STUDIES ON BIOACTIVE POLYSACCHARIDES OF HYBRID MUSHROOM

A SYNOPSIS

SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

(IN CHEMISTRY)

TO

VIDYASAGAR UNIVERSITY

BY

KOUSIK MAITY

DEPARTMENT OF CHEMISTRY & CHEMICAL TECHNOLOGY
VIDYASAGAR UNIVERSITY
MIDNAPORE 721102
WEST BENGAL, INDIA

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Synopsis

The important focus of the present thesis entitled ***"Studies on bioactive polysaccharides of hybrid mushroom"*** is to determine the structure, immunoenhancing property as well as antioxidant activity of different polysaccharides isolated from the fruiting bodies of an edible hybrid mushroom of *Pleurotus florida* and *Calocybe indica* variety APK2. The entire thesis is divided into five chapters.

Chapter-I describes the general introduction of carbohydrates, polysaccharides, mushroom, hybrid mushroom, mushroom polysaccharides and their biological activities.

Chapter-II represents the experimental methods, which were employed during the whole course of the research work.

Chapter-III is one of the major part of the thesis, which describes the isolation, purification, determination of the structure, immunological activity and as well as antioxidant study of the polysaccharide isolated from aqueous extract of the fruiting bodies of an edible hybrid mushroom of *Pleurotus florida* and *Calocybe indica* variety APK2. This work has been published in **International journal of biological macromolecules** 48 (2011) 304-310.

Chapter-IV, another major part of the thesis contains chemical analysis and study of immunoenhancing and antioxidant property of a glucan isolated from an alkaline extract of a somatic hybrid mushroom of *Pleurotus florida* and *Calocybe indica* variety APK2. This work has been published in **International journal of biological macromolecules** 49 (2011) 555-560.

Chapter-V is another key part of the thesis which deals with the isolation and characterization of the water-insoluble glucan, isolated from alkaline extract of a somatic hybrid mushroom of *Pleurotus florida* and *Calocybe indica* variety APK2. This work has been published in **Fitoterapia** 84 (2013) 15-21**.**

Chapter I: It represents the general introduction of carbohydrates, polysaccharides, mushroom, hybrid mushroom, mushroom polysaccharides along with their biological as well as antioxidant property. **Carbohydrates** are a major class of naturally occurring organic compounds which came by their name because they usually have the general formula $C_x(H_2O)_y$. It played the important role in establishment and evolution of the life on earth by creating a direct link between the sun and chemical energy. These are widely distributed both in animal and plant tissues. According to a more comprehensive definition by Robyt "Carbohydrates are polyhydroxy aldehydes or ketones or compounds that can be derived from them by oxidation, reduction or replacement of different functional groups." Carbohydrates are formed in green plants as the result of photosynthesis, which is the chemical combination or "fixation" of carbon dioxide and water by utilization of energy gained through absorption of visible light. They are classified into monosaccharide, disaccharide, oligosaccharide and polysaccharide. The great bulk of the carbohydrates in nature are present as polysaccharides, which have relatively large molecular weights.

The term **Mushroom** means "a macrofungus with a distinctive fruiting body which can be hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand". Since ancient time, mushrooms have been cultivated worldwide for their taste, nutritional supplements, and potential application as drugs. Mushroom polysaccharides are not only used against cancers of stomach, esophagus, lungs, and colons but also act as anti-inflammatory, antiviral (against AIDS), hypoglycaemic and antithrombotic agents. *Lentinus edodes* (**Lentinan / Shiitake, Japan**), *Schizophyllum commune* (**Schizophyllan**), *Agaricus blazei* (**Agarican, USA**) *Ganoderma lucidum* (**Lingzhi, China**) and *Grifola frondosa* (**Maitake, Japan**) have been used clinically as anti-tumor agents. Further

improvement of quality of edible mushrooms, **Hybrid mushrooms** is readily needed to be developed. It has long been established that, para-sexual hybridization is one of the ways to combine genetic characters across the species barrier. Different **Polysaccharides** are used as dietary fiber. Recent research revealed that chemically dietary fiber consists of non-starch polysaccharides such as arabinoxylans, cellulose, and many other plant components such as resistant starch, resistant dextrins, inulin, lignin, waxes, chitins, pectins, beta-glucans, and oligosaccharides. The immunomodulatory and anti-tumor properties are the key issues of **mushroom polysaccharides** for drawing the attention of chemist and immunobiologists. The biological activities and antioxidant property of polysaccharides depend on the size of molecule, branching rate and form. So, it is very important to determine the exact structure of the polysaccharides, isolated from mushroom.

