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Candida albicans Hyphal Mannan is Structurally Distinct from Yeast Mannan

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Candida albicans Hyphal Mannan is Structurally Distinct from Yeast Mannan

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by

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ABSTRACT

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C. albicans is a polymorphic fungal pathogen which has the ability to shift from yeast to hyphae. *C. albicans* cell wall is composed of glucan, chitin, mannoprotein and mannan. It is not possible, using standard extraction methods, to isolate mannan from *C. albicans* hyphae. To isolate hyphal mannan, we developed a simplified alkali extraction method. Using this method it was determined that hyphal mannan has a much lower molecular weight, a smaller polymer distribution and altered conformation structure when compared to yeast mannan. The hyphal mannan was found to contain little to no acid-labile portion with only α-Man-PO_4 groups and no long chains of β-1, 2-linked mannosyl repeat units, when compared to the yeast mannan. It was concluded that the *C. albicans* hyphal mannan is substantially different from the mannan found in the yeast form. This is an entirely new observation that extends the existing knowledge about the structural biology of *C. albicans* hyphae and may provide insights into the role of hyphae in pathogenesis.

DEDICATION

This research work is dedicated to God Almighty for his unconditional grace and favor, my mother, Joyce Owusuaa and my uncle Richard Owusu and George Kolog Gbinniya for their prayers and support, my sister and all my loved ones.

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LIST OF ABBREVIATIONS

NMR: Nuclear magnetic resonance

GPC: Gel Permeation Chromatography

MW: Molecular Weight

RU: Repeat Unit

COSY: Correlated Spectroscopy

CHAPTER 1

INTRODUCTION

Candida albicans

Candida albicans, an opportunistic pathogen, is the most commonly hospital-acquired fungal infection in critical care wards.[1-2] *C. albicans* infections of mucosal surfaces are common in otherwise healthy individuals.[3] However, the fungus can cause serious life threatening infections in immunosuppressed individuals.[3] Under normal conditions, *C. albicans* is a commensal organism which exists as part of the normal microbial flora in approximately half the world's population.[4] *C. albicans* have many virulence attributes that contribute to its general survival, including persistence and fitness within the host organism and other factors associated with adhesion, invasion, cell damage and induction of host responses.[5-7] The host defense mechanisms which hold C. albicans in a commensal (non-infectious) state include mechanical barriers that prevent fungal penetration such as the epithelial surfaces, soluble antimicrobial factors as well as the innate and adaptive cellular immune mechanisms.[4] Alterations in the physiological state of the host organism have been shown to turn this normally harmless commensal yeast into a pathogen capable of inflicting debilitating illness. This points both to the importance of host defense mechanisms in keeping *C. albicans* in the commensal state and the potential virulence of *C. albicans* when suitable conditions arise.[4] *C. albicans* can cause potentially fatal systemic infections due to their ability to break down mucosal barrier.[4] The fungus has several features that enables it to be virulent including hydrolytic enzymes and adhesions as well as the ability to undergo structural or morphological changes from the yeast form to the hyphae form in a process known as fungal dimorphism.[8-10]

Morphological Transition in *C. albicans*

The ability of *C. albicans* to shift between a single celled form called yeast (blastospore) and a filamentous form (both pseudohyphae and true hyphae) is critical to its pathogenicity.[11] In addition to this yeast-hyphal transition, there are a number of other natural occurring morphological forms that are characteristics of specific cellular functions.[12] These distinct morphologies include the opaque form, characteristic of mating-competent cells[13] the chlamydospores, characteristic of suboptimal growth conditions resulting in thicker cell wall[14] and the pseudohyphal form, which usually coexists with the hyphal and yeast forms in vegetative cultures and during infections.[11] Hyphal cells may promote invasion of the host tissue, but the yeast cells facilitate dissemination of the pathogen.[15-18] *C. albicans* morphogenesis is controlled by a complex network of signaling pathways that are commonly accompanied by the regulation of genes associated with the morphological states.[19] The shift from the yeast to hyphal morphology can be activated by various external factors such as serum, N-acetyl-D glucosamine, neutral pH, physiological temperature of 37 ^{o}C, high amount of CO_2, and nutrient starvation[12] such as amino acid starvation by the presence of serum. The morphogenic shift is also reported to be caused by stresses such as oxidative, nitrosative and osmotic stresses.[20]

The Cell Wall of *C. albicans*

The cell wall of *C. albicans* is composed of approximately 90 % carbohydrates and 10 % protein. The majority of the carbohydrates are found as branched glucose polymers (β-1, 3 and β-1, 6 –(β-glucan), unbranched polymers of β-1, 4 N-acetyl-D (chitin), and mannose polymers covalently bonded to proteins.[21] Studies on the composition of the cell wall of the fungus is generally based on chemical characteristics utilizing the solubility differences of the components

upon treatment with alkali and an acid.[22] A brief description of each carbohydrate component of the cell wall is presented below.

Cell Wall Chitin

Chitin, the second most abundant natural polysaccharide after cellulose, is composed of β-(1,4)-linked-2-acetamedo-2-deoxy-β-D-glucose[23] (N-acetylglucosamine). Chitin is a carbohydrate polymer that is commonly found in the exoskeletons of insects, spiders, and other arthropods.[24] The content of chitin varies from 22-44% in fungal cell walls, 3-5% in green algae, and 25-50 % in the cuticles of arthropods and mollusks.[25] It is often considered as a derivative of cellulose as it is structurally identical but it has acetamide groups ($-NH_2COCH_3$) at the C-2 positions[26] as shown in Figure 1. The use of chitin has become of great interest as a new functional biomaterial with great potential in many fields.[26] Chitin as well as its deacetylated form (chitosan) also participate in immune recognition, activation and attenuation.[27-28]

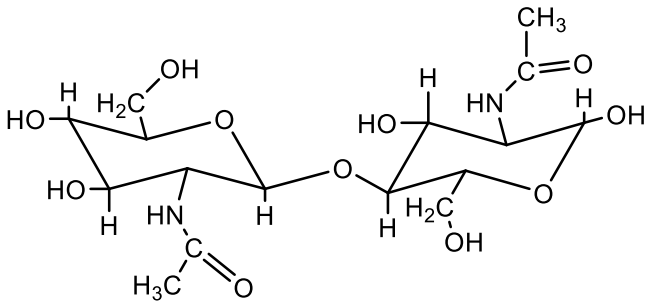

Figure 1: The structure of chitin which is composed of β-(1, 4)-linked-2-acetamedo-2-deoxy-β-D-glucose.[23]

Cell Wall Glucans

β-Glucans are structurally complex, insoluble glucose homopolymers, found in the cell wall of algae, bacteria and fungi.[29-30] In *C. albicans*, β-glucans are the major cell wall component, accounting for approximately 50-60 % of the total dry cell weight. Based on their different solubilities in basic and acidic solutions, *C. albicans* β-glucans have been categorized into an alkali-soluble polymer of low molecular weight and an acid-soluble, branched molecule. Both of which contain β-D-(1→6)-linked residues, including an alkali-acid insoluble, highly branched complex containing equivalent amounts of β-D-(1→6) and β-D-(1→3) linkages in a complex with chitin.[31]

While the basic molecular structure of β-glucans is relatively homogeneous, the type of bonding, its molecular weight as well as its molecular configuration may vary depending upon the microbial source.[32] Therapeutically, β-glucans are known for their immunomodulatory and antitumor properties.[33] The glucans on the cell wall is known to stimulate the immune system under conditions that enhance 1, 3-β-glucan exposure at the surface of the cell induce an increase in the amount of pro-inflammatory cytokines.[34] This enhanced glucan exposure can occur after exposure to echinocandins and during the progression of an infection as host enzymes act on the fungal cell surface.[35]

Cell Wall Mannans

Cell wall mannan accounts for approximately 40 % of the total carbohydrate composition of the cell wall.[21] *C. albicans* N-linked mannan is composed an α-1, 6-linked D-mannose repeats units with branches containing α-1, 2, α-1, 3,and β-1,6 and single α-1,6-linked mannose units and phosphodiester bonds.[36-37] The O-linked mannan is composed of either single or short

unbranched mannose monomers.[38] The cell wall mannan of *C. albicans* is composed of an acid-labile potion and an acid-stable portion. These two components are bridged by a phosphodiester group and some studies have shown that the acid-labile portion is sometimes significantly reduced.[39] The mannan layer covering the glucan is not strictly an 'immunological shield' since it is also recognized by a plethora of Pattern Recognition Receptors (PRRs). However, alterations to the mannan layer with subsequent exposure of β-1,3 glucan, enhances the immune and pro-inflammatory response.[3] The masking of glucans by mannans is thought to reduce recognition of the yeast by the innate immune system.[40] Figure 2 is a schematic representation of a representative cell wall mannan structure.

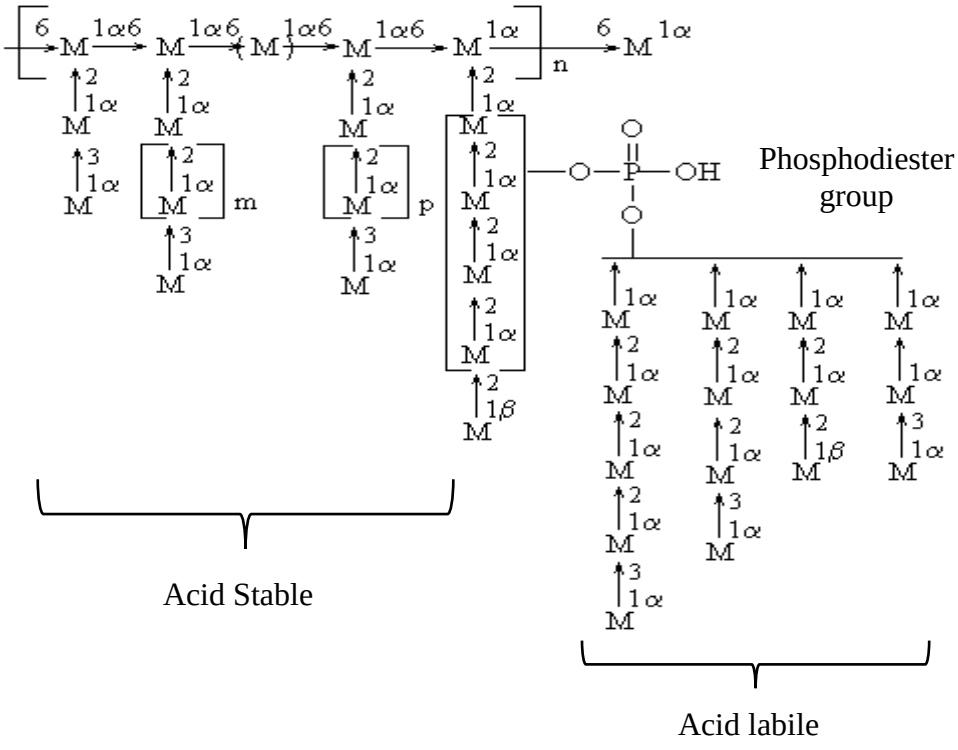

Figure 2. Schematic representation of cell wall mannan of *C. albicans* as described by Shibata *et al.*[39]

Evidence indicates that the cell wall-mannan of yeast is a linear polymer backbone consisting of α-(1→6)-linked D-mannopyranose units with short side chains of mannose units attached to the backbone mainly by α-(1→2)-linkages and to each other by both α-(1→2) and α-(1→3)-linkages.[41] It is known that some of the side chains are linked to the α-(1→6)-linked backbone by (1→3)-linkages.[41] It is reasonable to assume that some of these side chains may be branched, and some of the mannose units in the backbone are unsubstituted. All of the polysaccharides of the cell wall contribute to the immunological signature of *C. albicans.*[42] One of the important questions which remains to be answered is what makes one fungus commensal and another pathogenic. It is believed that differences in the fungal cell wall play an important role in determining whether a fungus is pathogenic. Differences in the structure and or composition of the cell-wall mannan, as well as other cell-surface components such as the protein and β-glucan are well known to affect the virulence of *Candida* species including *C. albicans.*[43]

Among the potential virulence factors of *C. albicans* as well as antigens, the significance of mannan is truly unique.[44] It is known to provide the antigenic variability that is most useful for species identification and subtyping as it may be the antigen that is most useful for rapid and early serodiagnosis of infection, and it has been the component chosen most often for studies of effects of Candida on immune function.[44] Mannan is known to stimulate or suppress cell-mediated and immune functions because the oligosaccharide fragments of mannan appear to be effective inhibitors of cell-mediated immunity.[44]

Extraction Methods of Fungal Mannan

Several extraction methods exist for the isolation of mannan from the cell wall of *C. albicans*. These methods utilize hot alkali, citrate buffer, hot water and/or an enzymatic digestion. When mannan is extracted with hot alkali[44] at very high concentrations, mannose serine and mannose threonine linkages as well as phosdiester and other peptide bonds are cleaved due to the basic pH. This leads to the loss of O-linked oligosaccharides and thus greatly affects the mannan's antigenicity and biological properties.[44] Extraction of cell wall mannan with neutral citrate buffer[44] or hot water[44] leads to the preservation of the carbohydrate component but may denature the protein due to the higher temperatures involved. Treatment of cells with zymolyase, which is a mixture of β-glucannase and proteinase, with a trace amounts of mannosidase optimally preserves the structure of the mannan.[45] It is worth mentioning that most of these methods have been used for the extraction of mannan from the yeast form of *C. albicans* and not the hyphal form as information about the extraction of mannan from the hyphal form is limited. Table 1 below details some methods previously employed for the extraction of cell wall mannan in yeasts. These methods provide a large amount of products but they all have their limitations.

