and volume was adjusted for the desired experiment. 1-4 g of dry yeast was extracted with boiling NaOH solution for 15 minutes at 100 ^{O}C with stirring in a 150 mL/500 mL Erlenmeyer flask with a loose fitting glass stopper. The solution was allowed to cool down for ~1 hour, it was then

centrifuged for 5 minutes @ 5000 rpm on the Sorvall Legend RT+ Centrifuge in 50 mL/250 mL Nalgene PPCO bottles. The residue was kept and the supernatant was discarded after all centrifugation steps. The residue was resuspended in 50 mL/200 mL DI water. It was then centrifuged for 5 minutes at 5000 rpm. The residue was kept. The residue was re-suspended in DI water and pH between 6.9 and 7.1 with 0.1 M HCl and 0.1 M NaOH.

Acid was prepared for the acid extraction step. The concentration was adjusted for the desired experiment. The yeast residue was extracted with boiling acid solution in a clean 125 mL/500 mL Erlenmeyer flask. A clean flask was used to avoid contamination from any remaining base in the first flask that could affect the pH of the acid extraction. The solution was allowed to cool down for ~1 hour, it was then centrifuged for 5 minutes @ 5000 rpm in 50 mL/250 mL Nalgene PPCO bottles.

The yeast residue after the acid extraction was then extracted with boiling ethanol solution (50 mL/200 mL) at 80 ^{o}C in same flask. The pellet after the ethanol boiling was difficult to centrifuge, so the speed of the centrifuge was increased to 7000 rpm. The residue after the ethanol extraction was resuspended in DI water 50 mL/200 mL in centrifuge bottle, it was then centrifuged and the residue was kept. The residue was washed with water by resuspended in DI water and boiled for 15 minutes. It was allowed to cool and then centrifuged. The residue was again suspended in DI water and the pH was adjusted between 6.9-7.1 with 0.1 M HCl and 0.1 M NaOH. The solution was centrifuged and the residue was kept. The residue was again washed with water by suspending it in DI water, and centrifuged. This step was repeated three times. The residue was resuspended in less than 15 mL DI water in a pre-weighed 50 mL conical tube. It was then frozen overnight at about -80 ^{o}C. The samples were lyophilized after it has been frozen. The samples were weighed after the lyophilization to determine the percent yield.

For the optimized extraction method, series of different set of extractions were made. Several experiments were conducted where the number of acid extractions, the acid concentration, base concentration, number of base extractions and number of ethanol extractions were varied as tabulated in Table 2. In method 1, the NaOH concentration was 1 N, the yeast was extracted three-times with boiling base solution and then adjusted to a pH~7. It was then followed by extraction three-times with boiling 1 N phosphoric acid solution. This was followed by three extractions with ethanol. In method 2, the concentration of the NaOH and H_3PO_4 was kept constant at 1 N, however the number of extractions was reduced. The yeast was extracted one-time with boiling base solution and the adjusted to a pH~7, it was then followed by a one-time acid extraction with boiling acid solution. This was followed by three-time extraction with ethanol. Method 3 varies with method 2 only in terms of the number of ethanol extractions. In method 3, the yeast was extracted one-time with the boiling base solution; it was adjusted to a pH~7 and then followed by a one-time acid extraction. This was followed by a one-time ethanol extraction. The concentration of both the acid and base was reduced to 0.1 N in methods 4 and 5. In method 4, the yeast was extracted one-time with 0.1 N base boiling solution, it was then adjusted to a pH~7. This was followed by a one-time extraction with 0.1 N acid boiling solution. After the acid extraction step, the yeast was finally extracted with a one-time ethanol boiling solution. Method 5 was very similar to method 4 with the exception of the number of ethanol extractions. In method 5, the yeast was extracted one-time with boiling base solution; it was then adjusted to pH~7 and then followed by a one-time extraction with boiling acid solution. This was then followed by a one-time extraction with boiling ethanol solution. Figure 5 shows a flow chart of the optimized acid/base extraction procedure.

Table 2. Tabulated set of conditions used for the isolation of glucan from *S. Cerevisiae*.

Method	**NaOH Conc. (N)**	**NaOH boilings**	**H_3PO_4 Conc. (N)**	**H_3PO_4 Boilings**	**Ethanol Boilings**
1	1	3	1	3	3
2	1	1	1	1	3
3	1	1	1	1	1
4	0.1	1	0.1	1	3
5	0.1	1	0.1	1	1

Methods 1 through 5 were repeated with smaller amounts of the sample. Approximately 1 g of the yeast was used instead of the typical 4 g used in the original analysis. All volumes were reduced from 200 mL to 50 mL.

^{1}H NMR Analysis

Approximately 18-22 mg of the dry sample was dissolved in 1 mL of d_6-DMSO in a 5 mL test tube. It was shaken several times to dissolve the sample. To ensure that the samples completely dissolved in the d_6-DMSO, the test tubes containing the solution was placed on a hot bath and the temperature was set to 80 ^{o}C for about half an hour. The glucan solution was shaken occasionally to ensure complete dissolution. About 2-4 drops of TFA was added to the solution in the test tube to shift the water peak further down field, this is done to improve the resolution of the spectra. The solution was then transferred into an NMR tube, and analyzed with a 400 MHz NMR.

During the analysis of the NMR spectra, whole base area integration was performed for the anomeric hydrogen which is around 4.5 ppm. Base area integrations were also performed for the side chain hydrogen 1, that is, H_1 SC (1-6) and H_6 SC (1-6) respectively. The branching frequency (BF) and side chain length (SC) were calculated from the values of the integrated area.

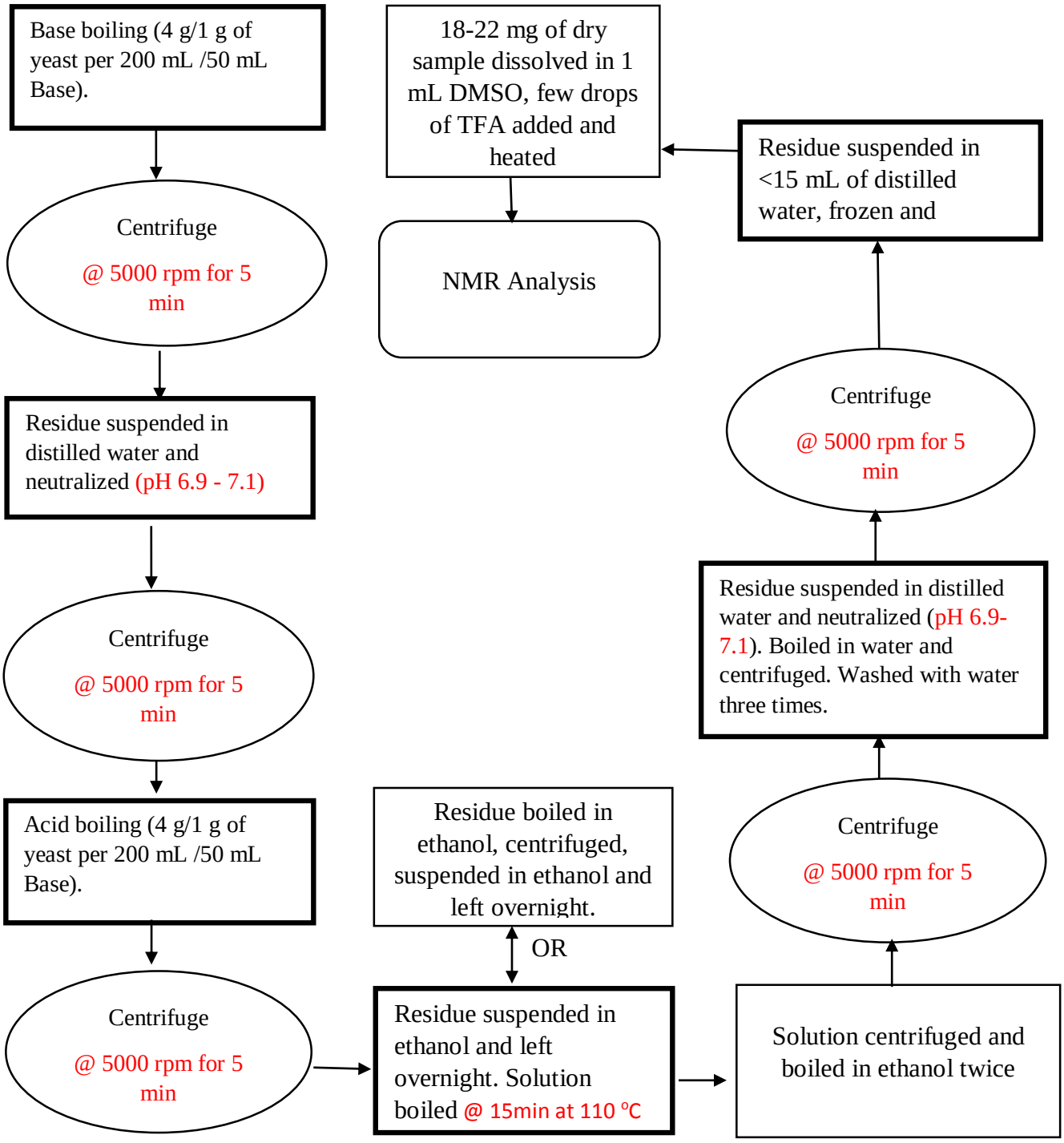

Figure 5. Schematic diagram of beta glucan isolation from yeast cell walls.

Titration Extraction Method

A titration method of extraction was performed. In the first sets of extraction, the concentration of the base was held constant at 0.1 N NaOH, and the concentration of the acid was reduced in each extraction. In the second sets of extraction, the concentration of the acid was held constant at 1 N H_3PO_4 and the concentration of the base was reduced in each extraction step. In all these extractions, the yeast was extracted one-time with a boiling base solution and then adjusted to a pH~7. This was followed by a one-time extraction with the boiling acid solution. It was finally extracted one-time with a boiling ethanol solution. Approximately 1 g of the dry sample was used with 50 mL of solution in each step. Below is the titration combination that was performed.

a. 0.1 N NaOH vs 1 N H_3PO_4

b. 0.1 N NaOH vs 0.1 N H_3PO_4

c. 0.1 N NaOH vs 0.01 N H_3PO_4

d. 0.1 N NaOH vs 0.001 N H_3PO_4

e. 1 N NaOH vs 1 N H_3PO_4

f. 0.1 N NaOH vs 1 N H_3PO_4

g. 0.01 N NaOH vs 1 N H_3PO4

h. 0.001 N NaOH vs 1 N H_3PO_4

Base Extraction with no Acid

Approximately 1 g of dry *S. Cerevisiae* was extracted one-time with a 50 mL 0.1 N NaOH boiling solution. It was then adjusted to a pH~7. The residue after the base extraction was extracted one-time with boiling ethanol solution. It was boiled and washed in the DI water three times. It was then dissolved in approximately 15 mL of DI water and kept in a plastic bottle in the freezer. The solution was lyophilized and analyzed with the NMR spectroscopy.