Chapter II: The methodologies that have been used during research tenure to determine the structure of polysaccharides have been discussed in this chapter. The immunoenhancing and antioxidant activities of polysaccharides depend on the size of molecule, branching rate and form. So, it is very important to determine the exact structure of the polysaccharides of the medicinal and edible mushrooms. The polysaccharide is purified using different chromatographic techniques. The accurate structure of the polysaccharides is determined using two types of methods:

(1) Chemical method that includes acid hydrolysis, methylation, and periodate oxidation studies.

(2) Spectroscopic method comprising of 1D (^{1}H, ^{13}C) and 2D NMR (**DQF-COSY**, **TOCSY, NOESY, ROESY, HMQC, HSQC**, and **HMBC**), UV-vis spectroscopy, and Gas Chromatography-Mass spectrometric experiment (**GLC-MS**).

Chapter III: This chapter consists of the isolation, purification, structural characterization and immunoenhancing as well as antioxidant properties of the polysaccharide isolated from the aqueous extract of the fruit bodies of somatic hybrid (PCH9FB), raised through intergeneric protoplast fusion between edible mushroom strains of *Pleurotus florida* and *Calocybe indica* var. APK2 by chemical and NMR analysis. Water-soluble polysaccharide isolated from the hot water extract of the fruit bodies (450g) of PCH9FB was cooled, filtered, and precipitated in alcohol. The residue was dialyzed, centrifuged, and freeze dried to yield 300 mg of crude polysaccharide, which on gel permeation chromatography through sepharose-6B using water as an eluant yielded only one homogeneous fraction. The pure polysaccharide (PS) showed specific rotation $[\alpha]_D$ +29.15 (*c* 0.104, H_2O, 28.7 °C) and the average molecular weight of the PS was estimated from a calibration curve prepared with standard dextrans and it was nearly 2.25×10^5 Da. The detailed structural studies of this PS was carried out on the basis of total acid hydrolysis, methylation analysis, periodate oxidation, and 2D-NMR (^1H, ^{13}C, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC) studies. On acid hydrolysis with 2M CF_3COOH, followed by alditol acetate preparation and analysis through gas-liquid chromatography (GC) the PS was found to contain glucose, galactose, and fucose in a molar ratio 2:2:1. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al. The polysaccharide was methylated according to the method of Ciucanu and Kerek. The GC and GC-MS analysis of the alditol acetates obtained from the hydrolyzate of the methylated polysaccharide revealed the presence of 1,5,6-tri-acetyl-2,3,4-tri-*O*-methyl galactitol (41.65%), 1,2,4,5,6-penta-acetyl-3-*O*-methyl glucitol (19.11%), 1,5-di-acetyl-2,3,4,6-tetra-*O*-methyl glucitol (21.54%), 1,5-di-acetyl-2,3,4-tri-*O*-methyl fucitol (17.71%), in a molar ratio of nearly 2:1:1:1. These results indicated that (1→6)-linked galactopyranosyl, (1→2,4,6)-linked glucopyranosyl, terminal glucopyranosyl and terminal fucopyranosyl moieties were present in a molar ratio of 2:1:1:1. The GC analysis of the alditol acetates of the periodate-oxidized, reduced, PS showed only glucose. The GC-MS analysis of the alditol acetates of the periodate-

oxidized, reduced, methylated PS showed 1,2,4,5,6-penta-acetyl-3-*O*-methyl glucitol. These results further confirmed the mode of linkages of the monosaccharide components present in the PS.