Table 1. Methods previously employed for the extraction of cell wall mannan[44]

Method	Conditions	Limitations
Hot alkali extraction	2 % KOH at 100 ^{o}C for 2 hours	Glycosyl-serine and threonine linkages, phosdiester linkages and the cleavage of some peptide bonds
Citrate buffer method	20 mM citrate buffer at pH 7 at 120 ^{o}C for 1hour, 30 minutes.	Protein is denatured
Hot water extraction	Distilled water at 140 ^{o}C for 2 hours	Protein is denatured
Enzyme treatment (zymolyase)	Phosphate buffer at pH 7.5 at 28 ^{o}C for 1 hour to 3 hours	High cost involved

It is also worth mentioning that no extraction method is completely selective for cell wall mannan[44]. Another step must be employed to enable a successful separation of mannan from other carbohydrates and protein components of the cell wall and cytoplasm. One of the most widely used methods is the Fehling method[46] which utilize Fehling solution. This approach exploit the ability of mannan to chelate and to be precipitated by the copper in Fehling solution. One limitation of this method is that some traces of copper remain bonded to the mannan even after repeated washings and reprecipitation with methanol-acetic acid mixture.[47]

Hypothesis

Cell wall mannan and mannoprotein from *C. albicans* has been previously extracted using a simplified but still harsh method.[48] This method, though useful for extracting mannan from the yeast form of the organism, has not been successful extracting mannan from the hyphal form. We hypothesized that *C. albicans* hyphal mannan is structurally less complex than the yeast mannan, which has prevented its isolation using standard methods as it is more easily degraded. To solve this problem, we have developed a new and simplified method for the extraction of hyphal mannan.

Research Aims

1. Develop a milder and an effective technique for the extraction of cell wall mannan from *C. albican* hyphae as well as the yeast.
2. To elucidate the structures as well as their molecular weight of the mannans isolated with Nuclear Magnetic Resonance and Gel Permeation Chromatography respectively.
3. Compare and contrast the molecular weight and polymer distribution of yeast and hyphal mannans.

CHAPTER 2

MATERIALS AND METHODS

Strains and Media

Candida albicans strain SC5314 was taken directly from frozen stock and passaged on YPD (1 % yeast extract, 2 % peptone, 2 % dextrose, and 2 % agar). For yeast morphology, strain SC5314 was inoculated into 2 L of YPD for growth at 30 ^{o}C for 18 h. For hyphal morphology, strain SC5314 was inoculated into 15 L medium 199 at pH of 7.5 (9.5 g M199 and 12.5 g Tris-HCL) at 1×10^{5} cells/mL for growth at 37 ^{o}C overnight for a well-developed hyphae. Fully developed hyphae were microscopically confirmed before harvesting each flask by filtration which typically yields 10-12 g hyphal cells before lypholization. Stock was received from Dr. Kruppa at the Quilin School of Medicine, ETSU.

Mannan Extraction

The isolation procedure employed for the mannan extraction includes the following: Briefly, approximately 4 g of yeast cells and 1.5 g of the hyphae cells were delipidated with 100 mL acetone for about 15 minutes. The samples were then centrifuged for about 10 minutes at 5000 rpm. The lipid free residue was boiled in either 50 mM NaOH (100 mL) or 50 mM H_3PO_4 (100 mL) for 15 minutes, allowed to cool, neutralized with small amounts of an acid and the cell debris was separated by centrifugation for 5 minutes at 5000 rpm. Methanol (4 volumes) of 50 mL each was added to precipitate the carbohydrate. The supernatant was separated from the precipitate. The mannan isolates were then frozen at -80 ^{o}C and lypholized to dryness. The new procedure for the extraction of both hyphal and yeast mannan of *C. albicans* is as shown in Figure 3 below.

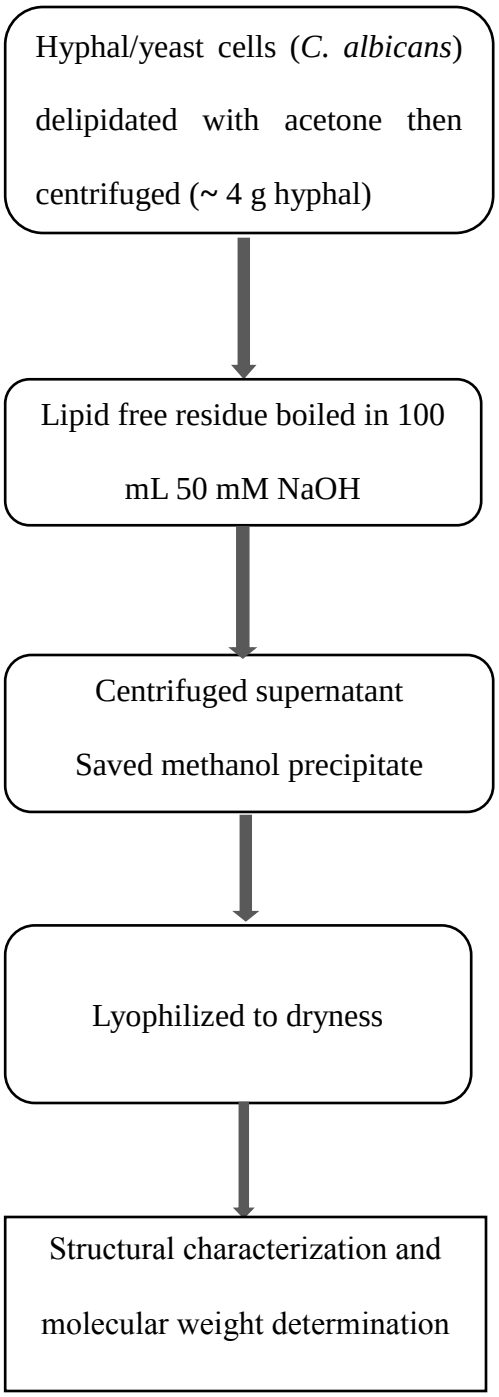

Figure 3: Method of isolation of mannan from C. albicans yeast and hyphae

NMR Analysis of Yeast and Hyphal Mannan

The 600 MHz NMR parameters developed by Kruppa *et al*[40] were employed for the analysis of the mannans in this study. Proton NMR spectra for mannan were collected on Bruker Avance III 600 NMR spectrometer using a CH cryoprobe operating at 333 K (60 ^{o}C) in 5-mm NMR tubes. Mannan (variable sample sizes ranging from 10 to 23 mg) was dissolved in a l mL D_2O (Cambridge Isotope Laboratories, 99.8+ % deuterated). Proton 1D and 2D NMR spectra including COSY, were obtained in this study. Chemical shift referencing was relative to Trimethylsilylpropionate (TMSP) at 0.0 ppm. NMR spectra at 600 MHz were collected and processed as follows: for 1D NMR, 256 30^{o} scans, 65,536 points, 20.5 ppm sweep width centered at 6.175 ppm, exponential apodization with 0.3 Hz broadening, and 1 s pulse delay. Mannan NMR spectra were processed using wxMacNUTS (2nd Generation NMR Utility Transform Software, Version 1.0.1, Acorn NMR, Inc.) on a Macintosh MacBook Pro running OSX version 10.5.8. Spectral comparisons in pairs are used to detect structural changes as indicated by changes in assigned peak intensities. For each set of comparisons, the spectra are height normalized to the largest peaks in each spectrum. The tallest peak in each spectrum at 5.067 ppm is assigned to the anomeric proton of α-D-(1-2)-linked mannosyl repeat units.

Multi-Detector Gel Permeation Chromatography Analysis of Cell Wall Mannan from Yeast and Hyphal *C. albicans*

The MW, polydispersity, polymer distribution and Mark-Houwink (α) values were obtained using a Viscotek/Malvern GPC system consisting of a GPCMax auto injector fitted to a TDA 305 detector (Viscotek, Houston, TX). The TDA contains a refractive index detector, a low angle laser light scattering detector, a right-angled laser light scattering detector, an intrinsic viscosity detector and a UV detector (λ = 254 nm). Three Waters Ultrahydrogel columns, i.e.

1200, 500 and 120, were fitted in series (Waters Corp., Milford, MA). The columns and detectors were maintained at 40°C within the TDA 305. The system was calibrated using Shodex P-82 pullulan standards (5000–800,000 Da) in mobile phase (Showa Denko distributed by Waters Corp.). Mannan samples were dissolved (3 mg/mL) in mobile phase (50 mM sodium nitrite, pH 7.6). The samples were incubated for ~15 min at ambient temperature, followed by sterile filtration (0.2 μm) and injected into the GPC (200 μL). The data were analyzed using Viscotek OmniSec software v. 4.6.1.354. Dn/dc was calculated using the OmniSec software (v. 4.6.1.354). Dn/dc for the mannan samples was determined to be 0.185.

Initially the data were analyzed using a single peak assignment in order to obtain an average Mw for the entire polymer distribution. Subsequently, the data were analyzed using multiple peak settings. Each peak was quantified and the data expressed as area under the refractive index curve-adjusted for calculated concentration. The percentage that each peak contributed to the total polymer distribution was calculated based on a total of 100 %. Replicate analysis of calibration standards indicated reproducibility of ± 3 %, which is well within the limits of the technique.

CHAPTER 3

RESULTS AND DISCUSSION

The structure of mannan from the yeast form of *C. albicans* is well known but there are very few reports on the structure and composition of the C. albicans hyphae mannan.[49] This is due, in part, to the fact that the classical method for mannan isolation from the yeast is not effective in isolating mannan from *C. albicans* hyphae.

In this study, our aim was to develop a method capable of isolating mannan from both the yeast and hyphal morphologies of *C. albicans*. Mannan was successfully isolated from the hyphal and yeast morphologies of *C. albicans* employing a simplified procedure which is described in Figure 3. This simplified method employs the use of a weak base concentration (50 mM) or the use of a weak acid (50 mM H_3PO_4). This is in contrast to the classical method which employs a stronger acid. In the classical method, yeast or hyphae are boiled for about 2 to 3 hours in an autoclave. In this novel method (Figure 3), the boiling times were significantly reduced to 15 minutes. This makes the whole extraction process more time and cost effective. Also, the likelihood of degradation in the native structure of the mannan was highly reduced because of the milder nature of the extraction procedure.

In this study both 1D and 2D NMR analyses were employed to elucidate the hyphal mannan structure. Also, previously published chemical shift assignments, characteristic of individual mannosyl motifs in specific side chains, were employed to correlate the groups that correspond to the specific resonances observed. From the analysis of chemical shifts of H-1 and H-2 for each crosspeak, we are able to assign unique mannosyl repeat units to each resonance in the 1D spectrum. Based upon those assignments and integration of the 1D spectrum, it was

possible to determine the level of different structural features present in the mannan products. By using this approach, it was possible to provide structural assignments both for the acid-stable and acid-labile mannan side chains. Based upon these assignments, detailed structural differences in isolated cell wall mannan from both the yeast and hyphae *C. albicans* were made based upon 600 MHz proton 1D NMR spectra.