Extraction with the Base and Acidified Ethanol

Approximately 1 g of dry *S. Cerevisiae* was extracted with a 50 mL 0.1 N NaOH boiling solution. After the residue had been adjusted to a pH of ~7, it was then extracted with boiling acidified ethanol (50 mL of ethanol with, few drops of 1 N H_3PO_4) and centrifuged. It was then re-suspended in DI water. The residue was boiled and washed in DI water after it had been adjusted to a pH of ~7. It was then dissolved in approximately 15 mL of DI water and kept in a plastic bottle in the freezer. The solution was lyophilized and analyzed with the NMR.

CHAPTER 3

RESULTS AND DISCUSSION

β-glucan isolated from *Saccharomyces cerevisiae* is a polysaccharide of D-glucose that has wide applications in the food and pharmaceutical industries. The classical method of glucan isolation from *S. cerevisiae* involves a repeated extraction of the yeast sample with boiling NaOH and HCl solutions. While this method is efficient in isolating the glucan from all other components of the cell wall, it is also known to degrade the glucan by evidence of higher BF and lower SC.

Over the years, many researchers have developed several methods to isolate glucan in order to try to retain more of the proposed native structure, and therefore potentially provide a more effective glucan therapeutic. One method that has been developed is the use of Savinase enzymes for the isolation. This method has been demonstrated to successively isolate glucans with higher yields; however, the Savinase enzymes employed are very expensive making the isolation process cost prohibitive.[43]

The goal of this research was to develop a milder and cost-effective extraction procedure for β-glucan. The requirements for this method is that it must successfully isolate the glucan from all other components of the cell wall, as well as limit the extent of the degradation by evidence of a lower BF and a higher SC. Another aim of this research was to determine if the number of ethanol extractions could be decreased to allow for a more efficient extraction. Reduction of the ethanol extractions could result in a reduced cost in the extraction of the glucan as well as an overall retention of more of the glucan's proposed native structure. The reported function of the ethanol is to remove all residual lipids that may remain after the acid and base

extractions.[43-45] It was also determined whether all extraction volumes could be reduced. Reduction of the extraction volumes would allow quick extractions of smaller volumes which will limit the costs associated with the repeated extractions required for the optimization of the extraction procedure.

During our studies, a milder, simpler, and cost-effective method for isolating glucan from *S. cerevisiae* has been developed. This method was successful in isolating the glucan from all other components of the cell by evidence of the NMR spectra, and also limited the extent of degradation by evidence of a lower BF and a higher SC. The breaching frequency (BF), was calculated to determine the average number of glucose units between each 1,6-□-glucan side chain. The side chain length (SC) was also calculated to determine the average length of each 1,6-β-glucan side chain.

Classical Extraction Method

Three-times 1 N NaOH/HCl and Ethanol Extractions

It was previously reported by Mueller and coworkers that the branching frequency and side length depend on the strength of the acid used.[23,39] Lowman and coworkers reported a branching frequency and side chain length of 198.8 and 0.37 respectively when using the classical extraction method, that is, three times extraction of the yeast with 1N NaOH, HCl, and ethanol.[45] Figure 6 represents the spectrum of glucan isolated from *S. cerevisiae* using the classical extraction method. This spectrum agrees with the results reported by Lowman and co-workers. The calculated BF and SC can be observed in Table 3 to be 196.5 ± 9.8 and 2.5 ± 0.4 respectively, with a yield of 2.9 %. Our observation confirmed the results reported by Lowman

and co-workers that using the classical method for isolating glucan from *S. cerevisiae* degrades the glucan extract to a large extent; hence, the glucan extracted does not reflect its true structure.

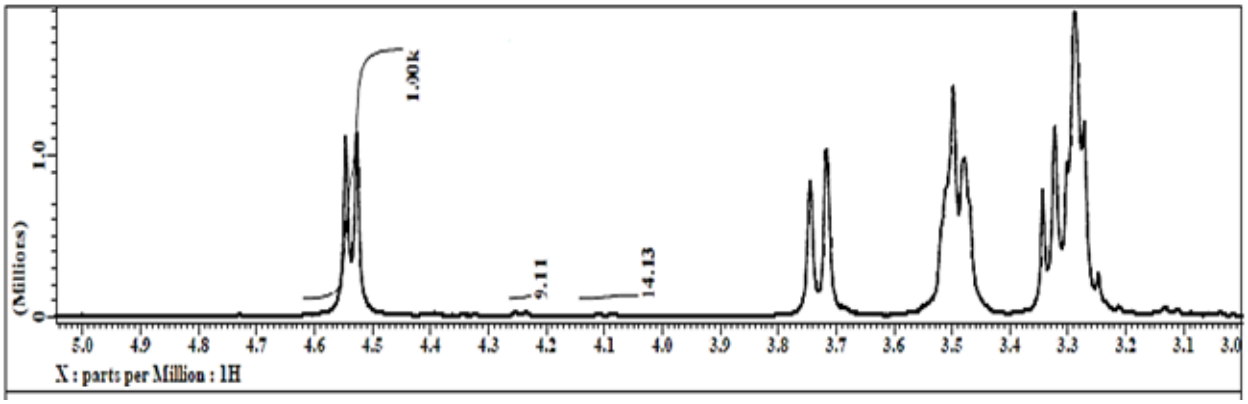

Figure 6. 1 D proton NMR spectrum of the (1→3,1→6)-β-glucan isolated from *S. cerevisiae* with three times extraction of the yeast with 1N NaOH, HCl and ethanol boiling solutions.

Table 3. Tabulation of structural statistical features and compositions observed using ^{1}H NMR spectroscopy for extracted glucans using classical method.

NaOH conc. (N)	HCl conc. (N)	NaOH / HCl boilings	Ethanol boilings	Branching Frequency (BF)	Side chain (SC)	Percent Yield
1	1	3	3	196.5 ± 9.8	2.5 ± 0.4	2.9 %
1	1	1	1	139.7 ± 6.8	2. 6± 0.2	3.2 %

<u>One-time 1 N NaOH/HCl and Ethanol Extractions</u>

After the confirmation of our initial experimental results that the classical extraction method degrades the glucan extract, we modified the classical method by reducing the number of

acid and base extractions. All boilings in this extraction procedure was reduced to one from the initial three. Figure 7 shows the H_1 NMR spectrum of glucan isolated with the modified classical method. This spectrum is very similar to the one obtained with the classical extraction method. They both have very small H_1 SC and H_6 SC peaks. The calculated BF and SC was determined to be 139.7 ± 6.8 and 2.6 ± 0.2, respectively, with a yield of 3.2 %. It was determined that by reducing the number of extractions, thus the exposure of the glucan to harsh acid and base, that the BF and SC was improved to a limited respect. This led us to a question of whether the type of reagents involved affected the BF and SC of the extracted glucan. Phosphoric acid was then investigated to determine if the use of a gentler acid (that is, a higher pKa 2.16 as compared to HCl which is -7) would yield any additional structural information.

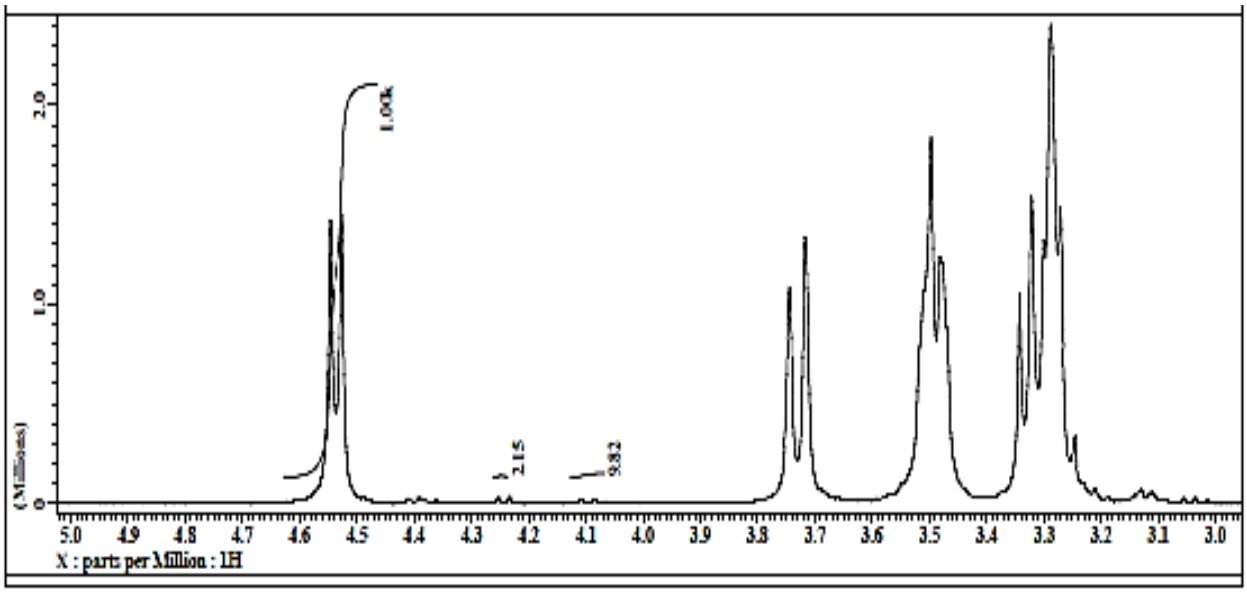

Figure 7. 1 D proton NMR spectrum of the (1→3,1→6)-β-glucan isolated from *S. cerevisiae* using one-time 1 N NaOH, HCl, and ethanol extraction.

Optimized Extraction Method

Three-times 1 N Acid/Base and Ethanol Extractions

After confirming from our experimental results that the classical method degrades the isolated glucan, it was imperative to develop a new method that will effectively isolate the glucan from *S. cerevisiae* as well as maintain its native structure. A milder acid (H_3PO_4) was employed in order to optimize the extraction method. Figure 8 shows the spectra of glucan isolated form *S. cerevisiae* using three-time acid/base, and ethanol extraction. Extraction of glucan using the optimized method of extraction, that is, three times extraction in 1 N NaOH, H_3PO_4 and ethanol degraded the glucan extract to a lesser extent. A meaningful increase in the branching frequency and the side chain length with the new method of extraction was observed. The glucan extracted with the optimized method gave a branching frequency and side chain length of 57.9 ± 1.6 and 2.7 ± 0.1, respectively. This observation confirmed the reported theory of Mueller and coworkers that, the degree of polymerization depends on the acid used and that using a milder acid potentially could result in increased branching frequency and side chain legth.[41,42] This is summarized in Table 4 below.