The proton NMR spectrum (500 MHz) of this PS at 27 °C contained four signals at 5.38, 5.05, 4.96, and 4.51 ppm for anomeric protons in a molar ratio of nearly 1:1:2:1. They were designated as **A**, **B**, **C**, and **D** according to their decreasing anomeric proton chemical shifts. In the ^{13}C NMR spectrum (125 MHz) at 27 °C, three signals were found in the anomeric region at δ 98.3, 100.3, and 103.3 in a ratio of nearly 3:1:1. Signals at δ 98.3 was assigned to anomeric carbons of **B** and **C** residues, signals at δ 100.3 and 103.3 were assigned to anomeric carbons of **A** and **D** residues, respectively. All the ^{1}H and ^{13}C signals were assigned from DQF-COSY, TOCSY, and HMQC experiments. From DQF-COSY experiment the proton coupling constants were measured.

In case of residue **A**, the anomeric proton chemical shift at 5.38 ppm, $J_{H\text{-}1,H\text{-}2}$ ~3.5 Hz, and $J_{C\text{-}1,H\text{-}1}$ ~170 Hz indicated that it was an α-anomer. Large coupling constants $J_{H\text{-}2,H\text{-}3}$ (~10 Hz) and $J_{H\text{-}3,H\text{-}4}$ (~10 Hz) for residue **A** indicated that it was a glucosyl moiety. The downfield shift of C-2 (77.1 ppm), C-4 (78.4 ppm), and C-6 (67.5 ppm) signals with respect to the standard values of methyl glycosides indicated that residue **A** was (1→2,4,6)-linked α-D-glucopyranose. The linking at C-6 was further confirmed from DEPT-135 spectrum.

Residue **B** was assigned as an L-fucopyranosyl unit, strongly supported by the appearance of a proton signal at δ 1.22 and a carbon signal at δ 16.1 for a CH_3 group, and small coupling constant $J_{H\text{-}3,H\text{-}4}$ (<3 Hz). The appearance of the anomeric proton signal for residue **B** at δ 5.05 ppm and the coupling constant value of $J_{H\text{-}1,H\text{-}2}$ (~3.75 Hz) clearly indicated that L-fucose was α-linked. This anomeric configuration was further confirmed by ^{1}H-^{13}C coupling constant $J_{C\text{-}1,H\text{-}1}$ ~171 Hz. The rest of carbon values in ^{13}C corresponded to the standard values of methyl glycosides indicating residue **B** was α-glycosidically linked terminal L-fucopyranosyl unit.

Residue **C** was assigned to (1→6)-linked α-D-galactopyranose. The galacto configuration was confirmed from the large coupling constant $J_{H\text{-}2,\,H\text{-}3}$ ~8 Hz and relatively

small $J_{H\text{-}3,H\text{-}4}$ ~3 Hz. The α-configuration of residue **C** was deduced from proton signal (δ 4.96) and coupling constants, $J_{H\text{-}1,H\text{-}2}$ ~3 Hz, and $J_{C\text{-}1,H\text{-}1}$ ~170 Hz. The anomeric carbon signal of residue **C** appeared at 98.3 ppm. The downfield shift of C-6 (δ 66.8) carbon signals with respect to standard values of methyl glycosides indicated that residue **C** was (1→6)-linked moiety which was further confirmed from DEPT-135 experiment.

In the case residue **D,** the anomeric proton signal at δ 4.51 ppm, and the coupling constants $J_{H\text{-}1,H\text{-}2}$~8.29 Hz and $J_{C\text{-}1,H\text{-}1}$~160 Hz indicated that it was a β-linked moiety. Large coupling constants $J_{H\text{-}2,\ H\text{-}3}$ and $J_{H\text{-}3,\ H\text{-}4}$ (~10 Hz) indicated its glucoconfiguration. Comparing with standard values of carbon signals it was concluded that residue **D** was terminal β-D-glucose. From the above experimental data the residues [**A – D**] were found to have following linkages;