Chemical Shift Analysis

Specifically the unique chemical shifts of the anomeric proton, H-1, and its neighboring proton, H-2, in specific mannosyl repeat units of isolated mannan side chain fragments to the chemical shifts of mannosyl repeat units in similar chemical environments in non-degraded, intact mannans were correlated. By this approach, it was possible to provide structural assignments both for the acid-stable and acid-labile mannan side chains without the time-consuming degradation and isolation of individual side chain fragments and detailed 2D NMR side chain structural characterization studies. Table 2 shows our chemical shift analysis from our data between proton (H-1) and proton (H-2) of the mannose units with respect to the isolated yeast mannans. From the 2D COSY correlations many structural features can be obtained. Mannans typically have a backbone composed of α-(1-6) mannose repeats units and a side chain made up of both α-(1-3 and 1-2) mannose repeats units. The chemical shifts at 5.154 ppm and 5.096 ppm (Tables 2 and 3) for both the yeast and hyphae respectively show the presence of repeat units along the backbone of α-(1-6) mannose repeats units.[40] For the structural motif Man β1-2**Man-**α1-P the anomeric proton, H-1 of α-Man-1-PO_4 which resonates at 5.572 ppm while H-2 resonates at 4.221 ppm for the yeast and is characteristic in yeast mannan of *C. albicans.*[40] This information is important because it defines the Man β1-2**Man-**α1-P structural motif in the acid labile portion of the yeast mannan.

The hyphal mannan extracted using this novel method did not exhibit chemical shift at 5.572 ppm for the H-1 ppm or at 4.221 ppm for the H-2 (Table 3) which according to Lowman et al[40] represent the presence of Mβ1-2**M**a1-P in the acid-labile portion. However, the shifts at 5.441 ppm (Table 3) show the presence of Man-PO_4 for the hyphae and is in line with one reported by Lowman et al.[40] This shows that the hyphae mannan's acid labile portion was gone or significantly reduced. Similarly other spectral regions in Table 3 can be defined for structural motifs containing α-Man and β-Man in the subregions Mb1-2**M**a1-2, Man-β1-2**Man-**β1-2Man-α1-2, **Man-**β1-2Man-α1-PO_4.

Table 2: NMR data and structural assignment of *C. albicans* yeast mannan from this research

H-1 (ppm)	H-2 (ppm)	Type	Structural Assignment
5.572	4.221	Yeast mannan	Mβ1-2**M**-α-1-P[a]
5.556	4.204	Yeast mannan	Mβ1-2(Mβ1-2)n**M**-α-1-P
5.373	4.108	Yeast mannan	*
5.294	4.113	Yeast mannan	M-α-1-2**M**-α-1-2
5.278	4.131	Yeast mannan	α-1-2M-α-1-2**M**-α-1-2
5.259	4.102	Yeast mannan	M-α-1-2(M-α-1-2)n**M**-α-1-2
5.192	3.647	Yeast mannan	*
5.183	4.281	Yeast mannan	Mβ1-2**M**-α-1-2
5.171	4.301	Yeast mannan	Mβ1-2**M**-α-1-2 *[c]

Table 2: (continued)

H-1 (ppm)	H-2 (ppm)	Type	Structural Assignment
5.166	4.269	Yeast mannan	Mβ1-2**M**-α-1-2
5.154	4.081	Yeast mannan	α-6(-2)M-α-1-6(Ma1-2)**M**-α-1-6(-2)M-α-1-6
4.941	4.029	Yeast mannan	Related to Mα-1-6*
4.926	4.020	Yeast mannan	*
4.856	4.261	Yeast mannan	Mβ1-2**M**β1-2Mβ1-2
4.854	4.183	Yeast mannan	Mβ1-2M**β**1-2Mβ1-2Mβ1-2(3)
4.833	4.084	Yeast mannan	**M**β1-2M-α-1-P
4.785	4.058	Yeast mannan	**M**β1-2M-α-1-2

[a] α = alpha; β = beta; M = mannan; mannosyl repeat unit used for the assignment is shown in BOLD;

P = phosphate linkage group,

[b] nd = crosspeak not detected, chemical shift taken from the 1D spectrum only

[c] * indicates uncertainty in the assignment

Table 3: NMR data and structural assignment of *C. albicans* hyphal mannan from this research

H-1 (ppm)	H-2 (ppm)	Type	Structural Assignment
5.441	3.661	Hyphal mannan	M-α-1-P
5.441	nd[b]	Hyphal mannan	
5.377	4.112	Hyphal mannan	α-1-2**M**-α-1-3M-α-1-2
5.376	4.107	Hyphal mannan	*
5.291	4.120	Hyphal mannan	*
5.289	4.121	Hyphal mannan	*
5.277	4.137	Hyphal mannan	*
5.277	4.133	Hyphal mannan	*
5.253	4.117	Hyphal mannan	*
5.192	3.656	Hyphal mannan	*
5.192	3.651	Hyphal mannan	*
5.192	3.608	Hyphal mannan	*
5.162	4.267	Hyphal mannan	*
5.162	4.266	Hyphal mannan	*
5.120	4.030	Hyphal mannan	*
5.096	4.018	Hyphal mannan	-6(M-α-1(-2M-α-1)n-2)**M**-α-1-
5.096	4.013	Hyphal mannan	*
5.071	4.077	Hyphal mannan	*
5.071	4.077	Hyphal mannan	*
5.055	4.208	Hyphal mannan	α-1-3**M**-α-1-2

Table 3: (continued)

H-1 (ppm)	H-2 (ppm)	Type	Structural Assignment
5.054	4.210	Hypha mannan	*
4.928	4.014	Hyphal mannan	M-α-1-6
4.928	4.012	Hyphal mannan	*
4.915	3.991	Hyphal mannan	α-1-6**M**-α-1-6
4.855	4.268	Hyphal mannan	*
4.855	4.265	Hyphal mannan	*
4.852	4.186	Hyphal mannan	*
4.850	4.162	Hyphal mannan	*

[a] α = alpha; β = beta; M = mannan; mannosyl repeat unit used for the assignment is shown in BOLD;

P = phosphate linkage group,

[b] nd = crosspeak not detected, chemical shift taken from the 1D spectrum only

[c] * indicates uncertainty in the assignment

Results from the 50 mM NaOH Extraction Method

Yeast and hyphae were extracted using 50 mM NaOH in 3 separate experiments. 600 MHz NMR spectra were collected for each of the extracted mannan samples. Figure 4 represents a typical spectra resulting from the mannan isolated from the yeast form of C. albicans. The resonances for both the acid-stable and the acid-labile portions of the carbohydrate were consistent with mannan isolated by the classical method. In each of the yeast mannan analyses,

Table 2 was employed to aid in the resonance assignments. The overlapping doublet resonances at 5.556 and 5.572 ppm are characteristic of -2Manα1- repeat units in short and long side chains attached to the phosphodiester group in the acid-labile portion[40] and this is observed in our isolates (Table 2). Resonances at 5.294, 5.278, 5.259, 5.183, 5.171, and 5.166 ppm indicate the presence of side chains containing -2Manα1- repeat units and these resonances are a close match to the one reported by Lowman et al.[40] Resonances at 5.154 and 5.072 ppm arise from -6-Manα1- repeat units in the backbone containing (1-2)-linked side chains[40] and this is also present in Figure 4 and Table 2. The resonance at 4.856 ppm is characteristic of multiple -2Man-β1- repeat units in a side chain and this is in close agreement with one reported by Lowman et al.[40] The resonance at 4.833 ppm is characteristic of the Manβ1- terminal repeat unit in a side chain of the acid-labile portion while the resonance at 4.785 ppm is characteristic of the same terminal repeat unit in the acid-stable portion[40] and these were observed in our data showing the presence of Manβ1-terminal repeat unit as shown in Table 2 and in Figure 4.

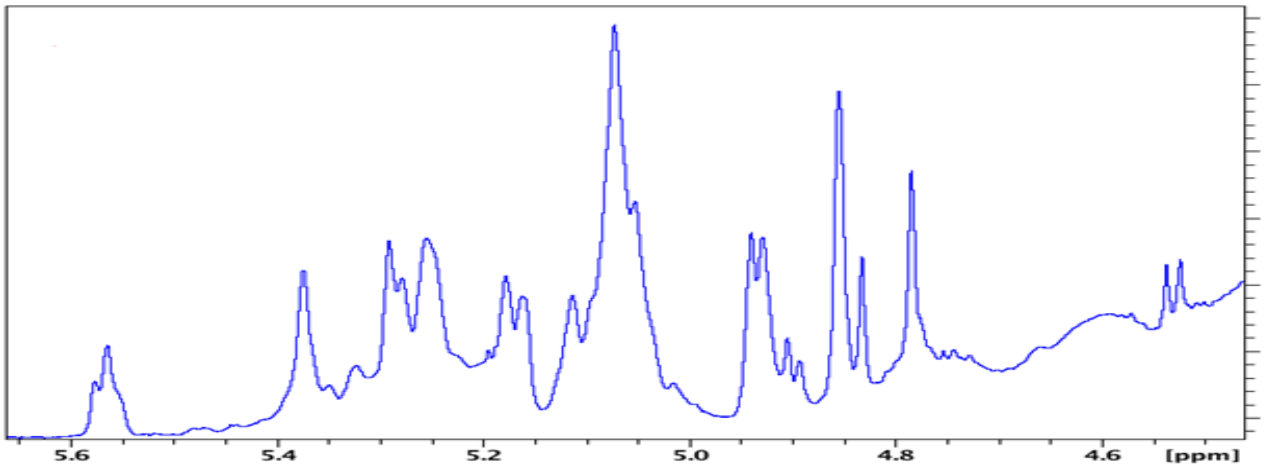

Figure 4: A typical NMR spectrum of the anomeric proton spectral region of *C. albicans* yeast mannan isolated with 50 mM NaOH

Our novel method with 50 mM NaOH resulted in the successful isolation of mannan from the hyphae form of C. albicans as shown in Figure 5. Analysis of Figure 5 shows that there is a complete loss of the long chain acid-labile portion, which is readily evident due to the complete absence of the doublet resonances 5.556 and 5.572 ppm. This demonstrates that the structure of the hyphal mannan is different from that of the yeast mannan. It was originally thought that the hyphal mannan would be longer and more complex than the yeast mannan but our data shows otherwise. Mannan extracted from hyphae with the novel method exhibits resonances for the acid-stable portion predominantly, but the resonances were reduced in height compared to the acid-stable portion of the yeast mannan. The acid-labile portion is only minimally observed or not observed at all in the hyphae (Table 3 for hyphae NMR assignments). The overlapping doublet resonances at 5.556 and 5.572 ppm characteristic of -2Manα1- repeat units in short and long side chains attached to the phosphodiester group in the acid-labile portion of the yeast mannan[40] are not present in the hyphae spectra. A very small doublet resonance at 5.551 ppm (arrow in Figure 5) for a Manα1- repeat unit attached to the phosphodiester linkage is observed in some of the hyphae spectra suggesting that the mild NaOH conditions may be hydrolyzing some or all of the unique, smaller acid-labile portions of the hyphal mannan. Clearly the acid-labile portion was structurally different in the hyphae compared to the yeast mannan. In addition, several of the long-chain repeat units characteristic of content in the yeast mannan were not observed in the hyphal mannan suggesting the presence of different, shorter side chain structures in the acid-stable portion for the hyphae compared to the yeast.

