

<http://www.pjbs.org>

**PJBS**

ISSN 1028-8880

**Pakistan  
Journal of Biological Sciences**

**ANSI***net*

Asian Network for Scientific Information  
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

## Exopolysaccharides Production by *Pleurotus pulmonarius*: Factors Affecting Formation and Their Structures

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**Abstract:** The optimization of exopolysaccharides (EPS) produced by *Pleurotus pulmonarius* was carried out. Malt extract at 1.5 % (w/v) showed the highest EPS production after 10 days of submerged incubation and initial pH 5.5. 5.5% (w/v) glucose and 0.4% (w/v) potassium nitrate as sole carbon and nitrogen sources were the best stimulators for EPS production respectively. Suitable incubation temperature was found to be 30°C. At the end of these series of experiments we have obtained (280 mg/100 ml) of EPS. The physicochemical characterization of EPS was studied and the structure was confirmed by the infrared spectroscopic analysis (IR).

**Key words:** Exopolysaccharides structure, production, *Pleurotus pulmonarius*

### INTRODUCTION

During the past three decades, many polysaccharides and polysaccharides-protein complexes have been isolated from fungi, algae, lichens and plants<sup>[1]</sup>. Furthermore, most of them which have various physiological activities were originated from fungi, especially mushrooms<sup>[2-5]</sup>.

Mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical products. In particular and most importantly for modern medicine, they represent an unlimited source of polysaccharides with antitumor and immunostimulating properties. Many Basidiomycetes mushrooms contain biologically active polysaccharides<sup>[6]</sup>.

*Pleurotus* species are promising as medicinal mushroom, exhibiting hematological, antiviral, antitumor, antibiotic, antibacterial and immunomodulating activities<sup>[5]</sup>.

The production of exopolysaccharides is sensitive to many factors such as temperature, pH, incubation periods, carbon sources and nitrogen sources<sup>[7-10]</sup>.

This work describes the best physical and chemical factors for production of exopolysaccharides by *P. pulmonarius* and the chemical structures of these polysaccharides.

### MATERIALS AND METHODS

**Inoculum:** *Pleurotus pulmonarius* was supplied from Consultative Commet Company of mushroom cultivation-

Egypt (CCCM). It was maintained on Potato Dextrose Agar (PDA) slopes at 5°C and is regularly subcultured at three months intervals.

#### Fermentation media

**Barley extract medium:** 100 g barley grains boiled in water bath for 30 min. and filter then complete to 1 L.

**El-Naghy medium:** El-Naghy *et al.*<sup>[11]</sup> (g/100 ml): Glucose, 1.0; NaNO<sub>3</sub>, 0.2; yeast extract, 0.05; KH<sub>2</sub>PO<sub>4</sub>, 0.1; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.05 and KCl, 0.05.

**Malt extract medium (g/100 ml):** Malt extract, 2.0

**Potato dextrose medium (g/100 ml):** Potato, 20 and Dextrose, 2.

**Peptone yeast extract medium:** Wang *et al.*<sup>[12]</sup> (g/100 ml): Glucose, 2.0; peptone, 0.2 and yeast extract, 0.6.

**Potato carrot medium (g/100 ml):** Grated potato, 2.0 and grated carrot, 2.0.

**Sabaroud's dextrose medium (g/100 ml):** Dextrose, 4.0 and peptone, 1.0

**Shieh and War medium:** Shieh and Ware<sup>[13]</sup> (g/100 ml) Glucose, 1.0; malt extract, 0.3; yeast extract, 0.3 and peptone, 0.05

**Culture process and exopolysaccharides production:**

0.7 mm diameter agar discs were cut with a sterilized cork borer from the edge of 7 days old culture grown on PDA plates at 28°C. 4 discs are used to inoculate 100 ml liquid media in 500 ml Erlenmeyer flask. Autoclaving was carried out at (15 p.s.i.; 121°C) for 10 min, pH was initially adjusted to 6 using 1N HCl or 1N NaOH for all media. Dry weights of both EPS and biomass were determined after 7 days incubation periods. Studies on of the effect of factors affecting EPS and biomass production by *P. pulmonarius* are studied using shaking flask culture on rotary incubator shaker (LAB-LiNe<sup>®</sup>- Incubator-Shaker) at 150 rpm and 33±2°C in 500 ml flask containing 100 ml of the medium. Biomass dry weights were determined by drying the mycelial mat with a filter paper at 80°C for 48 h and weigh again.