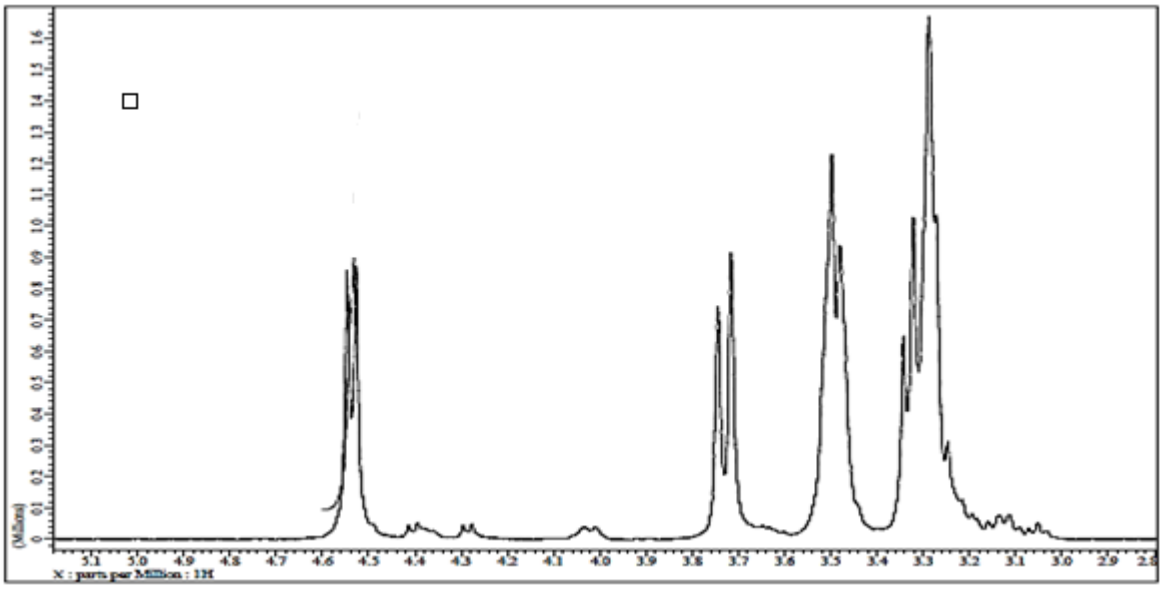

Figure 8. 1 D proton NMR spectrum of the (1→3,1→6)-β-glucan isolated from *S. cerevisiae* with three-times extraction of the yeast with 1 N $NaOH/H_3PO_4$ and ethanol boiling solutions.

One-time 1 N Acid/Base, three-times vs one-time Ethanol Extractions

During the optimization of the extraction method, it was investigated whether the number of base and acid extractions could be decreased from three to one. When milder conditions were employed, that is, the number of extractions was reduced from three times to one time, the glucan extracted had an improved degree of polymerization and a higher side chain length. As reported by Mueller and coworkers, milder acids gave improved branching frequency and side chain length.[38,39] Interestingly, we observed from our work that reducing the number of extractions produced improved spectra. Use of a single extraction of 1 N acid and base improved the branching frequency and the length of the side chains were 28.7 ± 1.2 and 4.1 ± 0.2, respectively, with a yield of 7.9 %.

Another aim of this research was to determine if the number of ethanol extractions could be decreased to allow for a more efficient extraction while retaining a high-quality glucan product. Reduction of the ethanol extractions would result in a reduced cost in the extraction procedure as well as an overall retention of more of the glucan's native structure. Figure 9 A is a one-time ethanol extract and Figure 9 B is a three-time ethanol extract. Comparing the one-time to the three-time ethanol extract, it was observed that both conditions produced similar results. The extract with one-time ethanol produced branching frequency and side chain of 28.7 ± 1.2 and 4.1 ± 0.2, respectively with a yield of 7.9 %. Similarly, the extract with three times ethanol produced a branching frequency and side chain length of 30.2 ± 2.0 and 4.3 ± 0.2, respectively with a yield of 7.8 %. This means that the number of ethanol extractions could be reduced from three to one, with both conditions producing comparable results. This is summarized in Table 4 below.

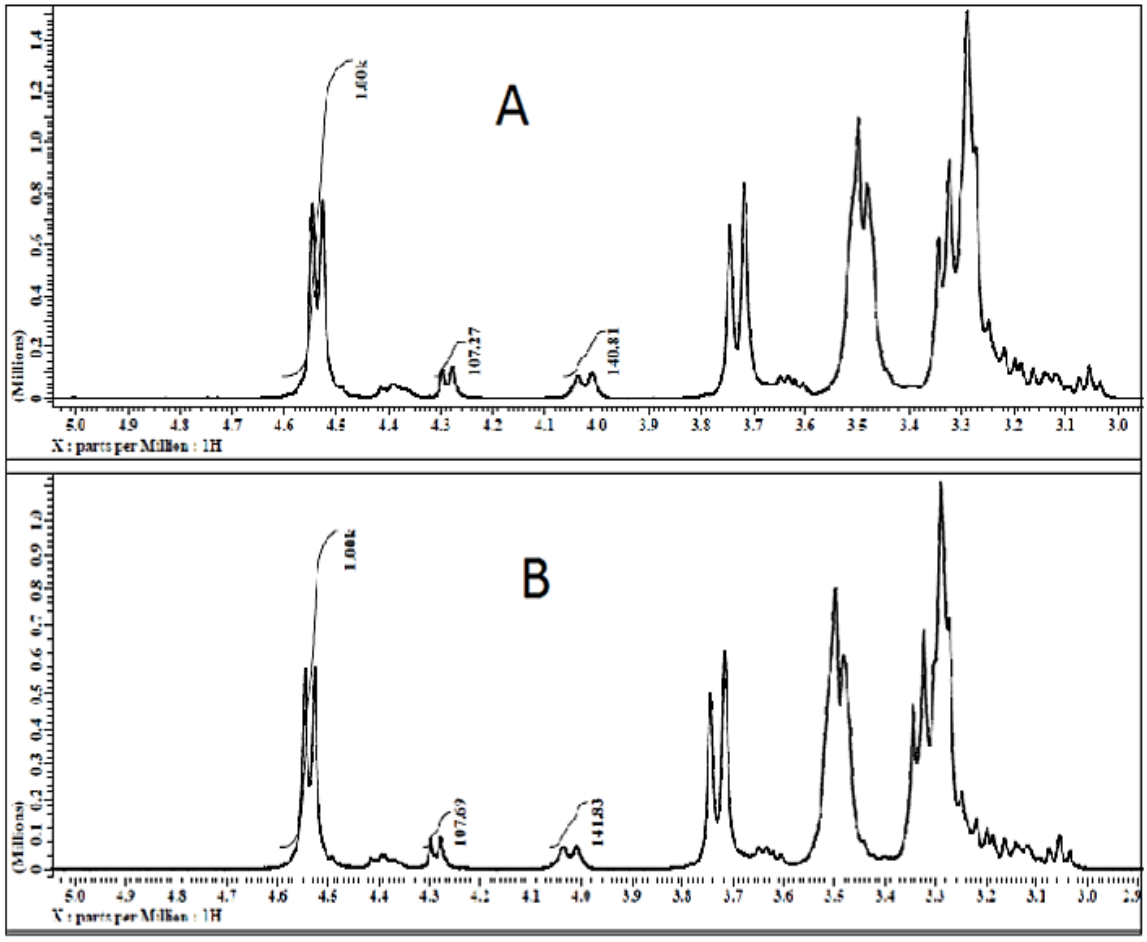

Figure 9. 1 D proton NMR spectrum of the (1→3, 1→6)-β-glucan isolated from *Saccharomyces cerevisiae* with one-time extraction of the yeast with 1 N NaOH, H_3PO_4 boiling solutions. **A** represents an extraction of the yeast with one-time ethanol boiling solution, and **B** represents an extraction of the yeast with three times ethanol boiling solutions.

One-time 0.1 N Acid/Base, three-times vs one-time Ethanol Extractions

In order to verify if one extraction with ethanol was sufficient, it was important to repeat the process with additional extraction conditions. The concentration of the base and acid was reduced to 0.1 N and the use of one and three ethanol extractions were compared as shown in Figure 10. There was an improvement in the values of the branching frequency and side chain length when the concentration of the acid/base was reduced. The branching frequency and side chain length was determined to be 18.6 ± 0.7 and 5.1 ± 0.1, respectively, when one ethanol extraction was conducted. This was comparable to the extraction procedure when ethanol was employed three times which gave a branching frequency and side chain of 25.1 ± 0.3 and 6.2 ± 0.1 respectively. This work supported the theory that the number of ethanol extractions could be reduced while still resulting in a pure glucan product. When it was observed that the reduction in base and acid concentration resulted in an improved BF and SC, the project changed focus to determine how low in terms of the concentration of the acid and base can we go before the glucan product began to exhibit impurities. This is summarized in Table 4 below.

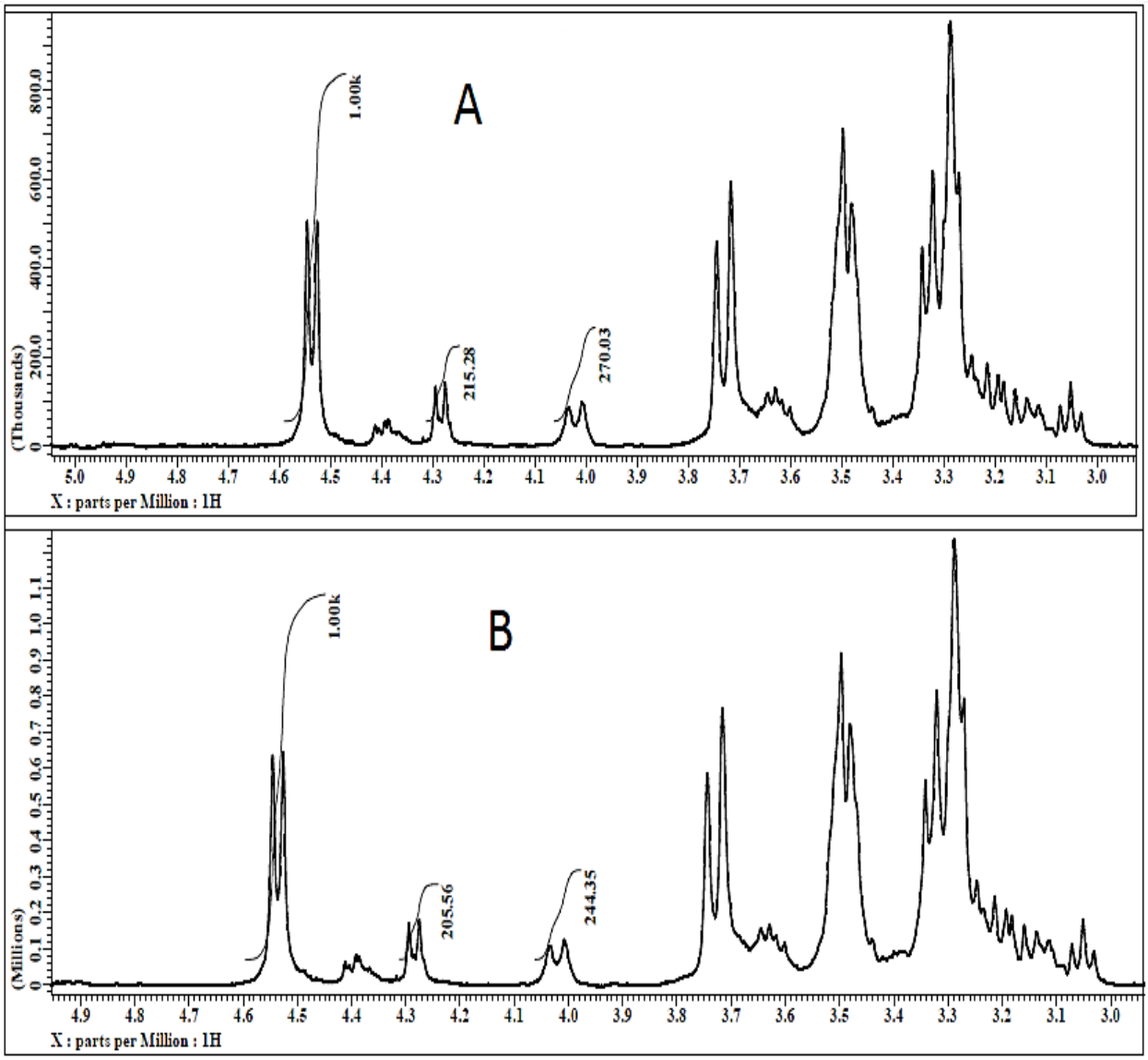

Figure 10. 1 D proton NMR spectrum of the (1→3, 1→6)-β-glucan isolated from *Saccharomyces cerevisiae* with one-time extraction of the yeast with 0.1 N NaOH, H_3PO_4 boiling solutions. **A** represents an extraction of the yeast with one-time ethanol boiling solution, and **B** represents an extraction of the yeast with three times ethanol boiling solutions.