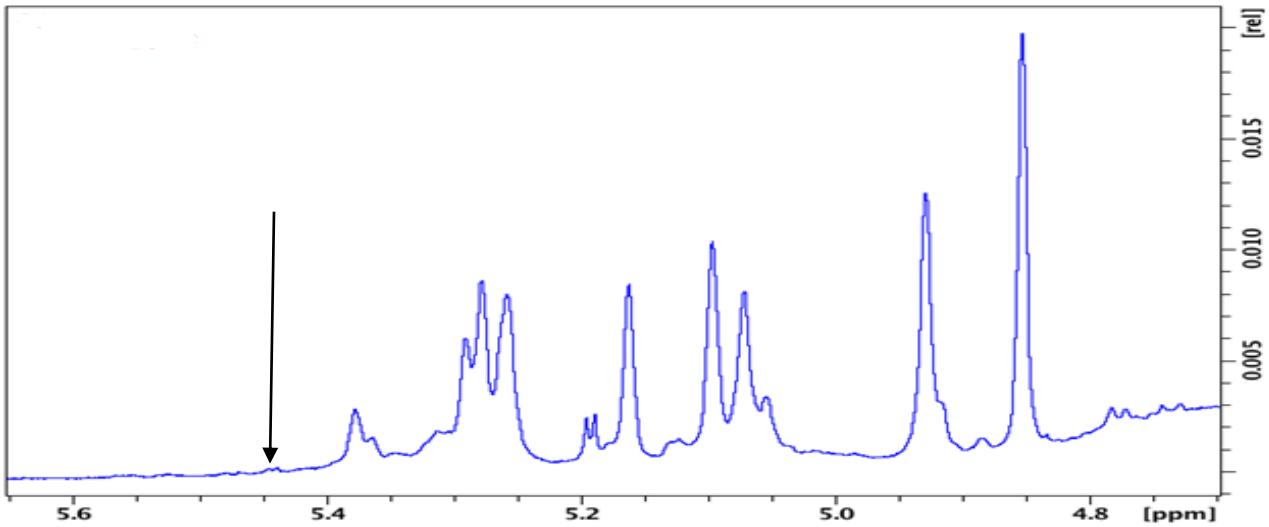

Figure 5: Proton anomeric region of *C. albicans* hyphal mannan isolated with 50 mM NaOH. The black arrow indicates a Manα1- repeat unit attached to the phosphodiester linkage.

All replicate extraction experiments provided similar spectra for the mannan isolated from yeast and hyphae supporting our conclusion that the novel extraction protocol did not impact the structural results reported here for yeast and hyphal mannan. While the NMR spectra from each of the different extraction experiments showed similar isolates with slight variations in composition, there was no evidence of any major differences in the chain compositions.

2D COSY NMR Analysis

The 2D COSY NMR (Two Dimensional Correlated Spectroscopy) spectrum of the full carbohydrate region for yeast and hyphae mannan is shown in the left hand of Figures 6 and 7. The expanded region (red square) shows the individual crosspeaks for correlations between neighboring H-1 and H-2 for each unique mannosyl repeat unit. From the analysis of chemical shifts of H-1 and H-2 for each crosspeak, it was possible to assign unique mannosyl repeat units to each resonance in the 1D spectrum as described in the chemical shift analysis above. Based

upon those assignments and integration of the 1D spectrum, the level of the various structural features can be estimated. A comparison of Figures 6 and 7 shows that yeast mannan is more complex than that of the hyphae mannan. which may be due to the absence of the acid-labile portion in the hyphal mannan indicated by the disapperance of the peak at 5.556 ppm**.**

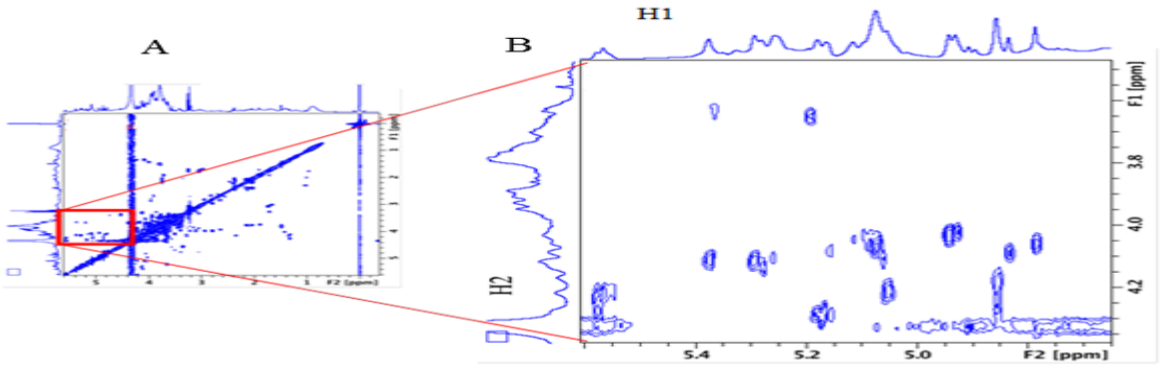

Figure 6. 2D COSY 600 MHz NMR spectrum of yeast mannan expanded to show detailed correlations between the anomeric proton spectral region and the rest of the carbohydrate spectral region

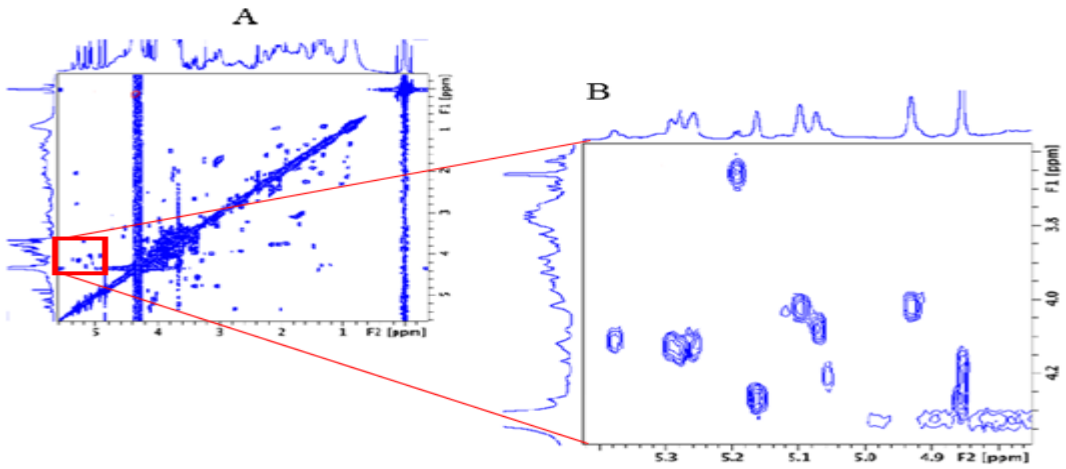

Figure 7. 2D COSY 600 MHz NMR spectrum of hyphae mannan expanded to show detailed correlations between the anomeric proton spectral region and the rest of the carbohydrate spectral region

Results from the 50 mM H_3PO_4 Extraction Scheme

Phosphoric acid (H_3PO_4) was investigated as a possible substitute for sodium hydroxide for the extraction of mannan from the cell wall of the yeast and hyphal *C. albicans*. The use of an acid for the extraction procedure has the potential to deplete or take away the acid labile portion. Therefore the use of an acid should have no or little effect on the structure since there will be no acid labile portion for it to deplete in the first place.

The mannan resulting from the hyphal cell wall through the use of 50 mM H_3PO_4 is shown in Figure 8. Again the overlapping doublet resonances at 5.556 ppm and 5.572 ppm characteristic of the -2Manα1- repeat units in the yeast[40] were not observed in the mannan spectra. This supports the results that were observed for mannan extracted using 50 mM NaOH. There were slight differences such as the small doublet at resonance 5.551 ppm observed in Figure 5 which was not observed in the acid-extracted mannans for hyphae. The spectra for the samples are shown in Figure 8. For the spectra in Figures 8B and 8C respectively, there was a large amount of mannose monomer as seen from the large peak at 4.22 ppm.

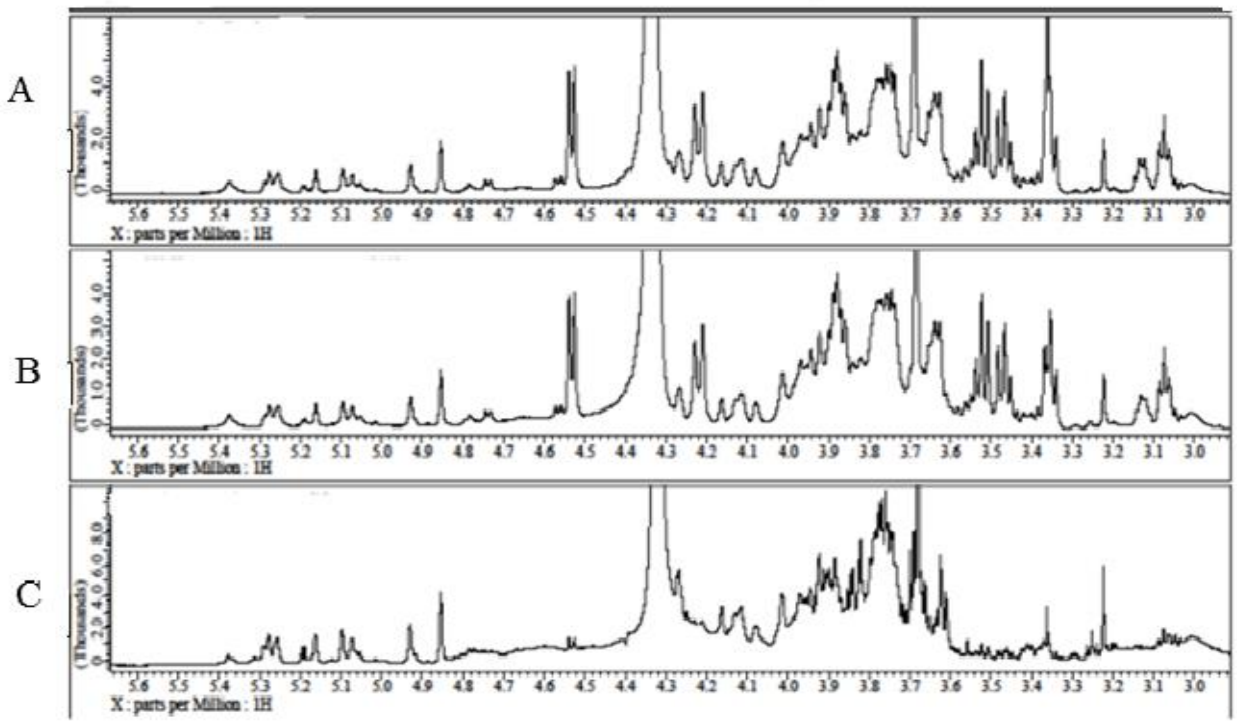

Figure 8. Comparison of the 600 MHz proton NMR spectra of mannans isolated with 50 mM $H3PO_4$ from yeast and hyphae *C. albicans*. Spectra for Figures 8A, 8B and 8C are all hyphae mannan from the same extraction.

The extraction of mannan from the yeast form of *C. albicans* is shown in Figure 9. For the yeast, it was discovered that the mannan in Figure 9C was just a residue while the spectrum for Figures 9A, 9B and 9D did resemble a good yeast mannan. The overlapping doublet resonances at 5.556 and 5.572 ppm, characteristic of -2Manα1- repeat units in short and long side chains attached to the phosphate diester group in the acid-labile portion in the yeast[40] (Table 2) were present. Figure 9A and Figure 9B were not great mannan samples when compared to the mannan from both the yeast and hyphae isolated using 50 mM NaOH solution, due to the presence of a large amount of material called glucan, but with the large doublet peak at 4.22 ppm, this may actually be a monosaccharide instead of a polymer. In short, even though the acid-extracted

mannan also had the same NMR fingerprint as the base extracted mannan, it does not work effectively well as compared to the base extracted mannan because materials like glucans start to appear in the spectrum when the extraction solution as an acid. Even the peak at 5.200 ppm in Figure 9D is reduced compared to that of Figures 9A and 9B.