Residue A: (1→2,4,6)-linked α-D-glucopyranose

Residue B: α- linked terminal L-fucopyranose

Residue C: (1→6)-linked α-D-galactopyranose

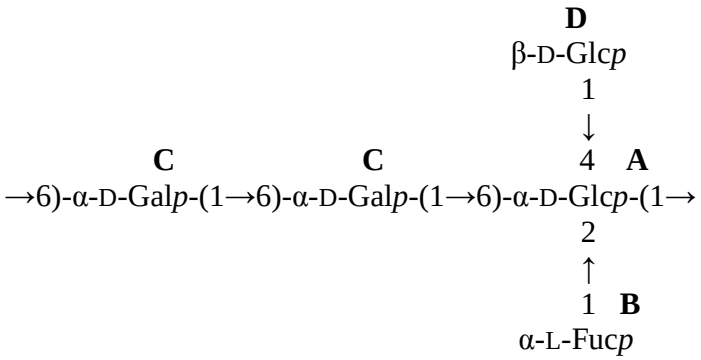
Residue D: β-linked terminal D-glucopyranose

The sequence of glycosyl residues of the polysaccharide was determined from NOESY as well as ROESY experiments. In NOESY experiment, the inter-residual contacts from **A**H-1 to **C**H-6a and H-6b, **B**H-1 to **A**H-2, **C**H-1 to **A**H-6a and H-6b, and **D**H-1 to **A**H-4 along with other intra-residual contacts established the following sequences;

A (1→6) **C**; **B** (1→2) **A**; **C** (1→6) **A**; **D** (1→4) **A**

The sequence was further confirmed by the HMBC experiment. The cross-peaks were found between H-1 (δ 5.38) of residue **A** and C-6 (δ 66.8) of residue **C** (**A** H-1, **C** C-6); C-1 (δ 100.3) of residue **A** and H-6a (δ 3.65) and H-6b (δ 3.67) of residue **C** (**A** C-1, **C** H-6a; **A** C-1, **C** H-6b). Similarly, cross-peaks were found between H-1(δ 4.96) of residue **C** and C-6 (δ 67.5) of residue **A** (**C** H-1, **A** C-6); C-1 (δ 98.3) of residue **C** and H-6a (δ 3.75) and H-6b (δ 3.92) of residue **A** (**C** C-1, **A** H-6a; **C** C-1, **A** H-6b).The cross peaks were also found between H-1 (δ 5.05) of residue **B** and C-2 (δ 77.1) of residue **A** (**B** H-1, **A** C-2); C-1 (δ 98.3) of residue **B** and H-2 (δ 3.61) of residue **A** (**B** C-1, **A** H-2). Cross-peaks were

observed between H-1 (δ 4.51) of residue **D** and C-4 (δ 78.4) of residue **A** (**D** H-1, **A** C-4) as well as C-1 (δ 103.3) of residue **D** and H-4 (δ 3.64) of residue **A** (**D** C-1, **A** H-4). Moreover some intra-residual couplings were also obtained in HMBC spectrum. The HMBC and NOESY connectivities clearly supported the presence of pentasaccharide repeating unit in the PS isolated from the hybrid mushroom PCH9FB as;



Macrophage activation of the polysaccharide was observed in vitro. Upon treatment with different concentrations of the polysaccharide an enhanced production of NO was observed in a dose-dependent manner with maximum production of 16.0 µM NO per 5×10^5 macrophages at 200 µg/mL of the polysaccharide. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the polysaccharide by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. At 25 µg/mL of the polysaccharide, splenocyte proliferation index was found maximum as compared to other concentrations. Hence, 25 µg/mL of the polysaccharide can be considered as efficient splenocyte stimulator. Again 10 and 25 µg/mL of this sample showed maximum effect on thymocyte stimulation.

DPPH scavenging activity was performed by different concentrations of polysaccharide. A maximum of 65 % DPPH radical scavenging activity was observed at 8 mg/mL of the polysaccharide. The EC_{50} value of the polysaccharide for DPPH radicals was 6.2 mg/mL. The scavenging activity of the polysaccharide increased steadily from 2-8 mg/mL, while it reached a maximum plateau from 0.5-2 mg/mL for ascorbic acid, which indicates the scavenging activity of polysaccharide against DPPH radical was less than that of ascorbic acid.