**EPS quantification:** Samples from shake flasks were separated and concentrated to a small volume (10-15 ml) using a rotatory evaporator under reduced pressure at 40°C using vacuum pump according to Gao *et al.*<sup>[14]</sup>. The EPS was precipitated from the supernatant by addition of one volume of 95% (v/v) aqueous ethyl alcohol. The mixture is agitated during addition of alcohol to prevent local high concentration of the precipitate and left over night at 4°C before being centrifuged at 7000 rpm for 20 min. The precipitate was collected on a preweighed membrane filter (0.7 µm) pore size and dried at 80°C to constant weight. The dry weight of mycelium was measured after repeated washing of the mycelial pellets obtained and then dried to constant weight at 90°C for 12 h. EPS was extracted according to Ohno *et al.*<sup>[15]</sup>.

**Physicochemical studies:** EPS was dissolved in water at 60°C for 8 h and the residues after centrifugation could be dissolved in dimethylsulfoxide (DMSO) at 60°C. Monomer composition was determined after complete acid hydrolysis with 5 M trifluoroacetic acid (TFA) for 16 h at 100°C<sup>[16]</sup>. The hydrolysate was then analyzed for their sugar content. The identification of monosaccharides composition of EPS was determined by using a part of acid hydrolysate to react with phenyl hydrazine by the method of Wistler<sup>[17]</sup> and melting points of sugar osazone were then measured. Neutralized hydrolysate was examined by chromatography on Whatman No. 1 paper and periodates oxidation was carried out according to the method of Farag *et al.*<sup>[18]</sup>. The spectra were obtained from 1 mg of the substances and 300 mg of potassium bromide (KBr) using Matson 5000 FTIR –Spectrometer. Total carbohydrates were determined as anhydroglucose by the phenol sulfuric acid method according to Dubois *et al.*<sup>[19]</sup>.

**Protein estimation:** Protein content in the crude EPS preparation was determined by the method of Bradford<sup>[20]</sup> with bovine serum albumin (BSA) as the standard.

**RESULTS**

The effect of different types of media showed that among the eight tested media, malt medium stimulated the highest yield of EPS (14.5 mg/100 ml) and peptone, yeast extract medium supported the highest biomass yield which was 1767 mg/100 ml (Table 1). However, the effect of different malt concentrations showed that the maximal EPS yield (16 mg/100 ml) was achieved only at 1.5% malt extract which wasn't significantly different (P< 0.05) from 2% malt concentration and any further increase in malt concentration cause reduction in EPS production. The maximal biomass yield (1010 mg/100 ml) was achieved at 3% malt concentration (Fig. 1).

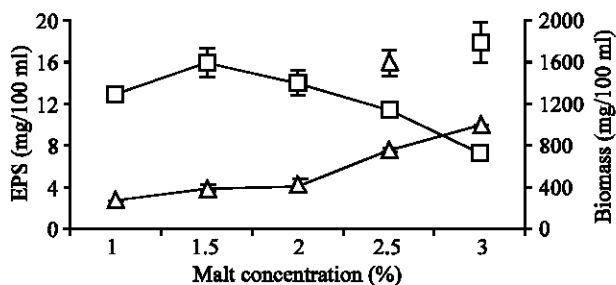


Fig. 1: Effect of different malt concentrations on EPS and biomass production. Values are shown as mean of triplicate SD (mean SD). Vertical bars represent LSD at p< 0.05

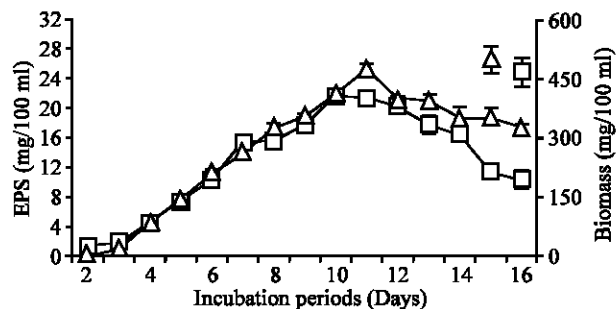


Fig. 2: Effect of different incubation periods on EPS and biomass production. Values are shown as mean of triplicate SD (mean SD). Vertical bars represent LSD at p< 0.05

The maximum EPS yield (20 mg/100 ml) was achieved at the 10th day of incubation period (Fig. 2). EPS production from the periods of 8th to 14th day hadn't any significant difference (P<0.05) from that of the 10th day.