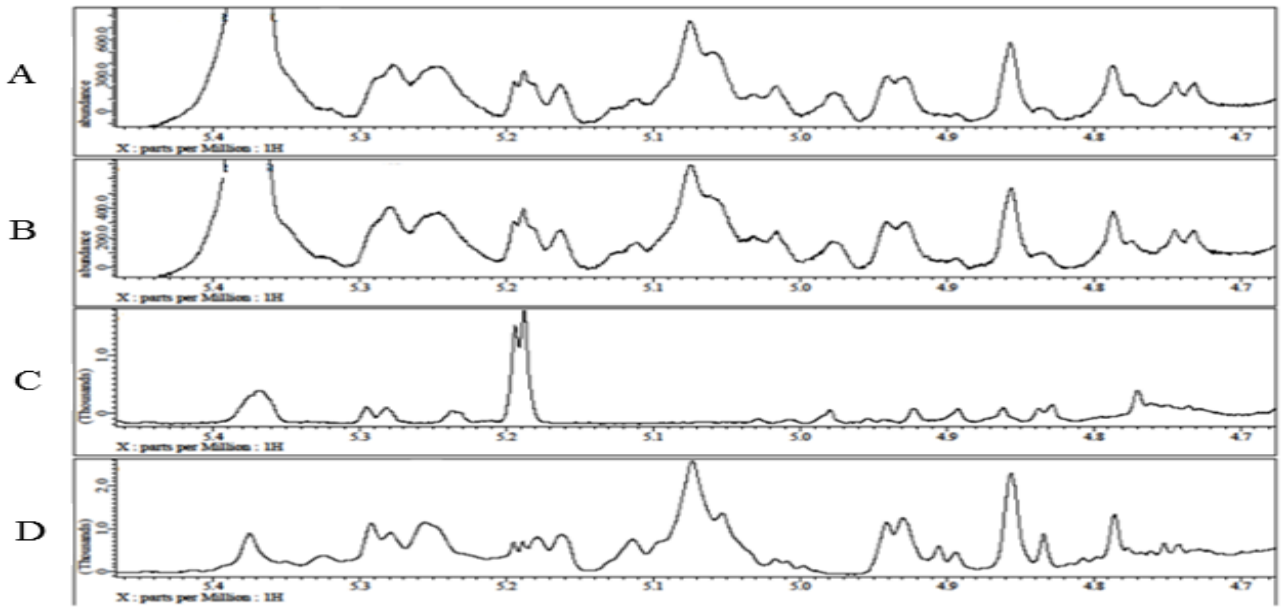

Figure 9. Comparison of the 600 MHz proton NMR spectra of mannans isolated with 50 mM H_3PO_4 from yeast and hyphae *C. albicans*. Figure 9A is just a residue, while Figures 9B, 9C and 9D are all yeast mannan from the same extraction.

Distinct Structural Differences Between Yeast and Hyphal Mannan

From this work, it was confirmed quantitatively, that the hyphal mannan is significantly different from the yeast mannan. Table 4 compares structural features of yeast and hyphal mannans. The structures of these two mannans are clearly different. The acid labile portion of the hyphal mannan contains only one mannosyl repeat unit attached to the phosphate diester linkage in place of the longer chains observed in the yeast mannan.

Also, the composition of the acid stable portion is different between the two mannan isolates. The percentages in Table 4 were generated for RU (Repeat Unit) composition of the side chains in the yeast and the hyphae. A comparison was made in terms of the percent of each side chain RU type relative to the total amount of RU's, total of side chain and back bone RU's. For example, as the acid stable portion is about 94 % in the yeast mannan, it was found out that it was more that 99 % in the case of the hyphae mannan. Also, while the acid labile portion of the yeast was 6 %, it was only about 1 % in the case of the hyphae mannan. This is a confirmation that the acid labile portion of the hyphae mannan is either completely missing or significantly reduced. Again from Table 4, while the yeast mannan contained about 33 % dimers (Mβ1-2Mα1-PO_4) and 65 % trimers (Mβ1-$(2M\beta1)_n$2Mα1-PO_4) and long chain acid labile portions, the hyphae did not contain any at all, represented by 0 %.

However, the hyphal mannan contained about 100 % Mα1-PO_4 while only 2 % was seen in the case of the yeast mannan. The significance in the structure of both the yeast and hyphae mannan is also clearly evident in the case of the backbone to side-chain ratios. It was discovered that the hyphae mannan is only about 28 % in terms of the backbone to side chain ratios relative to the yeast mannan that is 4.3:10 compared to 15:10. This quantitative information is very important because it shows the actual amount of the different units in the mannan polymer of both the *C. albican* yeast and hyphae.

Table 4: A quantitative comparison of hyphae and yeast mannan from Candida albicans

Structural Information	**Yeast**	**Hyphal**
Acid Stable Portion	94 %	➢ 99 %
Acid Labile Portion	6 %	< 1 %
	Acid Labile Portion	
Mα1-PO_4	2 %	100 %
Mβ1-2Mα1-PO_4	33 %	0 %
Mβ1-$(2M\beta1)_n$2Mα1-PO_4	65 %	0 %
	Acid Stable Portion	
1-3Mα1-2 in side chains	12 %	7 %
-2Mα1- in side chains	17 %	41 %
Mβ1-2Mα1-2	5 %	< 1 %
Mβ1-2Mβ1-2Mα1-	5 %	24 %
Backbone-to-Side chain Ratio	15:10	4.3:10

The unique structural differences in the yeast and hyphae mannans were put together in a form of a diagram and are presented in Figure 10. It is evident from Figure 10 that while the yeast mannan has some considerable amount of the acid-labile portion present, the hyphae mannan has just about a fraction of its acid-labile portion present which is true for all the extracted mannans.

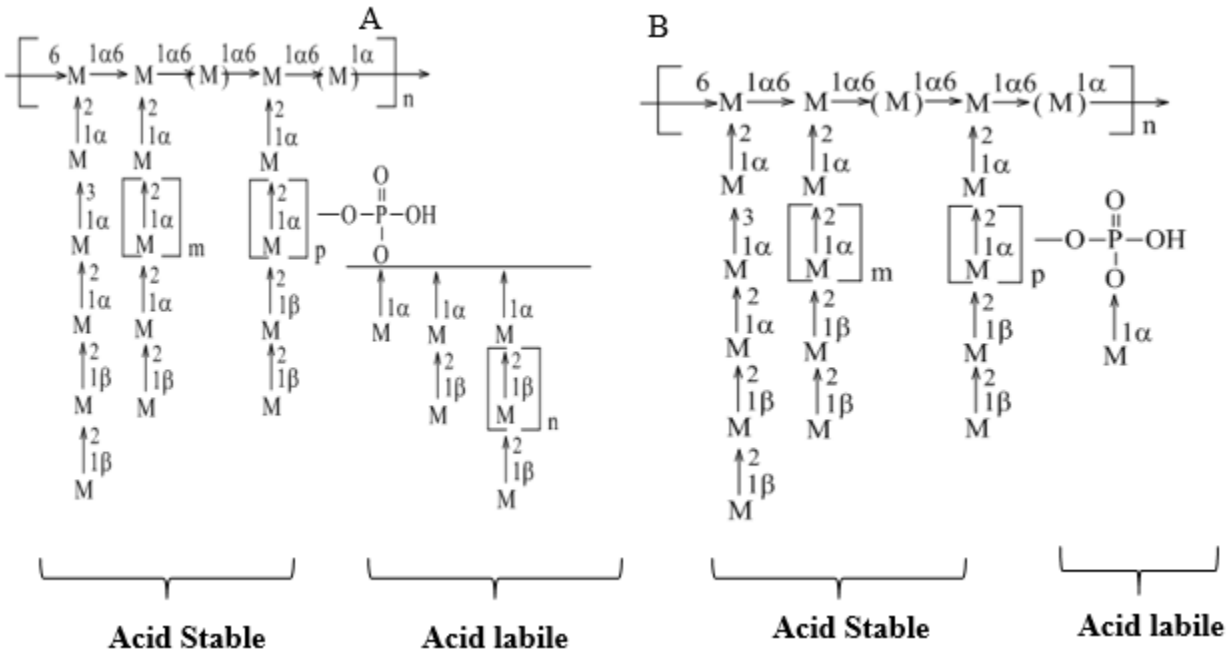

Figure 10: Diagrammatic presentation of the structural differences in yeast (10A) and hyphae (10B) mannans isolated.

GPC Analysis on Mannans Isolated with 50 mM NaOH

Gel Permeation Chromatography depends solely on the molecular size of the polymer. GPC is a powerful tool for determining the size and molecular weight of a polymer as well as the polymer distribution in solution. GPC was employed to confirm MW differences between hyphae and yeast mannan. NMR indicates a difference in the composition that should provide a large difference in MW for these two sources of mannan. Table 5 shows molecular weight as well as the polydispersity we obtained for both yeast and hyphae mannan isolated with 50 mM NaOH.

The hyphae mannan was found to be about 70 % smaller in terms of molecular weights than the yeast mannan and as well as narrower polydispersity than the yeast mannan. Figure 11 shows the polymer distribution for the yeast and hyphae mannans. The GPC data correlate well with the NMR data as smaller molecular weights for the hyphal mannan maybe a result of the significant or complete loss of the acid-labile as was already shown by the NMR data.

Table 5: Chromatographic analysis of *C. albicans* yeast and hyphae mannan with 50 mM NaOH

	***C. albicans* yeast Reference mannan**	***C. albicans* yeast mannan**	***C. albicans* hyphae mannan**
MW x 10^5 (D)	8.76	6.50	1.15
Polydispersity (MW/Mn)	6.30	5.90	1.70

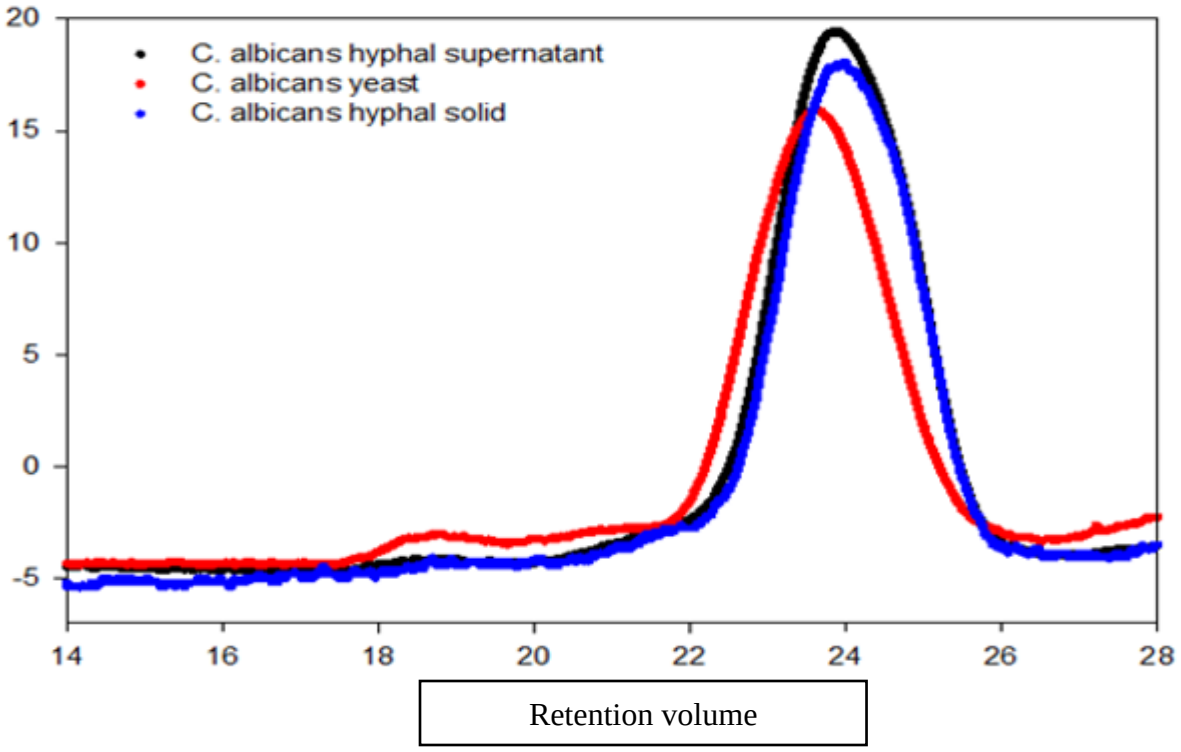

Figure 11. Polymer distribution of *C. albicans* yeast and hyphal mannan from *C. albicans* SC5314 with 50 mM NaOH solution.

The refractive index detector (solid blue, black and red) displays the sample concentration as a function of elution volume, which provides information on polymer distribution. The blue and black lines are for the hyphal mannan while the red is for the yeast.

It was found that the hyphal mannan molecular weight was 1.15×10^5 D while that of the yeast mannan was 6.5×10^5 D. Therefore the hyphae mannan is about 70 % smaller than the yeast mannan. This difference in the molecular weights between the yeast and hyphal mannan correlates with the loss of the acid-labile portion in the hyphal mannan of *C. albicans*.