Chapter-IV: This chapter describes the structural characterization and the study of immunological and antioxidant properties of a glucan isolated from the alkaline extraction of the fruit bodies of somatic hybrid mushroom of *Plourotus florida* and *Calocybe indica* var. APK2. The hot alkaline-extract of fresh fruit bodies (500 g) of PCH9FB was cooled, filtered, and precipitated in alcohol. The residue was dialyzed, centrifuged, and freeze dried to yield 395 mg of crude polysaccharide, which on column-chromatography through sepharose-6B using water as an eluant yielded only one homogeneous fraction. The pure polysaccharide (PS) showed specific rotation $[\alpha]_D$ +27.15 (*c* 0.1, H_2O, 26.0 °C) and the average molecular weight of the PS was estimated as ~ 1.98×10^5 Da. The detailed structural studies of this PS was carried out on the basis of total acid hydrolysis, methylation analysis, periodate oxidation, and 2D-NMR (^1H, ^{13}C, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC) studies.

Acid hydrolysis of the PS followed by sugar analysis using GC revealed the presence of only glucose as a monosaccharide constituent. Another part of the hydrolyzed product was set aside for paper chromatographic study, which also showed the presence of glucose only indicating that the polysaccharide was a glucan. The absolute configuration of the monosaccharide was determined by the method of Gerwig et al and found to be D configuration. The PS was methylated according to the method of Ciucanu and Kerek. The GC and GC-MS analysis of the alditol acetates of the methylated product showed the presence of 1,5,6-tri-acetyl-2,3,4-tri-*O*-methyl-glucitol (48.34%), 1,4,5-tri-acetyl-2,3,6-tri-*O*-methyl-glucitol (17.21%), 1,4,5,6-tetra-acetyl-2,3-di-*O*-methyl-glucitol (16.27%), 1,5-di-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol (18.18%). These results indicated that (1→6)-linked, (1→4)-linked, (1→4,6)-linked and terminal-glucopyranosyl residues were present in the glucan in a molar ratio of nearly 3:1:1:1. The GC analysis of the alditol acetates of periodate-oxidized, reduced PS showed total disappearance of sugar residues. This result confirmed the mode of linkages of the monosaccharide components present in the glucan.

The ^1H NMR spectrum (500 MHz) of the glucan at 27 °C contained four signals at 5.38, 4.95, 4.51, and 4.50 ppm for anomeric protons in a molar ratio of nearly 1:1:1:3. They

were designated as **A**, **B**, **C**, and **D** according to their decreasing anomeric proton chemical shifts. In the ^{13}C NMR spectrum, four signals were found in the anomeric region at δ 98.3, 100.2, 103.3, and 103.4 in a ratio of nearly 1:1:1:3. On the basis of HMQC spectrum the signals at δ 100.2, 98.3, 103.3, and 103.4 were assigned to anomeric carbons of residue **A**, **B**, **C**, and **D** respectively. All the ^{1}H and ^{13}C signals were assigned from DQF-COSY, TOCSY, and HMQC experiments. The proton coupling constants were measured from DQF-COSY experiment. Based on the coupling constants, $J_{H\text{-}1,H\text{-}2}$ ~3.5 Hz and $J_{C\text{-}1,H\text{-}1}$ ~170 Hz the residues **A** and **B** were established as α-anomer. Large coupling constants $J_{H\text{-}2,H\text{-}3}$ ~10 Hz and $J_{H\text{-}3,H\text{-}4}$ ~10 Hz indicated that both were glucosyl moiety. In residue **A**, all carbon chemical shift values matched with standard values of methyl glycosides. So it was non-reducing end α-D-glucopyranosyl residue. On the other hand, in residue **B** the downfield shift of C-4 (δ 76.4) with respect to standard value of methyl glycoside indicated that residue **B** was (1→4)-linked α-D-glucopyranosyl moiety. Residue **C** and **D** were established as β-anomer from coupling constant values $J_{H\text{-}1,H\text{-}2}$ ~8.5 Hz, and $J_{C\text{-}1,H\text{-}1}$ ~160 Hz. Coupling constant values of $J_{H\text{-}2,H\text{-}3}$ (~10.0 Hz) and $J_{H\text{-}3,H\text{-}4}$ (~9.5 Hz) revealed that both were glucosyl moiety. The downfield shift of C-4 and C-6 of residue **C** indicated that it was (1→4,6)-linked β-D-glucopyranosyl moiety. The downfield shift of C-6 of residue **D** indicated that it was present as (1→6)-linked β-D-glucopyranosyl residue. The linking at C-6 of both the residues, **C** and **D** was confirmed from DEPT-135 spectrum. From the above experimental data the residues [**A – D**] were found to have following linkages;

