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Biological Activity of Extracellular and Intracellular Polysaccharides from *Pleurotus tuber-regium* Hybrid and Mutant Strains

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Abstract *Pleurotus tuber-regium* (Fr.) Singer (1951) is a unique sclerotium-forming edible and medicinal mushroom. Interestingly, both the sclerotium and mushroom are edible and are often used for curing various ailments. Previous studies have focused on the antimicrobial and antioxidant activity of the extracellular polysaccharide (EPS) from wild *P. tuber-regium*. There has been no report on the intracellular polysaccharide (IPS) of the wild mycelia, likewise there is very meager information on the improvement of the perceived potentials of *P. tuber-regium*. This research study analysed the EPS and IPS fractions of *P. tuber-regium* hybrid and mutant strains. The antimicrobial potential of the IPS and EPS fractions, their scavenging activity on 1, 1-diphenyl–2picryhydrazyl (DPPH) and Hydroxyl radicals were also determined. Both IPS and EPS fractions of *P. tuber-regium* hybrids and mutants showed increased DPPH and hydroxyl scavenging activity over the wild *P. tuber-regium* with an EC_{50} mostly < 1 mg/ml. The antimicrobial activity of the IPS from a mutant strain had an IC_{50} of 15.6 mg/ml compared to the wild type (18.75 mg /ml). This study showed that selected mutant and hybrids of *P. tuber-regium* had increased radical scavenging activity indicating potentially increased biological activity that could offer increased benefit as a neutraceutical.

Keywords: *antioxidant, antimicrobial, Pleurotus species, sclerotium, medicinal mushrooms, Lentinus tuber-regium, neutraceutical*

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1. Introduction

The exposure of human cells to reactive oxygen species (ROS) is almost continuous, as they are constantly produced endogenously in the human body via normal metabolism of aerobic cells. Some of the free radicals in cells include; superoxide anion, hydroxyl radical, non-radical molecules like hydrogen peroxide and singlet oxygen. At high concentrations, ROS are extremely dangerous to living organisms; they are however neutralized by cellular antioxidant defenses, either enzymatically or non-enzymatically. In the event of loss of activity by these regulators, the uncontrolled production of these free radicals has been linked to disease conditions such as rheumatoid arthritis, cancer, diabetes, cardiovascular diseases and many others [15]. They are known to be the major cause of various chronic and degenerative diseases including; coronary heart disease, inflammation, and stroke. Hydroxyl radicals on the other hand are responsible for aging, tissue damage and lipid peroxidation, and could influence the onset of some degenerative diseases [2].

Previous studies established that mushrooms are quite elaborate in their radical scavenging activity. Exopolysaccharide fractions were characterized by Zheng *et al*., [11] from submerged fermentation of *Boletus aereus* and attributed the highest antioxidant activity recorded in one of the fractions to its molecular weight and monosaccharide composition. The antioxidant activity of *Pleurotus* HK 37 was studied by Muthangya *et al*., [15] and detected a EC_{50} of less than 0.09 mg/ml and it was reported that the activity seems to depend on the substrate type used for cultivation. Sun et al.,2013 showed that the radical scavenging activity of the EPS from *P. eryngii* at 400 mg/ml were 59.3 %, 38.69 % and 66.36 % on hydroxyl, super oxide anion and 1,1 – diphenylhydrazyl (DPPH) radicals respectively. The extracts of *Termitomyces clypeatus, T. robustus, Lentinus subnudus* and *Lenzite species* had a comparable scavenging effect on hydroxyl radicals as butylated hydroxytoluene (BHT) used as control. It was also observed that these extracts were able to inhibit the growth of indicator organisms at concentrations between 12.5 mg/ml to 100 mg/ml [18]. Adebayo et al., [1] reported that most antioxidant and antimicrobial activities exhibited by fungi, especially Pleurotus spp can be linked to phytochemicals.