Constant Base with Reduced Acid Concentration

A titration of extraction conditions was performed to determine the optimum of conditions that produced the glucan with the most native structure. In the first set of extractions, the base was kept constant at 0.1 N while the acid was reduced from 1 N to 0.001 N. It was observed that, reducing the acid did not have a major impact on the structure of the glucan extracted. In fact, all the acid conditions (1-0.001 N) resulted in very similar values for BF and SC. It was concluded from analyzing Figure 11 that the acid concentration did not have a major impact on the glucan product and: therefore, led us to speculate that the acid extraction was not required to yield glucan product that is free of other components of the cell wall. Additionally it became very important to determine how essential the base step was to the extraction procedure. This is summarized in Table 4 below.

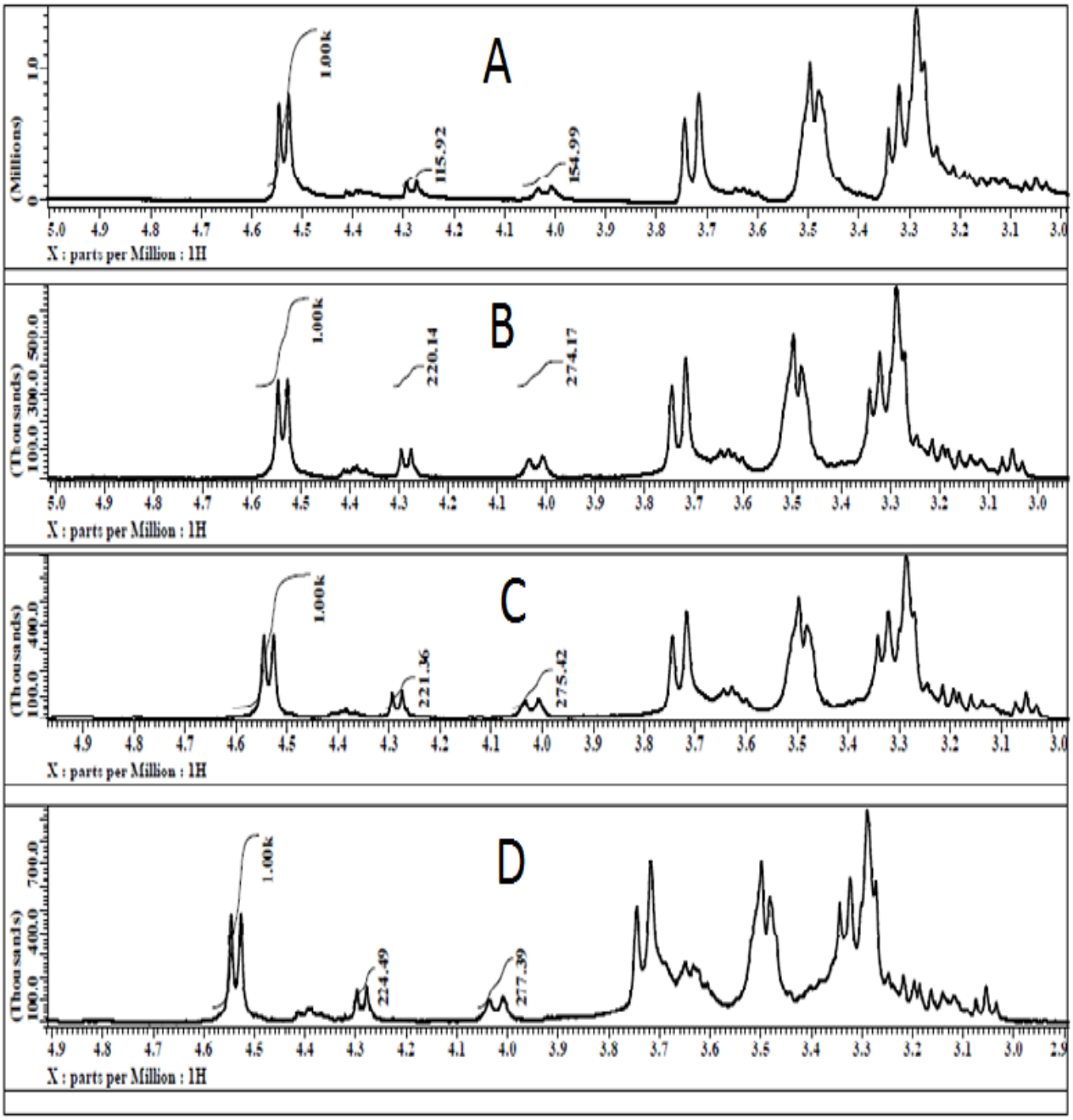

Figure 11. 1 D proton NMR spectra of the (1→3,1→6)-β-glucan isolated from *S. cerevisiae* with a one-time extraction of the yeast with 0.1 N NaOH and ethanol boiling solution. **A, B, C,** and **D** represent an extraction of the yeast with 1 N, 0.1 N, 0.01 N, and 0.001 N H_3PO_4 boiling solutions, respectively.

Constant Acid with Reduced Base Concentration

In order to determine the impact the base had on the whole extraction procedure, a titration method of extraction where the concentration of the acid was held constant and the concentration of the base was reduced was required. The acid concentration was held at 1 N while the NaOH concentration was reduced from 0.1 to 0.001 N. This set of experiments presented an interesting result. The extraction of a clean, free from impurities, glucan product was not successful when the concentration of the base was reduced below 0.1 N. From **Figure 12** it can be observed that the shape of the peaks within the NMR spectra changed dramatically when the concentration of the base was reduced to 0.01 N and 0.001 N respectively. The isolation of the glucan from other components of the cell wall was only successful with the 0.1 N base. From **Figure 12 B** and **C**, it can be determined that the glucan extracted contain other components of the cell wall. The H_1 and H_6 peaks cannot be seen, and therefore the spectra could not be interpreted for branching frequency and side chain length. This was most likely a result of remaining impurities such as mannan, chitin, proteins, and lipids. This is because other components like chitin and mannan are not soluble in the solvent used for the analysis, that is, d_6-DMSO. This chitin and mannan will act as particulate matter and change the shape of the NMR spectra. We convinced that the shape of the peak in **Figure 12 B** and **C** were not coursed by external particulate matter because a new NMR tube was used for each analysis. After observing that the acid had little or no effect on the extraction procedure while the base appeared to be essential, we became interested in determining the real purpose of the acid during the extraction procedure. To do this, further extractions were performed in the absence of acid.

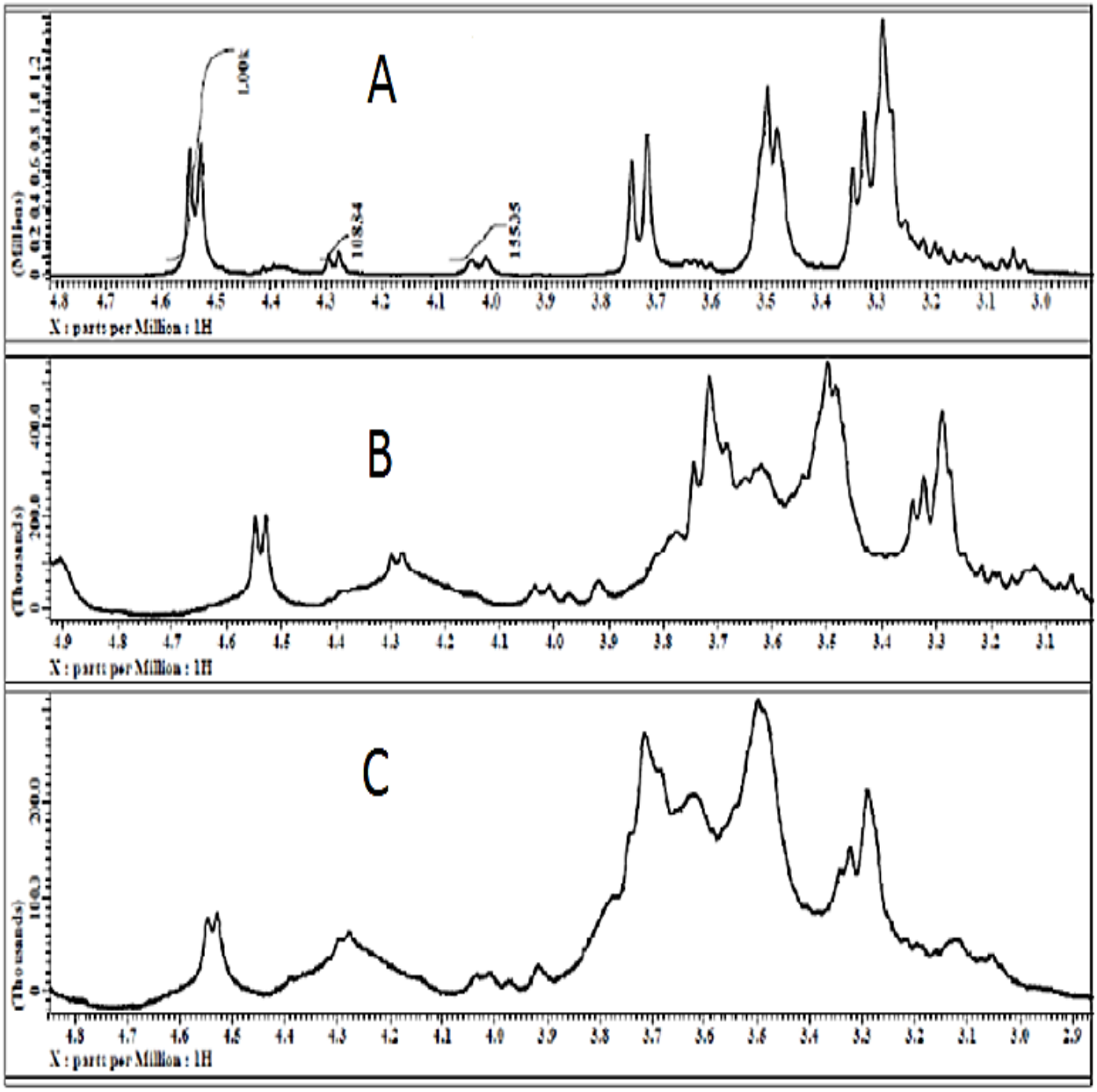

Figure 12. 1 D proton NMR spectra of the (1→3,1→6)-β-glucan isolated from *S. cerevisiae* with a one-time extraction of the yeast with 0.1 N H_3PO_4 and ethanol boiling solutions. **A**, **B**, and **C** represent an extraction of the yeast with 0.1 N, 0.01 N and 0.001 N NaOH boiling solutions, respectively.