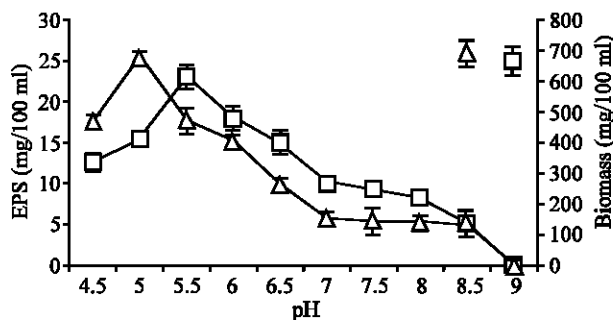


Fig. 3: Effect of different pH values on EPS and biomass production. Values are shown as mean of triplicate SD (mean SD). Vertical bars represent LSD at  $p < 0.05$

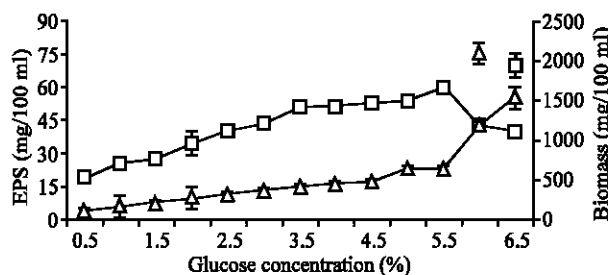


Fig. 4: Effect of different glucose concentration on EPS and biomass production. Values are shown as mean of triplicate SD (mean SD). Vertical bars represent LSD at  $p < 0.05$

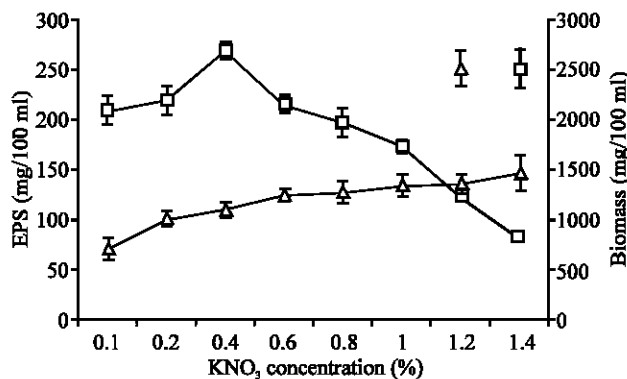


Fig. 5: Effect of different KNO<sub>3</sub> on EPS and biomass production. Values are shown as mean of triplicate SD (mean SD). Vertical bars represent LSD at  $p < 0.05$

However, within the period of the 1st to the 7th day, there was a progressively significant increase in EPS production however, from 14th to 16th day there was a highly significant decrease ( $P < 0.05$ ) in EPS production.

Maximum EPS ( $0.23 \text{ g L}^{-1}$ ) was obtained in cultures grown at final pH 5.5 and maximum biomass ( $6.78 \text{ g L}^{-1}$ ) was obtained at final pH of 5.0 (Fig. 3)

To examine the effect of carbon sources on the production of mycelial biomass and EPS, various carbon sources were provided at  $20 \text{ g L}^{-1}$  in the basal medium. As shown in Table 2, a high level of EPS and mycelial biomass was obtained when glucose and glycerol were used respectively as carbon sources.

The maximum EPS production was ( $60 \text{ mg/100 ml}$ ) at 5.5% glucose concentration then declined gradually at 6 and 6.5%. Fig. 4 showed that there was an increase in biomass with increasing glucose concentration. The maximum amount of biomass ( $1550 \text{ mg/100 ml}$ ) was recorded at 6.5% glucose concentration.

The effect of different nitrogen sources (Table 3) for the EPS production was studied. Statistically, it was clear that potassium nitrate surpassed all the nitrogen sources tested in stimulating EPS production ( $263.9 \text{ mg/100 ml}$ ), followed by asparagine and glutamic acid, which was also insignificantly better ( $p < 0.05$ ) than potassium nitrate. Peptone and yeast extract supplemented media achieved the highest biomass production followed by asparagine and glutamic acid.