GPC Analysis on Mannans Isolated with 50 mM H_3PO_4

Gel permeation chromatographic analysis was conducted on the mannan extracted with 50 mM H_3PO_4. Table 6 shows the results we obtained. Though it is clear from the Table 6 that there is not a significant difference between the yeast and hyphae mannan, one conclusion we can draw from this experiment is that the hyphal mannans were smaller than the yeast mannan in terms of the molecular weights. The similarity in molecular weights of the yeast and hyphal mannans may be as a result of the depletion of the acid labile portion of the yeast mannan by the acid which therefore reduces its size to almost that of the hyphal mannan, example 4.53×10^5 D for hyphae compared to 4.99×10^5 D for the yeast.

Table 6: GPC analysis of yeast and hyphae mannan with 50 mM H_3PO_4

Sample ID	**Molecular Weight ($\times10^5$)**	**Polydispersity (MW/Mn)**	**% Recovery**
Hyphal mannan	4.53	1.46	10.7
Hyphal mannan	2.95	8.45	7.3
Hyphal mannan	2.94	4.6	12.0
Yeast mannan	4.99	1.3	7.2
Yeast mannan	3.61	5.7	4.75
Yeast mannan	5.41	13.4	2.2

Dialysis Experiment

In both the yeast and hyphal base extracted mannans, the information or results from both the NMR and the GPC data showed the presence of low molecular weight materials. To remove the low molecular weight materials dialysis was employed with a 1000 molecular weight cut off membrane. Both NMR and GPC data from the dialysis experiments are as shown in Figure 12. It was determined that the dialysate bath samples A and B were mostly proteins with smaller amounts of sugar monomers. The mannan sample from the yeast, A, was determined to contain both glucosamine and mannose but did not contain glucose sugars. However, the hyphae samples contained all three monomers glucose, glucosamine and mannose units.

For the dialysis experiment, about 10 mg of each of the yeast and hyphae mannans were weighed and distilled water was added to dissolve it and was then put in a dialysis tube with a 1000 molecular weight cut off. It was then placed in a 200 mL beaker containing distilled water. It was stirred overnight and the water in the beaker as well as the solid residue left behind in the dialysis tube were frozen to -80 ^{o}C, lyophilized to dryness and finally GPC and ^{1}H NMR analysis was performed on them.

The presence of the glucosamine might be a result of the hydrolysis of chitin by NaOH during the mannan isolation process. The absence of glucose in the yeast isolate is interesting as it might be as a result of the isolation scheme. From the NMR spectra for Figure 12C and Figure 12D, it was determined that both mannans in Figure 12 which represents the yeast and hyphae mannan isolates respectively had considerable amounts of protein and the anomeric proton regions are consistent with the mannan structures previously seen for both the yeast and hyphae and that there were no significant changes observed in the mannan structures. Figure 12E is the

NMR spectrum for water insoluble mannan from the hyphae ad it appears to be predominantly protein with no evidence for mannan components in significant amounts.

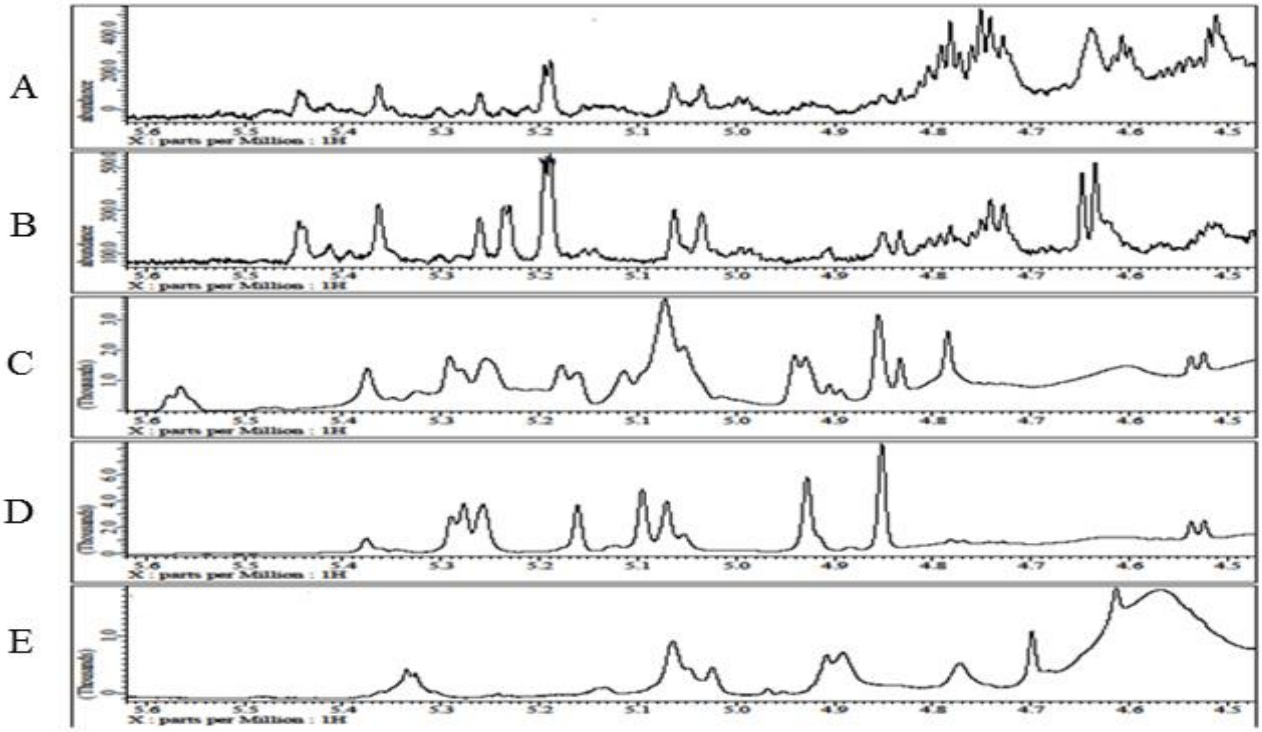

Figure 12. Comparison of the 600 MHz proton NMR spectra of mannans isolated with 50 mM $H3PO_4$ from yeast and hyphae *C. albicans*. Spectrum 12B, 12E and 12D are all hyphae mannan whiles 12A, and 12C are for the yeast mannan

GPC Analysis on Both Yeast and Hyphae Dialyzed Mannans

Comparing the molecular weights of the yeast and hyphae mannans in Table 7, it is clear that the hyphal mannan is smaller than the yeast mannan. From the data, it was estimated that the molecular weight of hyphal mannan is 47 % lower than yeast mannan. Also, the hydrodynamic volume of hyphal mannan is about 22 % smaller than yeast mannan, indicating that hyphal mannan is a smaller molecule in solution. Interestingly, the Rh values indicate that hyphal

mannan has a more rigid structure than yeast mannan. This suggests that the absence of the acid labile portion in hyphal mannan may impact the flexibility of the hyphal mannan. The polydispersity and the intrinsic viscosity were identical in both samples. The Mark Houwink (a) value (which is the slope of linear relationship between log intrinsic viscosity and log molecular weight) was determined to be about 147 % higher in hyphal mannan as compared to the yeast mannan.

Table 7: GPC analysis on both dialyzed yeast and hyphae mannan with 50 mM NaOH

	Yeast mannan	**Hyphal mannan**	**% change from yeast mannan**
Mw[a]	2.61×10^5	1.36×10^5	-47.96
MW/Mn[b]	2.58	2.50	-1.92
IV[c]	0.20	0.19	-7.17
Rh[d]	7.77	6.04	-22.24
Mark-Houwink (a)[e]	0.461	1.12	142.95

a. Mw = molecular weight in Daltons.

b. Polydispersity reflects the polymer distribution.

c. Intrinsic viscosity is a measure of a polymer's contribution to the viscosity of a solution.

d. Hydrodynamic volume is the volume of a polymer in solution.

α - slope of the linear relationship between log intrinsic viscosity and log molecular mass ([η]

A GPC chromatogram for yeast and hyphal mannans was obtained as presented. The red plot represents the C. albicans hyphal mannan while the black plot is yeast mannan. In Figure 13, the red plot is shifted to the right indicating a lower molecular weight polymer as compared to the black plot. This is an indication that the yeast mannan is of higher molecular weight than the hyphal mannan and this is also consistent with the data reported in this thesis.

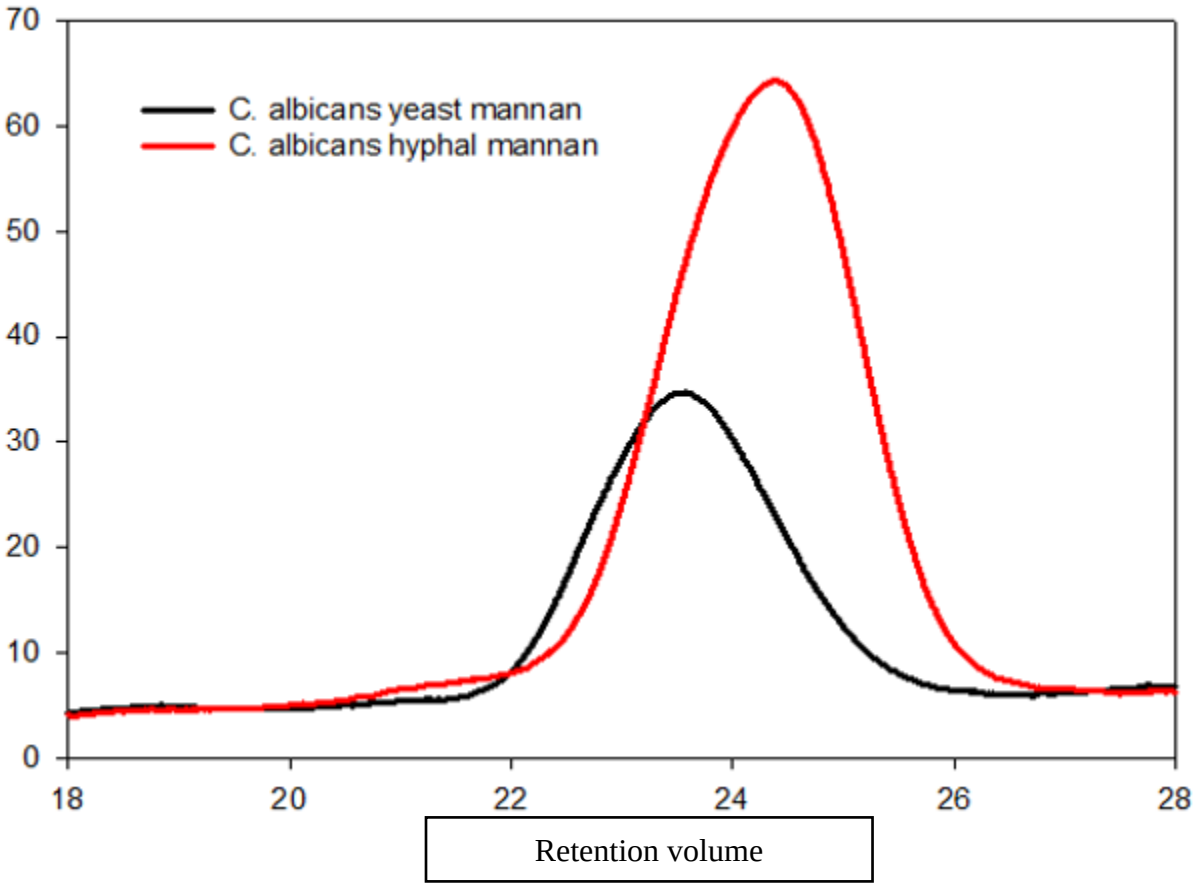

Figure 13. Polymer distribution of *C. albicans* yeast and hyphal mannan from *C. albicans* SC5314 using 50 mM H_3PO_4 solution. The chromatograms were produced by high performance GPC analysis in aqueous solution

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

The New Method for Mannan Isolation

A new method for the isolation of mannan from *C albicans* hyphae was developed in this work. The new method enabled isolation of mannan from hyphae where the classical isolations scheme did not allow for isolation of significant quantities of mannan. Using the new extraction method, it was possible to isolate both yeast and hyphal mannans. It was determined that the structure of mannan from the hyphal cell wall is less complex than the mannan from the yeast cell wall. This work provided a rapid mannan isolation method which was successful for the both the yeast and hyphae morphologies of *C. albicans* and may potentially be employed on many other yeast species as well. To the best of our knowledge, this is the first successful isolation of mannan from the hyphal morphology of *C. albicans,* in quantities sufficient to allow for the elucidation of novel structural information. As the method was successful with both morphologies, it allowed the direct comparison of the mannan structure.