No Acid/Acidified Ethanol Extraction

It was determined from this work that the acid concentration had very little impact on the purity of the extracted glucan, and therefore could be omitted from the extraction process. In order to accurately determine the function of the acid on the extraction procedure, an extraction was performed with two distinct conditions. First, an extraction with only the base, with no acid, was performed. Second, an extraction with the base and acidified ethanol was also performed.

The NMR spectra of the glucan resulting from these conditions had H_1 and H_6 peaks clearly observed as shown in Figure 13; the branching frequency and side chain length were determined. For the extraction without acid, the BF and SC was determined to be 17.3 ± 0.6 and 5.3 ± 0.3 respectively, it had a relatively higher yield of 11.5 % as compared to the extraction that was made with the acidified ethanol. The extraction with the acidified ethanol had a BF and SC of 17.6 ± 0.1 and 5.5 ± 0.1, respectively with a yield of 10.1 %. Both spectra had clearly defined peaks and were interpretable; however, a new peak was observed at 5.1 ppm. After a careful analysis of this new peak it was determined to represent glycogen. This was confirmed by comparing the glucan spectra to that of pure glycogen from Bovine liver that was obtained from literature. This observation explains why there was an increase in the percent yield for the extraction with no acid. The base extraction alone was not enough to remove all traces of glycogen, so when the extraction was made with no acid, significant amount of glycogen remained in the glucan extract and this contributed to the total percent yield. Glycogen is essentially a polysaccharide found in animal and human cells; it serves as the primary storage form of energy in the cell. It presents in the glucan extract makes it impure. This set of extractions demonstrated that the real impact of acid on the extraction was to remove the

glycogen. Even though it may seem not to have any effect on the extraction procedure, it is required to remove all traces of residual glycogen to produce a very pure glucan extract.

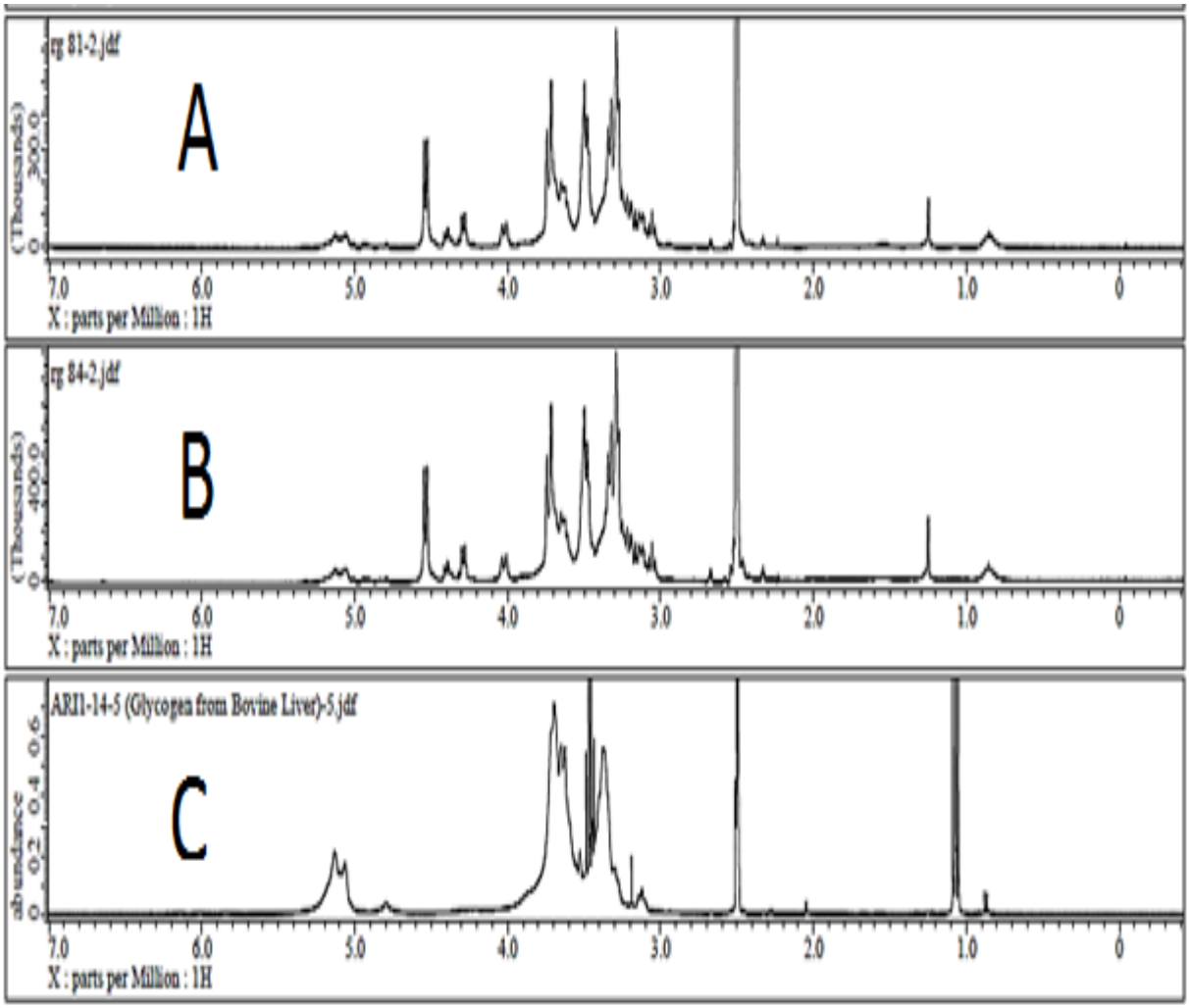

Figure 13. 1 D proton NMR spectra of (1→3,1→6)-β-glucan isolated from Saccharomyces cerevisiae with a one-time extraction of the yeast with 0.1 N NaOH boiling solutions. **A** represents extraction of the yeast with only the base and ethanol with no acid. **B** represents extraction of the yeast with the base and acidified ethanol. **C** represents NMR spectrum of glycogen isolated from bovine liver.

Table 4. Tabulation of structural statistical features and compositions observed using ^{1}H NMR spectroscopy for extracted glucans using optimized method.

NaOH conc. (N)	**H_3PO_4 conc. (N)**	**NaOH / H_3PO_4 times**	**Ethanol Times**	**Branching Frequency (BF)**	**Side chain (SC)**	**Percent Yield**
1	1	3	3	57.9 ± 1.6	2.7 ± 0.1	4.9 %
1	1	1	3	30.2 ± 2.0	4.3 ± 0.2	7.8 %
1	1	1	1	28.7 ± 1.2	4.1 ± 0.2	7.9 %
0.1	0.1	1	3	25.1 ± 0.3	6.2 ± 0.1	9.2 %
0.1	0.1	1	1	18.6 ± 0.7	5.1 ± 0.1	9.9 %
0.1	1	1	1	21.9 ± 0.6	3.5 ± 0.1	7.9 %
0.1	0.1	1	1	18.4 ± 0.2	5.1 ± 0.1	10.3 %
0.1	0.01	1	1	18.7 ± 0.2	5.2 ± 0.1	10.9 %
0.1	0.001	1	1	18.8 ± 0.4	5.2 ± 0.1	11.1 %
0.1	1	1	1	23.2 ± 2.0	3.6 ± 0.3	7.1 %
0.01	1	1	1	—	—	21.1%
0.1	No acid	1	1	17.3 ± 0.6	5.3 ± 0.3	11.5 %
0.1	Acidified ethanol	1	Acidified Ethanol	17.6 ± 0.1	5.5 ± 0.1	10.1 %

CHAPTER 4

CONCLUSION AND FUTURE WORK

Classical Extraction Method

The classical extraction method degraded the glucan extract to a large extent. The conditions used for the classical extraction method was three times extraction of the yeast with a 1 N NaOH boiling solution, followed by three times extraction with 1 N HCl boiling solution, and then finally extracted three times with boiling ethanol. This yielded a BF of 196.5 ± 9.8 and a SC of 2.5 ± 0.4 with a poor yield of 2.9 %. The NMR spectra for the product of this method looked distorted and were difficult to interpret. No further studies like the linkage analysis could be done with this extract because it was too degraded.

Reduction of the Number of Acid/Base Extractions for the Classical Method

Reducing the number of extractions for the classical extraction procedure had no significant effect on the glucan extract. Classically, the yeast was extracted three times with 1 N NaOH boiling solution, followed by three times extraction with 1 N HCl boiling solution, and then finally extracted three times with boiling ethanol. When the number of extraction was reduced, that is, the yeast extracted one time with 1 N NaOH boiling solution, followed by one time extraction with 1N HCl boiling solution, and then followed by a 1 time extraction with boiling ethanol, it resulted in a BF of 139.7 ± 6.8 and SC of 2.6 ± 0.2. This method also produced a very low yield of 3.2 %. The NMR spectrum for the product of this method looked distorted and was difficult to interpret. No further studies could be completed with this extract because it had been too degraded.

Optimized Extraction Method

When the acid was varied for the classical extraction method, improved values of BF and SC was obtained. Using H_3PO_4 for the yeast extraction produced an NMR spectrum that was easily interpretation. The BF and SC were observed to be 57.9 ± 1.6 and 2.7 ± 0.1, respectively. The method also resulted in an increase in the glucan yield, that is, 4.9 %.

Reduction of the Number of Base/Acid Extractions

Reducing the number of extractions from three to one with our optimized extraction conditions produced clearly distinctive and interpretable NMR spectra. When the yeast was extracted one-time with 1 N NaOH boiling solution, followed by one-time extraction with 1 N H_3PO_4 and followed by one time extraction with boiling ethanol, an improved BF and SC of 28.7 ± 1.2 and 4.1 ± 0.2, respectively was obtained. The yield also increased for this method to 7.9 %.