The most pronounced effect on EPS production (Fig. 5) was observed with 0.4% (w/v) potassium nitrate concentration with a yield of ( $268 \text{ mg/100 ml}$ ). However within the range between 0.1 to 0.8%, there was no any significant difference between them and 0.4%. When potassium nitrate was added into the media at the level of 0.1-1.4% g N, the maximum mycelial yield increased from 706 to  $1470 \text{ mg/100 ml}$ .

The optimum temperatures for mycelial biomass and EPS production ( $280 \text{ mg/100 ml}$ ) were found at  $30^\circ\text{C}$ .

EPS extracted showed very low solubility in water. A minor portion will be dissolved only in water at  $60^\circ\text{C}$  for 8 h. The residual insoluble major portion was dissolved in (DMSO) at  $60^\circ\text{C}$  for 2 h. Both two portions were acid hydrolyzed and the hydrolyzate was reacted with phenylhydrazine phenylthya zone contains glucose, mannose and galactose. The melting points of the minor portion were in the range of (Phenylethrazone, m.p  $185\text{-}201^\circ\text{C}$ ). Also, this portion was analyzed for protein, which gave 2.8%. In addition, the major portion appeared to be constituted mostly of glucose residue (glucan) and showed (Phenylethrazone, m.p  $170\text{-}172^\circ\text{C}$ ). No protein was detected in this portion. The percentage of polysaccharide per gram crude sample was calculated to be 96.8%. The periodate oxidation data imply the presence of (1,6) and (1,4)- $\beta$  linkages. The EPS samples were oxidized and consumed 0.5 mole of periodate with production of formic acid.

Table 1: Effect of different types of media on EPS and biomass production by *P. pulmonarius*

Type of Media	EPS (mg/100 ml ± SD)	Biomass (mg/100 ml ± SD)
Barley Extract	4.6±1.0	200.5±48
El-Naghy	11.6±1.0	297±3
Malt Extract	14.5±1.5	313±18
Potato Dextrose	9.0±0.22	505±82
Peptone Yeast extract	2.4±1.0	1767±120
Potato Carrot Extract	12.5±1.6	238±3
Sabaroud's	6.1±0.6	364±163
Sheih and Ware	1.8±0.2	690.4±132

Table 2: Effect of different carbon sources on EPS and biomass production

Carbon source	EPS (mg/100 ml ± SD)	Biomass (mg/100 ml ± SD)
Arabinose	15.5±3.5	460.8±46
Galactose	12.1±2.3	4481±1.1
Glucose	49.1±8.9	676±23.6
Glucouronic	11.8±1	316±62.3
Glycerol	42.5±7.5	520.7±14.3
Lactose	10.1±1.9	333±12.5
Mannose	16±0.8	411.7±45.5
Raffinose	13.5±1.5	157±31.5
Sorbose	30.5±5.5	308.9±14.3
Starch	28.3±2	573±80
Sucrose	18.5±0.5	699±43.3
Xylose	22.5±3.5	547±40.5

Each result is the means of 3 replicate ±SD

Table 3: effect of different nitrogen sources on EPS and biomass production by *P. pulmonarius*

Nitrogen source	EPS (mg/100 ml ± SD)	Biomass (mg/100 ml ± SD)
Ammoniumchloride	118.3±0.5	269.9±27
Ammoniumnitrate	146.5±1.9	247±4.9
Ammoniumphosphate	70.3±9.7	285±13.9
Ammoniumsulfate	129.1±22	614±72
Asparagine	253.3±12	847±121
Glutamicacid	221.1±24	817.5±19.5
Peptone	146.6±18.9	1163±9.1
Potassiumnitrate	263.9±31	635±63
Sodiumnitrate	49.4±9	636±88
Urea	61.4±8.5	134±7

Each result is the mean of three replicate ± standard deviation (±SD)

The infra red spectrum (IR) of the crude polysaccharide sample showed the band at 1000-1100  $\text{cm}^{-1}$  which are characteristic to glucan. The list of the bands at 750-950  $\text{cm}^{-1}$  intervals is present. In addition, the spectra showed the band around 890, 950, 1040 and 1150  $\text{cm}^{-1}$  revealed the (1,3)- $\beta$ -glucan linkages in addition to the bands in the region of 1375, 1255 and 1205  $\text{cm}^{-1}$ . Furthermore, small amounts of proteins are present (band at 1535  $\text{cm}^{-1}$ ).