Residue A: terminal α-D-glucopyranose

Residue B: (1→4)-linked α-D-glucopyranose

Residue C: (1→4,6)-linked β-D-glucopyranose

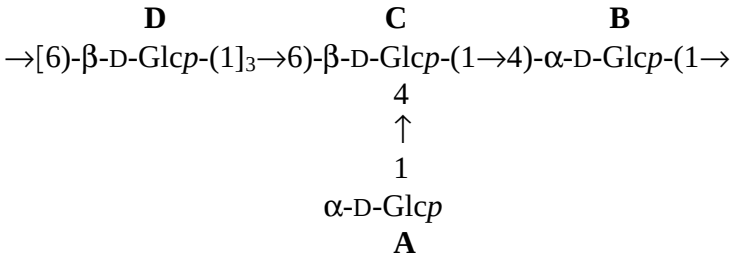
Residue D: (1→6)-linked β-D-glucopyranose

The sequence of glucosyl moieties was determined from NOESY as well as ROESY experiments followed by confirmation with an HMBC experiment. In NOESY experiment, the inter-residual contacts from **A**H-1 to **C**H-4, **B**H-1 to both **D**H-6a and **D**H-6b, **C**H-1 to

BH-4, **D**H-1 to both **C**H-6a and **C**H-6b, **D**H-1 to **D**H-6a and **D**H-6b established the following sequences;

A (1→4) **C**; **B** (1→6) **D**; **C** (1→4) **B**; **D** (1→6) **C**; **D** (1→6) **D**

The sequence was further confirmed by the HMBC experiment. The cross-peaks were found between H-1 of residue **A** and C-4 of residue **C** (**A** H-1, **C** C-4); C-1 of residue **A** and H-4 of residue **C** (**A** C-1, **C** H-4). Similarly, cross-peaks were found between H-1 of residue **B** and C-6 of residue **D** (**B** H-1, **D** C-6); C-1 of residue **B** and H-6a and H-6b of residue **D** (**B** C-1, **D** H-6a; **B** C-1, **D** H-6b). The cross-peaks were also found between H-1 of residue **C** and C-4 of residue **B** (**C** H-1, **B** C-4); C-1 of residue **C** and H-4 of residue **B** (**C** C-1, **B** H-4). Cross-peaks were observed between H-1 of residue **D** and C-6 of residue **C** (**D** H-1, **C** C-6); C-1 of residue **D** and both H-6a and H-6b of residue **C** (**D** C-1, **C** H-6a; **D** C-1, **C** H-6b). Similarly, Cross-peaks were observed between H-1 of residue **D** and C-6 of residue **D** (**D** H-1, **D** C-6); C-1 of residue **D** and both H-6a and H-6b of residue **D** (**D** C-1, **D** H-6a; **D** C-1, **D** H-6b). Moreover, some intra residual couplings were also obtained in HMBC experiment. The HMBC and NOESY connectivities clearly supported the presence of hexasaccharide repeating unit in the PS isolated from the hybrid mushroom PCH9FB as;



Macrophage activation of the glucan was observed in vitro. On treating different concentrations of the glucan an enhanced production of NO was observed with maximum production of 11.8 µM NO per 5×10^5 macrophages at 100 µg/mL of the glucan. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the glucan by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. Activation of splenocytes and thymocytes is an indicator of

immunoactivation. The splenocyte and thymocyte activation index were found maximum at 25 µg/mL of the glucan with reference to the standard LPS. Hence, 25 µg/mL of the glucan can be considered as efficient splenocyte and thymocyte stimulator. From these observations it was clear that this glucan can act as an efficient immunomodulator.