Reduction of the Number of Ethanol Extractions

One question was whether the number of ethanol extractions could be reduced and still result in a glucan product that is free from other components of the cell wall. When the yeast was extracted one-time with 1 N boiling base followed by a one-time extraction with 1 N boiling acid, and then extracted three times with boiling ethanol, a BF and SC of 30.2 ± 2.0 and 4.3 ± 0.2 was obtained. The yield for this extract was 7.8 %. When the acid and base conditions were held constant, but the yeast was only extracted one time in ethanol, a BF and SC of 28.7 ± 1.2 and 4.1 ± 0.2 with a yield of 7.9 % was obtained. Sticking with one time ethanol extraction was

confirmed when 0.1 N acid/base was employed. When extracted three times in ethanol a BF and SC of 21.5 ± 0.3 and 6.2 ± 0.1 with a yield of 9.2 % was observed while when extracted one time in ethanol a BF and SC which was 18.6 ± 0.7 and 5.1 ± 0.1, with a yield of 9.9 % was obtained. Based on these results, we confidently conclude that one-time ethanol extraction was sufficient to remove all lipids from the glucan extract.

Varying the Amount of Materials and Volume of Solutions

By repeating the above extraction procedure with one quarter of all materials, we were able to achieve comparable results as with the larger volumes. When 4 g of sample and 200 mL of all solutions was used with the yeast being extracted one time with boiling base solution, followed by one time extraction with boiling acid solution and then completed with one time extraction with boiling ethanol solution, a BF and SC of 28.7 ± 1.2 and 4.1 ± 0.2 with a yield of 7.9 % was obtained. When the extraction conditions was maintained but reducing the sample and all solutions to 1 g and 50 mL respectively, a BF and SC of 28.3 ± 1.1 and 4.6 ± 0.1 with a yield of 7.1 % was obtained. Therefore, we were able to successfully cut the cost and time of the extraction procedure by one quarter.

Constant Base with Reduced Acid Concentration

The titration method of extraction led us to an interesting observation. When the concentration of the base was held constant and the concentration of the acid was reduced, we found that the base extraction is the most important step in isolating glucan as it removes most of the proteins, as well as mannan, chitins etc. The acid actually had a minimal effect on the

procedure. Keeping the base constant at 0.1 N and reducing the acid from 1 N to 0.001 N had very little effect on the resulting glucan characteristics.

Constant Acid with Reduced Base Concentration

When the acid concentration was held constant while the base concentration was reduced an interesting result was observed. Reduction of the base to 0.001 N with 1 N acid gave us an NMR spectrum without enough data for analysis. This led us to the confirmation of our earlier conclusion that the base is the most important step in the glucan extraction procedure.

No Acid Extraction

It was believed that the acid had very little to no effect on the glucan extraction based on our previous observation. However, when the acid extraction step was omitted either with regular ethanol or acidified ethanol it was found that traces of glycogen remained in the extracted glucan. Therefore, while the acid has minimal effect on the extract, it is essential to remove the glycogen from the product.

Final Conclusion

It can be concluded that the optimum condition for glucan extraction from *S. cerevisiae*, was one-time extraction of the yeast with 0.1 N NaOH boiling solution, followed by one-time extraction with 0.1 N H_3PO_4 boiling solution, then followed by one-time extraction with boiling ethanol. This gave us the optimum BF and SC of 18.4 ± 0.2 and 5.1 ± 0.7, with a yield of 10.3 %.

Thus this research has led to a dramatic improvement in the yield of glucan from *S. cerevisiae.* Prior to this research, the BF and SC was 196.5 ± 9.8 and 2.5 ± 0.4 respectively for the classical method. The highest yield from the classical method was 2.9 %, and now the yield is 10.3 %.

Future Work

In the future, the structure of β-glucan isolated from *S. cerevisiae* using the recently optimized developed extraction method should be studied. These studies should be done on yeast from different fungal sources.

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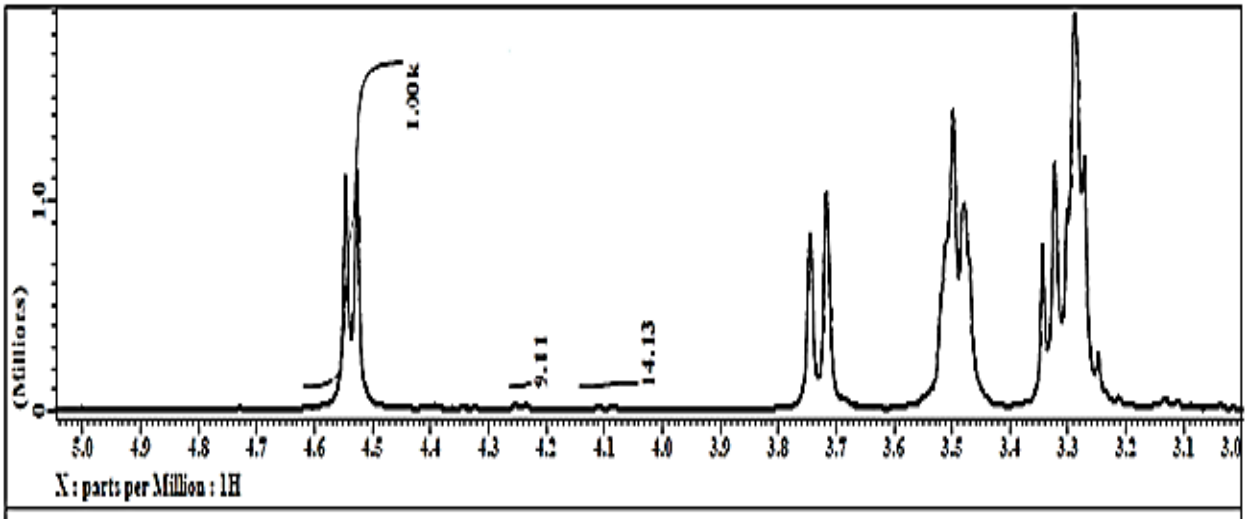
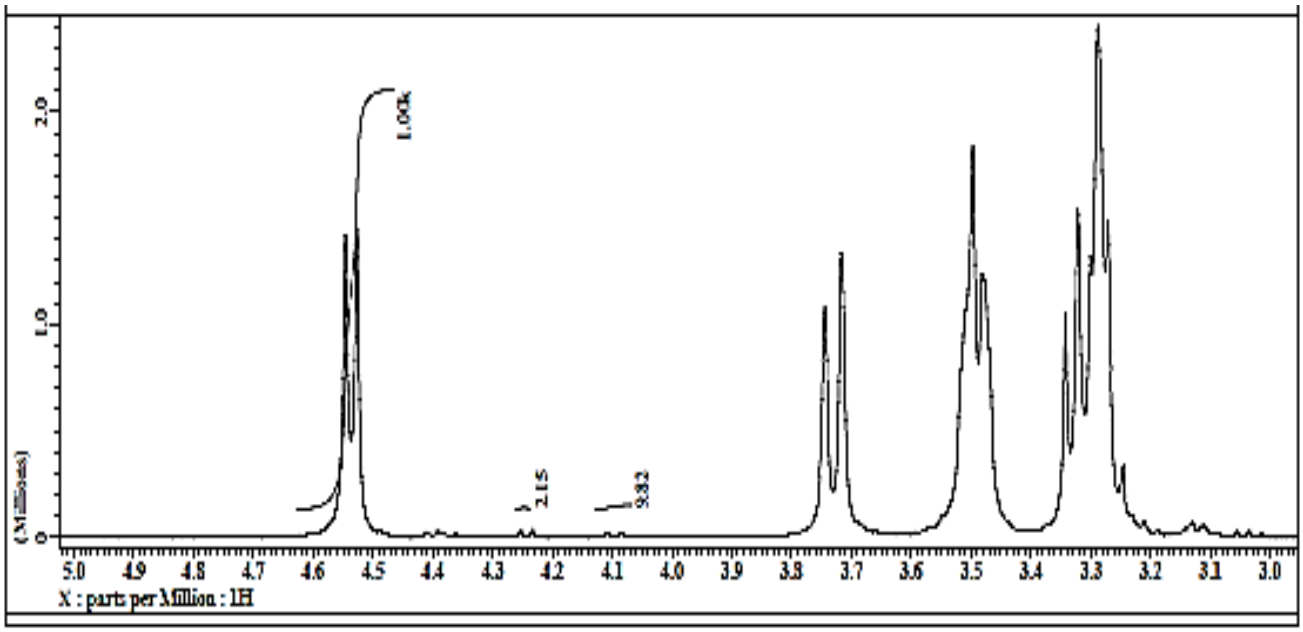
APPENDICES

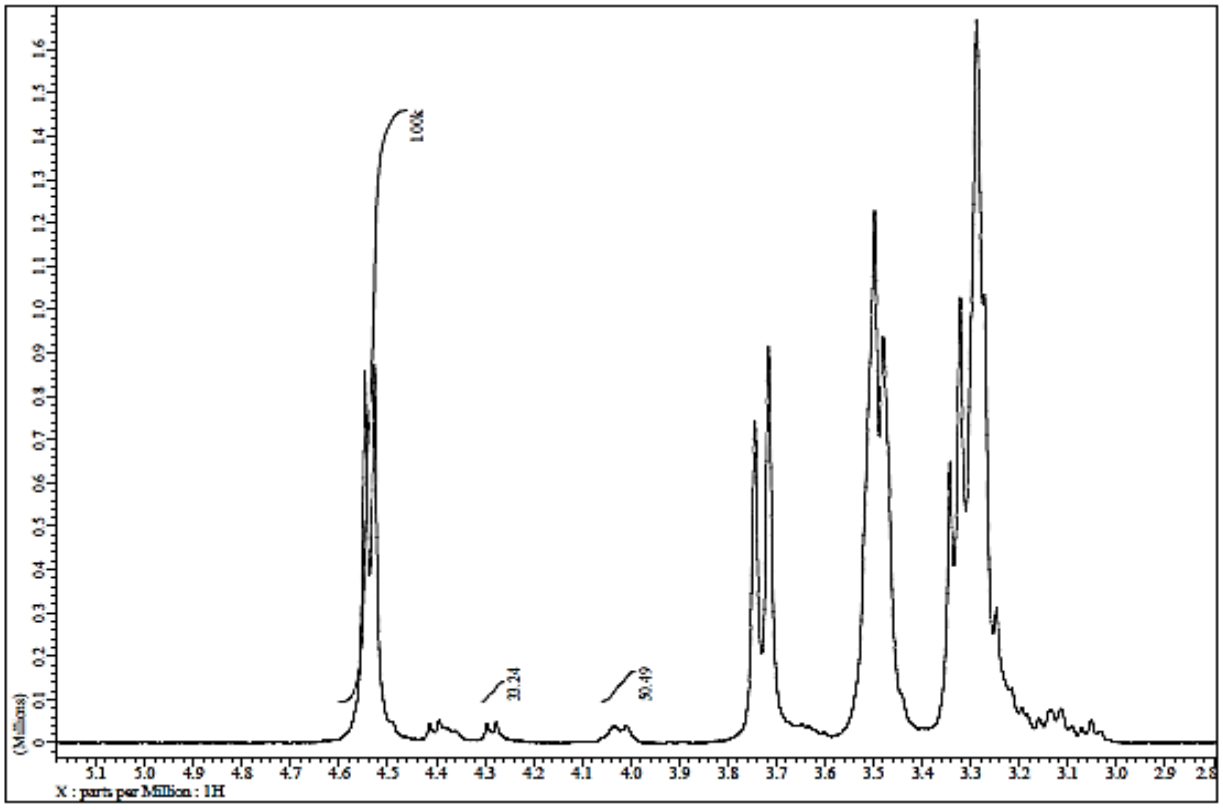
Appendix A: 1 D proton NMR spectrum of the (1→3,1→6)-β-glucan isolated from *S. cerevisiae* with three times extraction of the yeast with 1 N NaOH, HCl and ethanol boiling solutions.