## DISCUSSION

The growth of *P. pulmonarius* on submerged culture give rise to potential advantages of higher mycelial production in a compact space and shorter time with lesser chance of contamination<sup>[21,22]</sup>. The choice of mild

agitation rate of 150 rev/min is based on the fact that it was related to the compact morphological characterization at this adequate agitation intensity which in turn reflect the highest yield of EPS production. This observation was noticed latter by Park *et al.*<sup>[23]</sup> who concluded that the compactness of the pellets was found to be the most critical parameter affecting EPS biosynthesis and more compact pellets were formed at 150 rev/min with maximum EPS production by *Cordyceps militaris*.

High level of EPS production was achieved in malt extract medium. Kim *et al.*<sup>[10]</sup> obtained a maximum EPS production in potato malt peptone media for culture of various edible mushrooms they tested and reported that it may be due to presence of malt extract. It is interesting to note that the biomass yield in malt medium wasn't the best, however, Friel and McLoughlin<sup>[22]</sup> reported that good mycelial growth of *Agaricus bisporus* was enhanced by malt extract which was the key component in potato malt peptone medium they used. These results indicated that good mycelial growth seems not to be a determining factor for high production yield of EPS in *P. plumonarius*. The same conclusions have been detected by Sone *et al.*<sup>[24]</sup> for *Ganoderma lucidum*, (1995) and Kim *et al.*<sup>[10]</sup> for *Phellinus linteus*.

The results of incubation periods indicated that the production of EPS increased gradually to reach its maximal accumulation at the 10th day of incubation. The production of EPS increases with biomass suggesting that, it may be produced constitutively. After few days following the onset of EPS production, its level in the culture medium declined may be due to the secretion of  $\beta$  1,3 glucanases which was reported by Bes *et al.*<sup>[25]</sup> and Burns *et al.*<sup>[26]</sup> for the culture of *Phanerocheates chrysosporium* and *Plerutous florida*, respectively. Seviour and Hensegen<sup>[27]</sup> had shown that PS production by *Acremonium diospyri* was parallel with biomass over the first 10 days then ceased. Kim *et al.*<sup>[10]</sup> recorded 9 days for *Paecilomyces sinclairii* EPS production and 11 days for maximal biomass production which accord well with our work.

The pH value of the culture medium is a vital factor governs the production process of EPS. *P. plumonarius* appear to be able to grow over a wide range of pH values from 4.5-8.5 and showed that the pH optima for both EPS and biomass production are quite distinct. The results are similar to those reported for other EPS synthesizing fungi such as *A. pullulan*<sup>[28]</sup>, *Sclerotium gluconicum*<sup>[29]</sup> for which the pH optimum for growth has been shown to be lower than that for EPS synthesis. Also, pH 6 and 6.5 were optima for maximal EPS production by *Aspergillus flavipes* and *Aureobasidium pullulans*, respectively<sup>[30,31]</sup>.

*P. plumonarius* proved capabilities to use a large number of sugars, all of which stimulated PS production. Among 9 carbon sources screened in this study, glucose was the preferred carbon source, which cause production of maximal amounts of EPS. Our results were in agreement with, Michel *et al.*<sup>[32]</sup> and Burns *et al.*<sup>[26]</sup> by the culture of *Epicoccum purpurascens* and *Pleurotus florida*, respectively. However, sucrose stimulated the greatest biomass yields in *P. plumonarius*.

In the present investigation, we came to the conclusion that the profile of EPS production was not usually consistent with that of mycelial growth of *P. pulmonarius* for the same carbon source. Sucrose has been reported to stimulate EPS production in many studies<sup>[8,10,33]</sup>. In addition, mannose stimulated PS production by *Acremonium diospyri*<sup>[27]</sup> and ethanol as carbon source is used by Hamdy *et al.*<sup>[8]</sup> for PS production by *Fusarium accuminatum*.

Although, the mycelial growth was proportionally increased with the initial glucose concentration within the range from 1 to 6 g/100 ml, the EPS production wasn't significantly increased above 5.5 g/100 ml. These results were similar to the observation of Kim *et al.*<sup>[10]</sup> for culture of *Paecilomyces sinclairii*. In the present study, *P. pulmonarius* followed the trends reported by Sarkar *et al.*<sup>[35,36]</sup> for other filamentous fungi in that an initial rise in EPS with increasing glucose concentration, followed by a reduction in EPS at high glucose level.