A maximum of 69.17 % DPPH radical scavenging activity was observed at 8 mg/mL of the glucan (PS). The EC_{50} value of the PS for DPPH radicals was 4.0 mg/mL. The scavenging activity of the PS increased steadily from 2-8 mg/mL, while it reached a maximum plateau from 0.5 to 2 mg/mL for ascorbic acid.

Chapter-V: This chapter illustrates the structural characterization and study of immunoenhancing property of a water-insoluble glucan isolated from the alkaline extraction of the fruiting bodies of somatic hybrid mushroom PCH9FB. The hot alkaline extract of fruit bodies of somatic hybrid mushroom was cooled, centrifuged, and precipitated in ethanol. The residue was dialyzed until alkali free, centrifuged, and freeze-dried to yield 395 mg of PS-I (water soluble) and 850 mg of PS-II (water insoluble) crude polysaccharides. The PS-I was purified and presented in chapter IV. The molecular weight of the PS-II determined from a calibration curve prepared with standard dextrans as ~1.69 × 10^5 Da. Paper chromatographic analysis of the hydrolyzed PS-II was performed and showed only one spot of glucose. The presence of only glucose was further confirmed by the GC analysis of alditol acetates of the PS-II. Thus, above experiments confirmed that it was a glucan. The absolute configuration of the sugar units was determined as D according to Gerwig et al. The glucan was methylated according to the method of Ciucanu and Kerek. The GC and GC-MS analysis of the alditol acetates of the methylated product showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol (21.41%), 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-glucitol (61.30%), and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-glucitol (17.29%) in a ratio of nearly 1:3:1, respectively. These results indicated the presence of terminal, (1→3), and (1→3,6)-linked glucopyranosyl moieties, respectively. Further, GC-MS analysis of the alditol acetates of the periodate-oxidized, reduced, methylated polysaccharide showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-

glucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-glucitol in a ratio of nearly 3:1. The absence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol indicated that the terminal glucopyranosyl moiety was consumed during oxidation. This result confirmed the mode of linkages of the monosaccharide components present in the glucan.

The ^{1}H NMR spectrum (500MHz) of water-insoluble glucan at 30 °C in DMSO-d_6 showed four signals at 4.20, 4.49, 4.50, and 4.51 ppm for anomeric protons in a molar ratio of nearly 1:1:1:2. They were designated as **A**, **B**, **C**, and **D** according to their increasing anomeric proton chemical shifts. The ^{13}C NMR spectrum (125MHz) of the following glucan at 30 °C in DMSO-d_6 showed only one anomeric signal at 103.4 ppm. All the residues were established as β-anomer from coupling constant values $J_{\text{H-1, H-2}}$ ~8.5 Hz, and $J_{\text{C-1, H-1}}$ ~160 Hz. All the proton chemical shifts were assigned using DQF-COSY, TOCSY, and HSQC experiments. The C-6 linkage was confirmed from DEPT-135 experiment. The proton coupling constants were measured from DQF-COSY experiments.