C. albicans Yeast Mannan

In terms of the yeast mannan, it was found that the yeast mannan of *C. albicans* has both acid stable as well as acid labile portions. It has long side chains in the acid labile portion of the mannan. In addition to these features, it was discovered that the yeast mannan did not exhibit evidence for Ma1-PO_4 side chains in the acid labile portion. The average molecular weight for the yeast mannan was determined to be 6.5×10^{5} D and it has a higher polydispersity than that of the hyphae mannan.

C. albicans Hyphal Mannan

It was found that the hyphae mannan of *C. albicans* did not contain long side chains in the acid labile portion. In addition to this, it had very little or no acid labile portion containing only a trace amount of Ma1-PO_4 and also overall, the hyphal mannan has a narrower polydispersity than the yeast mannan. Its molecular weight was determined to be 1.15×10^5 D.

Comparison of Acid Versus Base Extraction Method

Using 50 mM NaOH or 50 mM H_3PO_4 solutions result in the isolation of hyphal mannan with a structure similar to hyphal mannan, that is, the hyphal mannan isolated in both cases show evidence of very little or the complete absence of acid-labile component while in both cases the acid stable portion was retained. Thus the hyphal mannan is structurally distinct from yeast mannan independent of the isolation procedure. However, the NaOH method provides a 'cleaner' isolate, which is preferable, that is, while the base extracted materials contains predominantly mannan, the acid extracted materials contains mannan as well as other unwanted macromolecules like chitin and lipids. The issue of low molecular weight materials which was observed in all the GPC data obtained was resolved using a 1000 MW cut off membrane in a dialysis experiment which yielded a clean mannan for analysis. Utilizing GPC and NMR techniques, it was discovered that the yeast mannan is still complex, and has a higher molecular weight than the hyphal mannan of *C. albicans*. In fact, it was determined that the hyphal mannan molecular weight is about 50 % less that of the yeast mannan. Specifically the yeast mannan has a molecular weight of 2.61×10^5 D while that of the hyphal mannan is 1.36×10^5 D. That is, the yeast mannan still has both the acid-labile and acid-stable portions intact but the hyphal mannan had only the acid-stable portion present.

Final Conclusion

A new method has been developed which is not only capable of isolating mannan from the yeast form of C. albicans, but it is also capable of isolating mannan from the hyphal *C. albicans*. This discovery has helped us to gain new insight into the structure and molecular weights of both the yeast and hyphal mannans. It was discovered that the hyphal mannan is structurally less complex than the yeast mannan in that, while the yeast mannan has both the acid-stable and the acid-labile portions present, the hyphal mannan has very small or sometimes not acid-labile portion present. The molecular weight as determined using the GPC was found to be 1.36×10^5 D for the hyphal mannan and also 2.61×10^5 D for the yeast mannan. The absence or significant reduction in the acid labile portion of *C. albicans* hyphal mannan may be crucial to the virulence and/or pathogenicity of this opportunistic fungus.

Future Work

Further experiments should be carried out to determine how the significant decrease or the complete loss of the acid labile portion in the hyphal mannan may contribute to its virulence nature. Also the Rh values suggests that the hyphal mannan has a more rigid structure which might have a significant impact on the flexibility of this material and hence the nature of this impact must also be investigated. Also, it is proposed that different concentrations of acid or base should be used to see whether more structural informations can be obtained and finally, the new method should be employed or tried on other pathogenic cells to see if it can be used to extract mannans from those species too.

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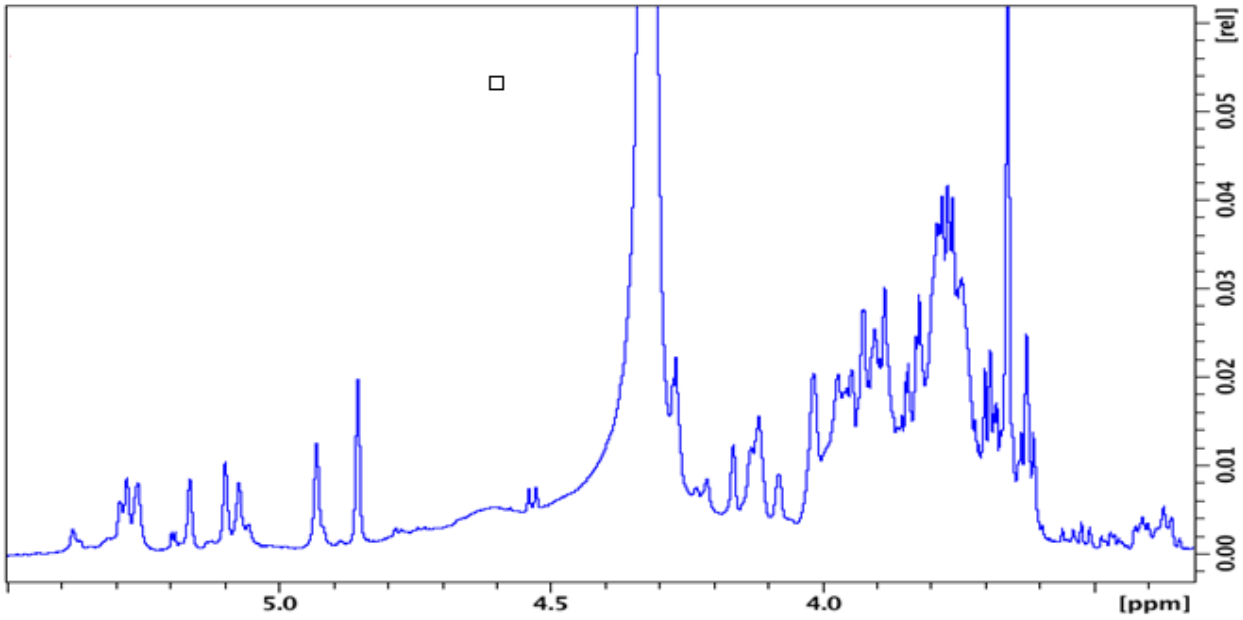
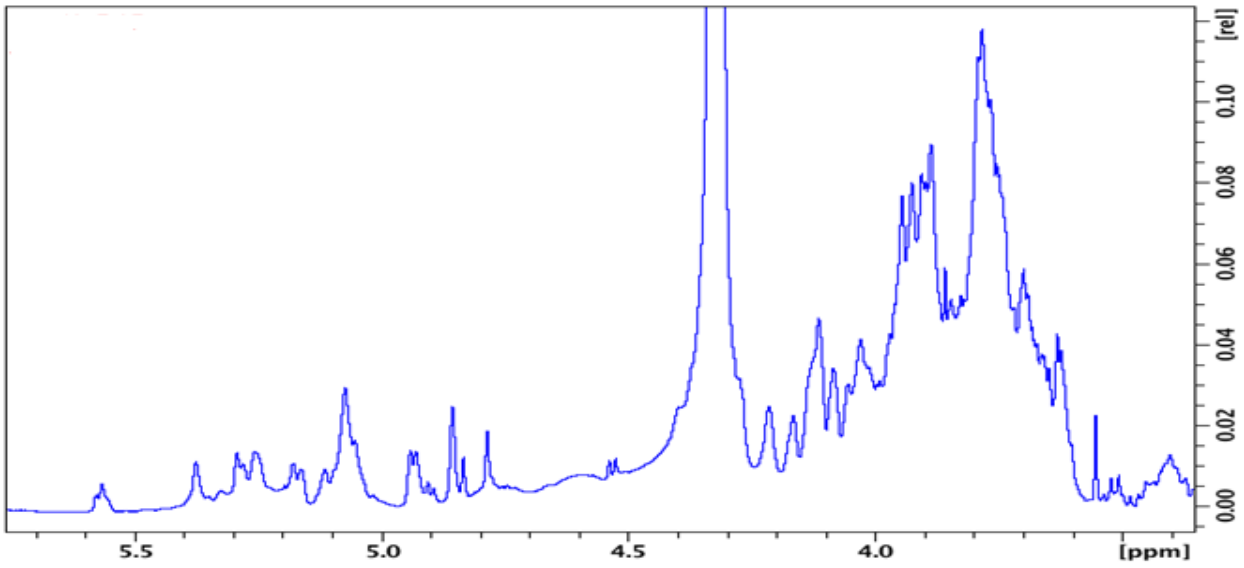
APPENDICES

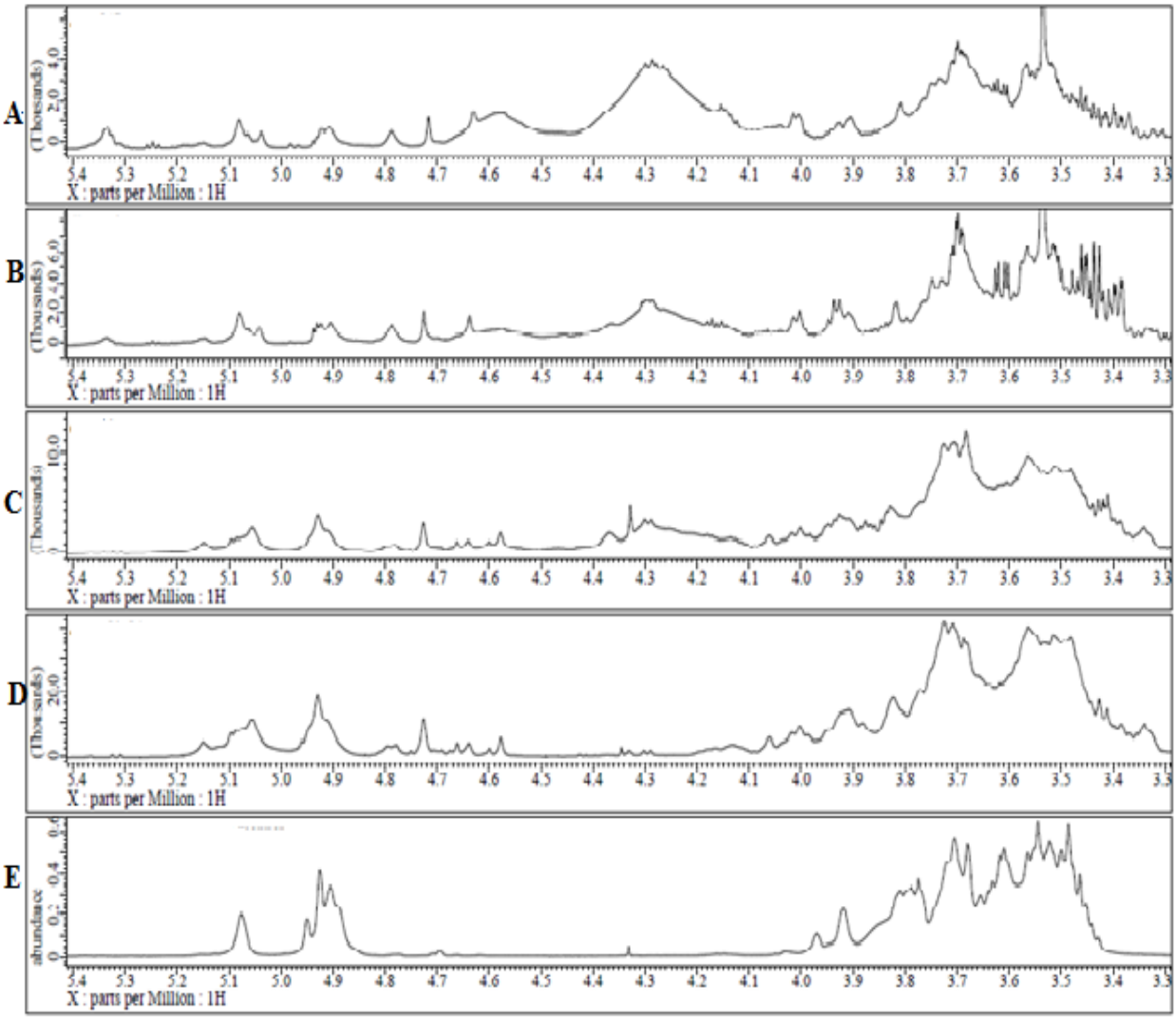
APPENDIX A: Proton NMR region of *C. albicans* hyphal mannan using 50 mm NaOH solution from the first replicates