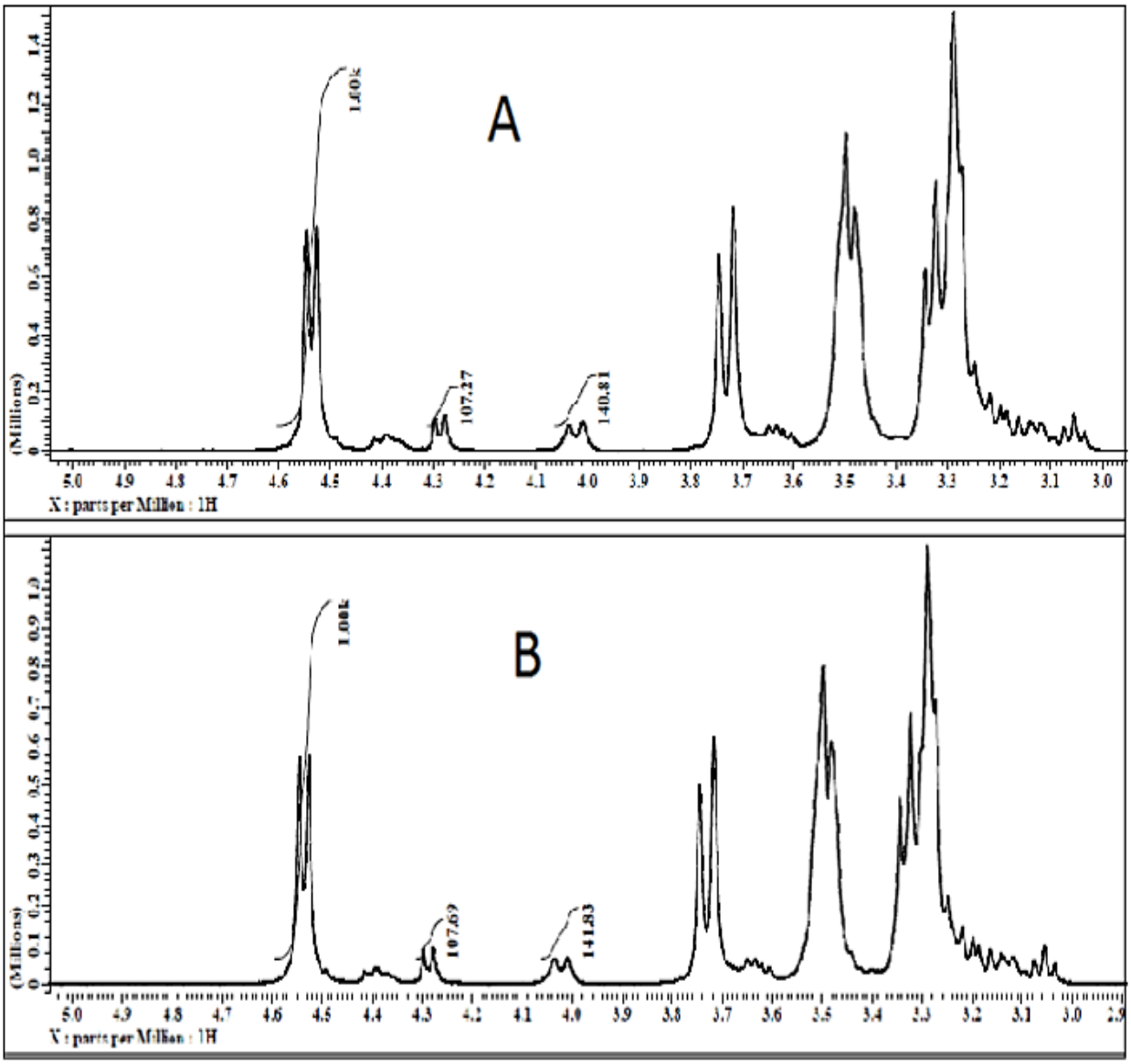
Appendix B: 1 D proton NMR spectrum of the (1→3,1→6)-β-glucan isolated from *S. cerevisiae* with one time extraction of the yeast with 1 N NaOH, HCl and ethanol boiling solutions.



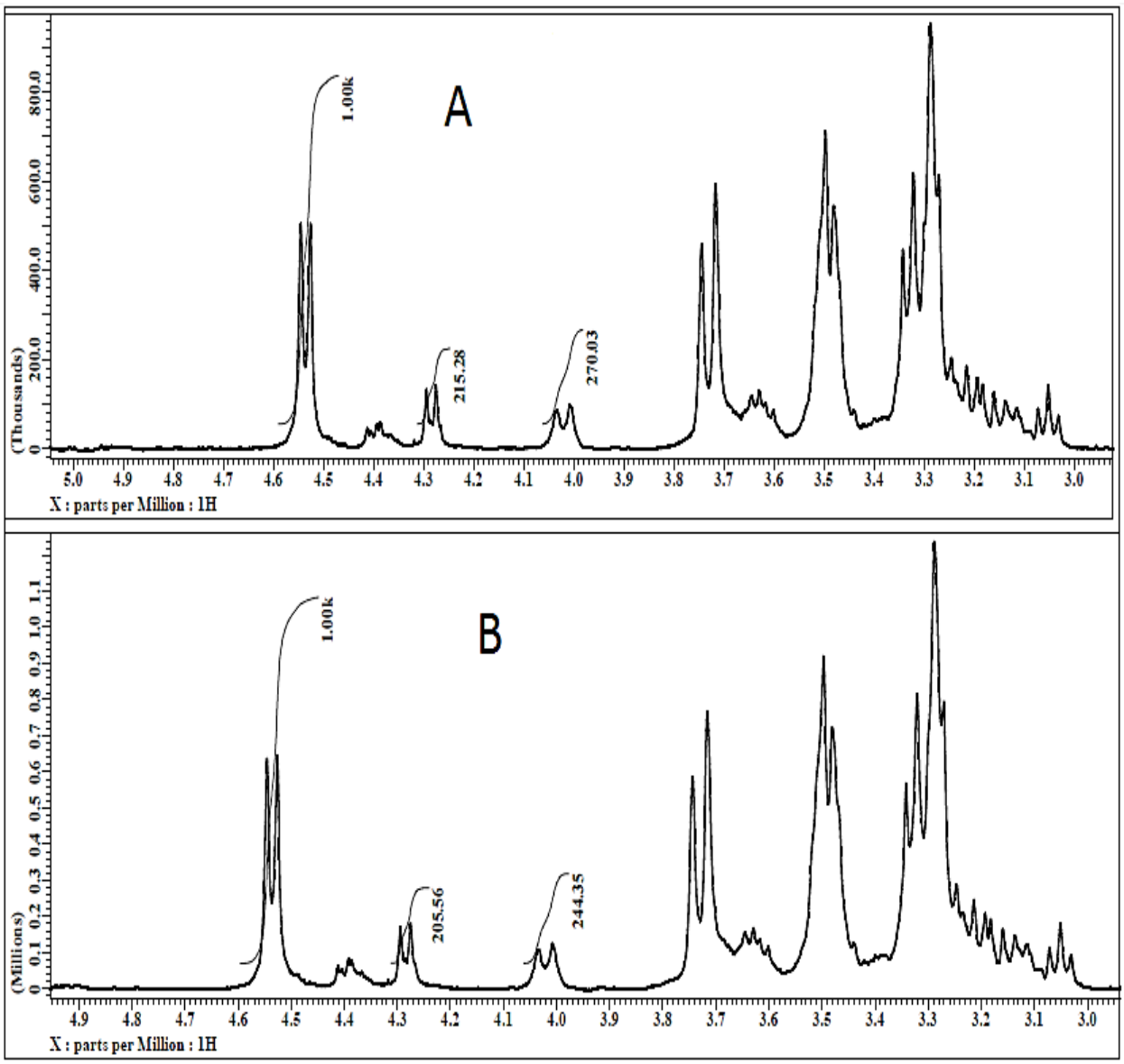
Appendix C: 1 D proton NMR spectrum of the (1→3,1→6)-β-glucan isolated from *S. cerevisiae* with three times extraction of the yeast with 1 N $NaOH/H_3PO_4$ and ethanol boiling solutions.



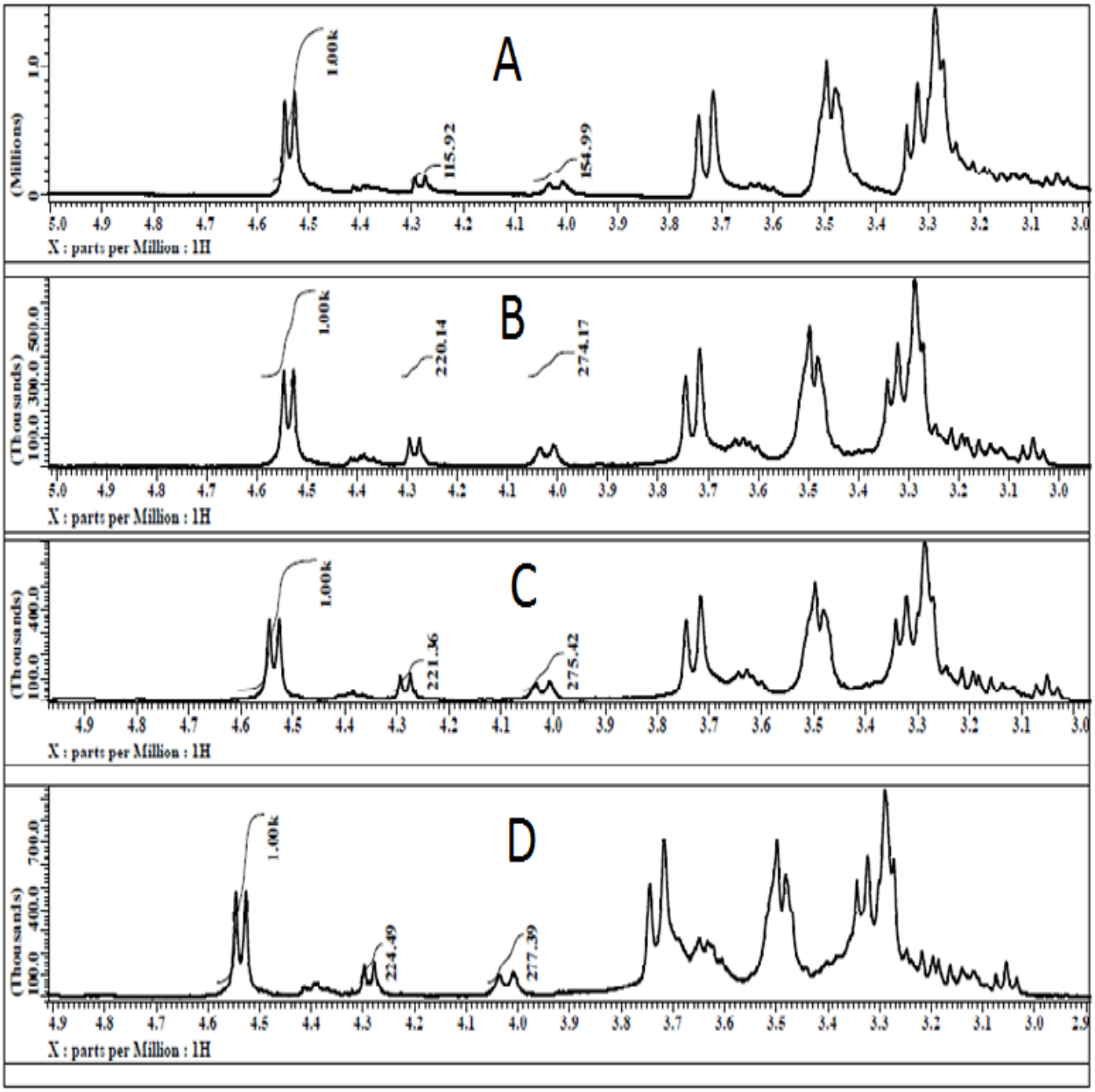
Appendix D: 1 D proton NMR spectrum of the (1→3, 1→6)-β-glucan isolated from *Saccharomyces cerevisiae* with one time extraction of the yeast with 1 N NaOH, H_3PO_4 boiling solutions. A represents an extraction of the yeast with one time ethanol boiling solution, and B represents an extraction of the yeast with three times ethanol boiling solutions.