The HSQC spectrum having C1/H1 cross-peaks at 103.4/4.20 ppm were assigned to the terminal unit **A** as reported earlier. All values from C-2 to C-6 of residue **A** corresponded nearly to the standard values of methyl glucoside. The C-1/H-1 cross-peaks at 103.4/4.49, 103.4/4.50, and 103.4/4.51 ppm were designated as **B**, **C**, and **D** and assigned to (1→3,6)-, (1→3)-, and another two (1→3)-β-D-glucopyranosyl moieties, respectively. The signal at 68.9 ppm of residue **B** was due to α-effect of glycosylation and assigned to C-6 of branched (1→3,6)-β-D-glucosyl moiety which corresponded to its proton peaks at 3.50 and 4.11 ppm in HSQC spectrum. The C-6 linkage was further confirmed from DEPT-135 experiment which showed the complete disappearance of the signal at 68.9 ppm but no appreciable downward displacement of the peak was observed in comparison with free C-6 of residues **A**, **C**, and **D**. Residue **B** showed upfield shift of C-5 carbon at 75.1 ppm compared to standard value (76.8 ppm) of methyl glucoside due to β effect at C-6 linking. The upfield chemical shifts of C-2 (73.0, 73.1, and 73.5 ppm) and C-4 (69.1 ppm) for residue **B**, **C**, and **D**, respectively were for C-3 linking. The peaks appearing at 86.4(X), 86.8(Y), and 87.4(Z) ppm in ratio of nearly (1:2:1) corresponded to C-3 linkages of **B**, two **D**, and one **C** residues indicating that the main chain consisted of four units. Since, **B** is the

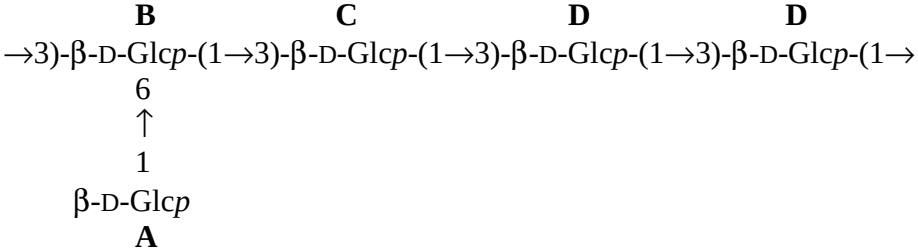
most rigid part of the backbone of the present glucan, its C-3 signal (86.4 ppm, X) appeared at the upfield region in comparison with the C-3 values of **C** and two **D** residues. The C-3 value (87.4 ppm, Z) of **C** residue attached glycosidically appeared 0.6 ppm downfield with respect to the two **D** residues (86.8 ppm, Y) due to the neighbouring effect of rigid part **B**. From the above experimental data the residues [**A – D**] were found to have following linkages;

Residue A: terminal β-D-glucopyranose
Residue B: (1→3,6)-linked β-D-glucopyranose
Residue C: (1→3)-linked β-D-glucopyranose
Residue D: (1→3)-linked β-D-glucopyranose

The sequence of glucosyl moieties was also determined from NOESY as well as ROESY experiments. In NOESY experiment, the inter residual contacts of **A**H-1/ **B**H-6a, **B**H-6b; **B**H-1/**C**H-3, **C**H-1/**D**H-3, **D**H-1/**B**H-3 indicated the following sequences;

A(1→6)**B**; **B**(1→3)**C**; **C**(1→3)**D**; **D**(1→3)**B**

Thus, the monosaccharide composition, methylation studies, and NMR experiments indicated that the repeating unit of the polysaccharide has a backbone of four (1→3)-linked β-D-glucopyranosyl units where one of them is substituted at *O*-6 position with β-D-glucopyranosyl moiety. Hence, the structure of the main repeating unit is established as:



The glucan was found to activate the macrophages. The stimulation of macrophages by glucan was studied by NO production in culture supernatant in vitro. On treating different concentrations of the glucan an enhanced production of NO was observed with maximum production of 13.6 µM NO per 5×10^5 macrophages at 100 µg/mL of the glucan. Activation of splenocytes and thymocytes is an indicator of immunoactivation. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the glucan by the MTT method. The splenocyte and thymocyte activation index were found maximum at 10 µg/mL of the glucan with reference to the standard LPS. Hence, 10 µg/mL of the glucan can be considered as efficient splenocyte and thymocyte stimulator. From these observations it was clear that this glucan can act as an efficient immunostimulator.