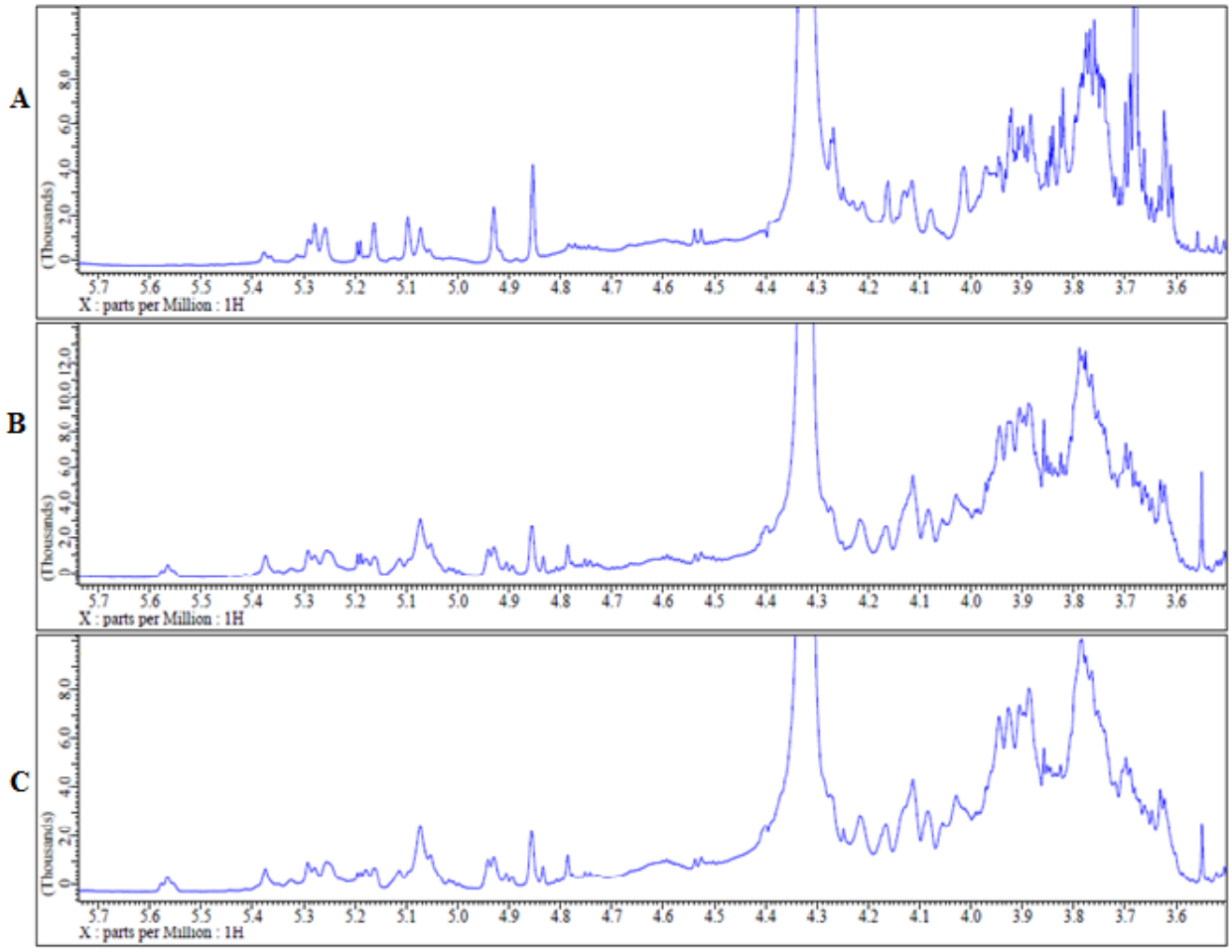
APPENDIX B: Proton NMR region of *C. albicans* yeast mannan using 50 mm NaOH solution for the first replicates.



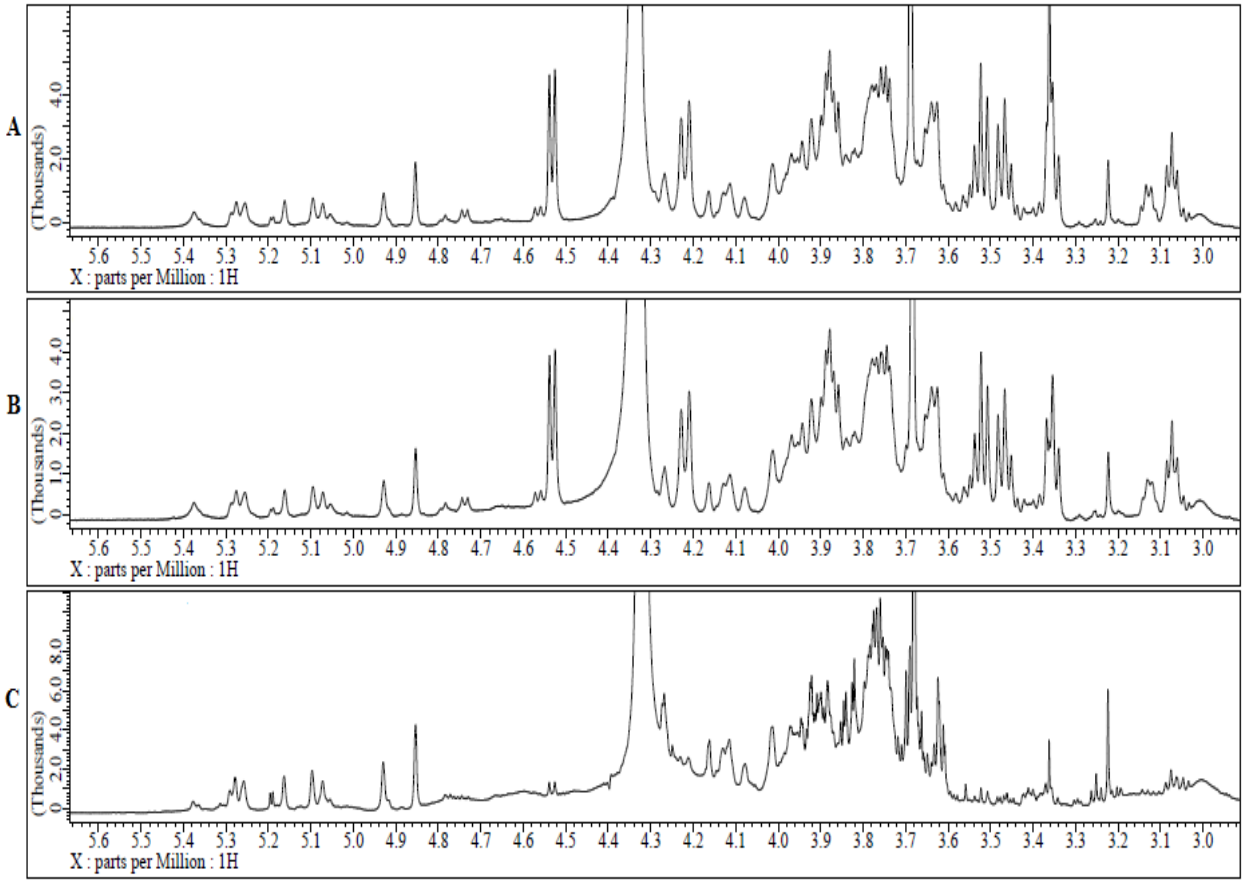
APPENDIX C: NMR spectrum for both *C. albicans* yeast and hyphal mannan using 50 mM NaOH solution from the second replicates. Spectrum A and B are for the yeast mannan whiles C and D are for the hyphal mannan. Spectra E is a standard yeast mannan from Sigma run in DMSO.



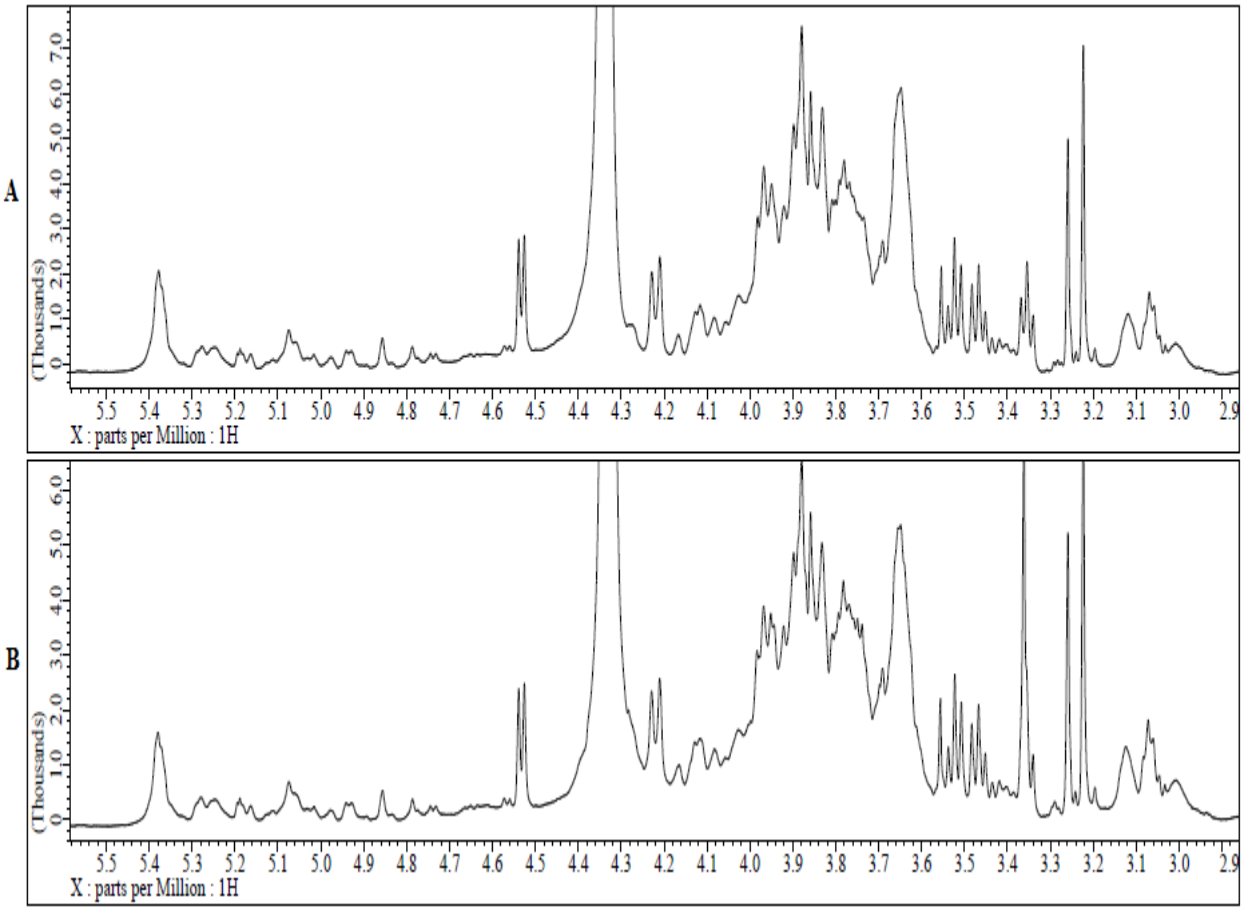
APPENDIX D: NMR spectrum for both *C. albicans* yeast and hyphal mannan using 50 mM NaOH solution from the third replicates. Spectrum A is for hyphal mannan whiles B and C are for the yeast mannan



APPENDIX E: NMR spectrum for *C. albicans* hyphal mannan using 50 mM H_3PO_4 solution from the fourth replicates. Spectrum A and B were obtained using the acid whiles spectrum C is from the extraction using 50 mM NaOH to serve as a comparison



APPENDIX F: NMR spectrum for *C. albicans* yeast mannan using 50 mM H_3PO_4 solution from the fourth replicates. Spectrum A and B both represents the yeast mannans obtained using the acid extraction method.



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