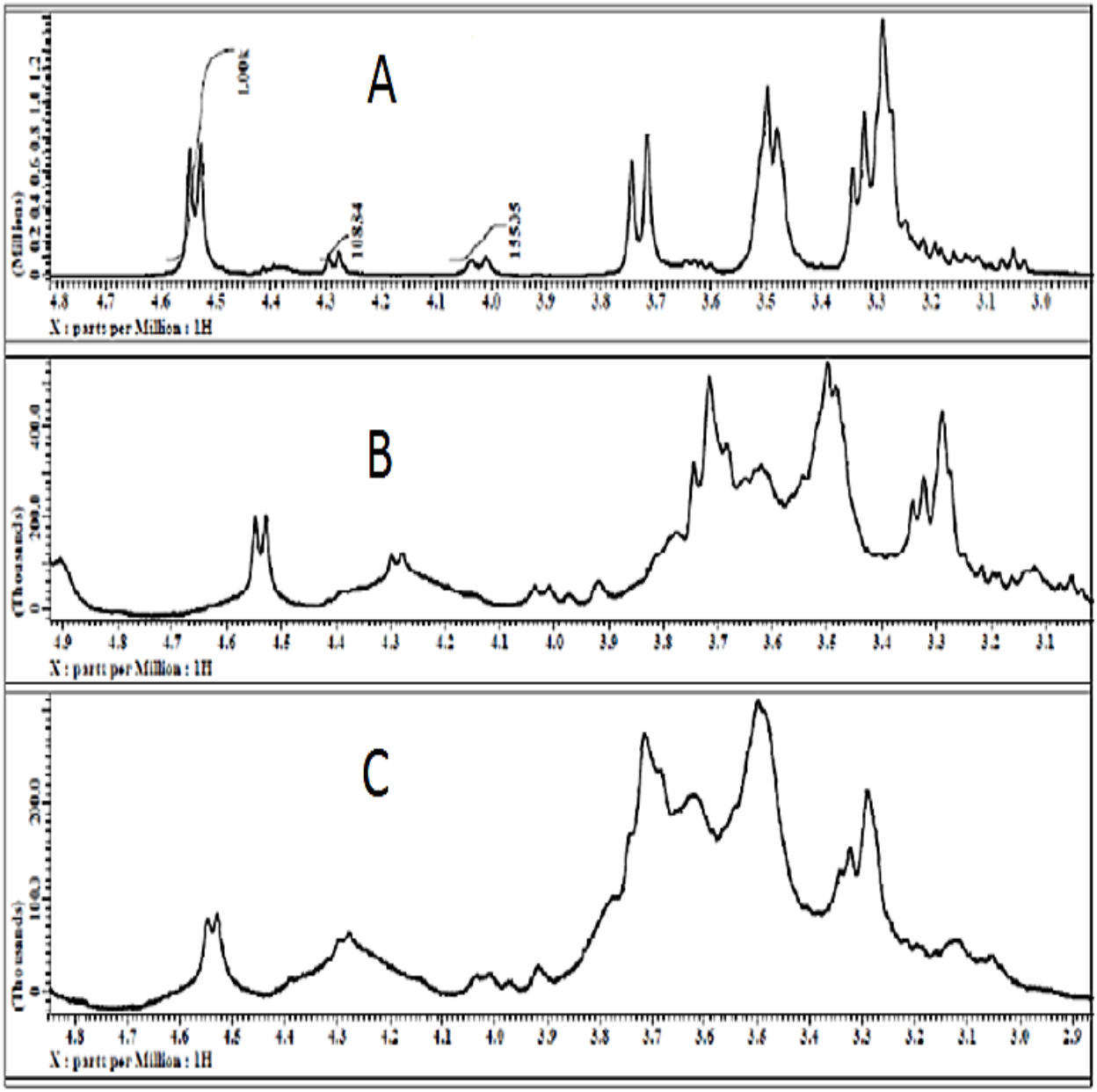
Appendix E: 1 D proton NMR spectrum of the (1→3, 1→6)-β-glucan isolated from *Saccharomyces cerevisiae* with one time extraction of the yeast with 0.1 N NaOH, H_3PO_4 boiling solutions. A represents an extraction of the yeast with one time ethanol boiling solution, and B represents an extraction of the yeast with three times ethanol boiling solutions.



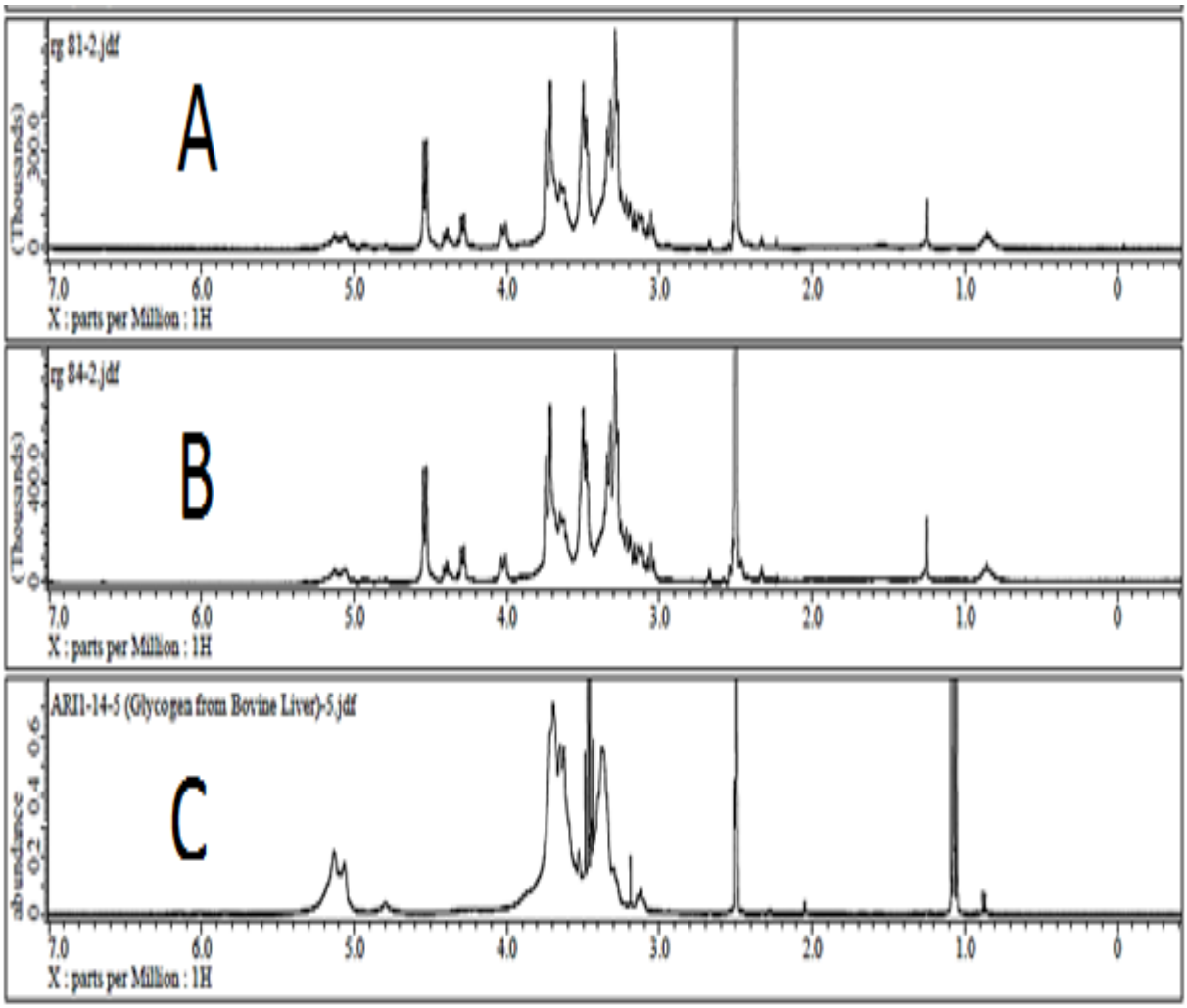
Appendix F: 1 D proton NMR spectra of the (1→3,1→6)-β-glucan isolated from *S. cerevisiae* with a one-time extraction of the yeast with 0.1 N NaOH and ethanol boiling solution. A, B, C, and D represent an extraction of the yeast with 1 N, 0.1 N, 0.01 N and 0.001 N H_3PO_4 boiling solutions respectively.



Appendix G: 1 D proton NMR spectra of the (1→3,1→6)-β-glucan isolated from *S. cerevisiae* with a one time extraction of the yeast with 0.1 N H_3PO_4 and boiling ethanol solutions. A, B, and C represent an extraction of the yeast with 0.1 N, 0.01 N and 0.001 N NaOH boiling solutions respectively.



Appendix H: 1 D proton NMR spectrum of the (1→3,1→6)-β-glucan isolated from *Saccharomyces cerevisiae* with a one-time extraction of the yeast with 0.1 N NaOH boiling solutions. A represent an extraction of the yeast with only the base and ethanol with no acid. B represents an extraction of the yeast with the base and acidified ethanol. C represents an NMR spectrum of glycogen isolated from bovine liver.



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