The results indicated that the carbon source can be utilized to improve the production yield of EPS and that good mycelial growth seems not to be a determining factor for high production yield of EPS in *P. pulmonarius*. Similar results were observed in *Phellinus linteus*<sup>[2,10]</sup>.

In studying of the effect of different nitrogen source on EPS and biomass production, it has been found that EPS yield was very dependent upon the type and the amount of nitrogen source provided for the organism. Potassium nitrate has been recorded as the best nitrogen source for EPS production by *P. pulmonarius*. These results were in a good accordance with the result obtained by Seviour and Kristiansen<sup>[37]</sup> for *Acremonium pullulan*, Michel *et al.*<sup>[32]</sup> for *Epicoccum purpurascens* and Stasinopoulos and Seviour<sup>[38]</sup> for *Acremonium persicinum*.

Nitrate also supported a considerable growth for *P. plumonarius* as nitrogen source. *Pleurotus Sajor-caju* and *Volvariella volvacea* were suggested to use nitrate as nitrogen source<sup>[39,40]</sup>.

All the literature agreed with the fact that EPS production occurred in N-limiting condition rather than C-limiting condition<sup>[26,41]</sup>. The mechanism of N-limiting

condition by Potassium nitrate can be explained on the basis of  $\text{NH}_4^+$  mechanism described in accordance with Seviour and Kristiansen<sup>[37]</sup> that the initial nitrate concentration appears to affect both the productions of biomass and EPS. The final biomass yield increases with the inlet nitrate and is considered to be a result of carbon accumulation by the cell. In addition, an increase in nitrate concentration enhances carbon flow into biomass formation with a corresponding decrease in PS level.

EPS biosynthesis varied considerably with the change of incubation temperature. The optimum temperature range of *P. plumonarius* EPS was found to be 30°C. This was consistent well with the finding of Hosni<sup>[42]</sup>, Zervakis *et al.*<sup>[9]</sup>, El-Fallal *et al.*<sup>[43]</sup> for growth and *P. pulmonarius* and Kim *et al.*<sup>[44]</sup> for growth and EPS production by *P. pulmonarius* and *Paecilomyces sinclairii*, respectively. Other workers have reported optimum temperature for growth of *Phellinus linteus* as 25-30°C<sup>[45]</sup>.

The complete characterization of this EPS was very difficult due to the insolubility of this crude polysaccharide. This problem was also described by Gutierrez *et al.*<sup>[16]</sup> who also expected that the insolubility of these polysaccharides be related to their high molecular weight (over 1000,000). Also, they showed that these polysaccharide fractions includes two portions a minor water-soluble fraction and this fraction is  $\alpha$ -(1-4)-linked glucose units and the structure of the second portion (which more than 80% of the total polysaccharide) appeared to showed  $\beta$ -(1-3)-linked glucose units.

The infrared spectroscopy has become a standard method for the analysis of extracted fungal polysaccharide<sup>[16,27,46]</sup>. Kacurakova *et al.*<sup>[47]</sup> found that the region between 1000-1500  $\text{cm}^{-1}$  in IR is really a carbohydrate "fingerprint" region by which each class of polysaccharides can be recognized. The list of the bands at 750-950  $\text{cm}^{-1}$  intervals which is assigned, to  $\beta$  and  $\alpha$  (1-3), (1-6)-glucan are characterized to the PS chart. Furthermore, bands around 890 and 1370  $\text{cm}^{-1}$  are revealed to  $\beta$ - configuration of the glucan linkages. Also, the band around 1040 is characteristically attributed to (1-3)-di-*O*-substituted glucose residues. In addition, band around 1150  $\text{cm}^{-1}$  is due to glucose (1-4)-di-*O*-disubstituted. The above assignments agree with those presented by other authors for other types of polysaccharides<sup>[46,48]</sup>.

There are many probabilities for oxidation the polysaccharide by periodate, we can find that only one-probability (1-6) glucan which produce formic acid and the (1-3)-linked glucan is extremely resistant to oxidation by periodate. From present results, the oxidation of

polysaccharide isolated from *P. pulmonarius* and detection of the formic acid can expect the presence of (1→6)-linked glucan. Based on the evidence described above, it can be concluded that the EPS of *P. pulmonarius* contain a backbone and side chain involving of β-1, 3 and β-1, 6 linked glucan.

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