Pleurotus tuber-regium (Fr.) Singer (1951), is an edible mushroom, rich in protein and medicinal. It belongs to the Basidiomycotina, mainly distributed in tropical and subtropical regions such as China, Australia, and Africa, especially Nigeria [17,24] and Ghana (Dzomeku, 2009). It was formally placed in the genus *Lentinus*, but more recently has been placed in the genus *Pleurotus*. *P. tuber-regium* is the only species, whether Pleurotus (Isikhuemhen and Nerud, 1999) or *Lentinus* in which the fruiting bodies arise from a sclerotium. This fungus is unique, with three morphologically different life forms viz; fruiting body, sclerotium and mycelia. The fruiting body has a typical scaled, infundibuliform pileus with a well-developed stipe, and decurrent gills [16]. Tissues are dimitic, composed of both generative and thick-walled skeletal hyphae [19]. Spores produced are cylindrical, smooth, and hyaline in appearance. The sclerotia of *P. tuber-regium*, a tuber-like structure is used by the fungus as a means to store food products and also gives rise to new fruiting bodies [16]. These sclerotia are characteristically dark brown with a white interior, spherical to ovoid in form. These structures enable P. tuber-regium to survive periods of drought, and support the fruiting of multiple basidiocarps over several growing seasons.

Each of these morphologically distinct forms has been associated with various bioactivity. The hot water extract of both mycelia and sclerotia had been shown to have strong in-vitro and in-vivo antitumor activities against HL-60 tumor cell culture and Sarcoma 180 solid tumor in BALB/c mice respectively [14]. The study of Huang et al., 2012 demonstrated the antihyperglycemic, antihyperlipidemic and antioxidant properties of *P. tuber-regium* extracellular polysaccharide in diabetic rat model and inferred that the EPS are medicinally important. Wu et al., [7] reported that water and alkali extract of *P. tuber-regium* sclerotium had excellent superoxide radical scavenging activity. Most of the previous research on this organism had focused on extracts from the sclerotium and fruiting body of this organism. To our knowledge, there has been no report on the antioxidant and antimicrobial potential of its biotechnologically improved variants. This present study evaluates the antioxidant and antimicrobial activity of the wild, mutant and hybrid strains of *P. tuber-regium*.

2. Materials and Method

P. tuber-regium sclerotium used for the induction of fruiting body was obtained from Oyo state, Nigeria. Tissue culture protocol was employed to obtain the mycelia isolates of *P. tuber-regium* (Pw) from its fruiting body. The DNA was extracted using established protocols and sequenced (GenBank Accession number - KP325382).

2.1. Hybridization and Mutation

Hybrids were formed via the anastomosis fusion of dikaryons. Briefly, the pairing was performed in 90 mm Petri dishes containing 20 ml of PDA, inoculated with 5 mm agar plug from actively growing (7 days culture) strains. The agar plugs of the monocultures of *P. tuber-regium* strains and other *Pleurotus* species (*P. sajor caju*: MBA-2345 *and P. pulmonarius*: MBA-2204) were placed 30 mm apart and incubated at 28°C until a well developed contact zone at juncture was established (10 days). A strip of mycelia was cut from the juncture and subcultured on fresh PDA plates. The hybrid formed was observed microscopically to confirm presence of anastomosis and clamp connections. An exponentially growing culture of *P. tuber-regium* was exposed to UV light with wavelenght 234 nm, for 180 mins. Mutants were selected at 30 mins interval and subcultured on MEA.

2.2. Exopolysaccharide Production and Purification through Submerged Fermentation

The medium used for the submerged cultivation of the wild, hybrids and mutants was constituted using yeast extract, 0.5 g/l; glucose, 20 g/l; MgSO4.7H2O, 0.6g/l; KH2PO4, 1.0g/l with the pH adjusted to 6.5. Mycelia plug of 5 mm was aseptically inoculated into each of the culture medium for the wild (Pw), mutant and hybrid strains, and placed in a shaker at 170 rpm for 12 days at 30°C. The mycelium was harvested from the culture broth by centrifuging at 4000 g for 20 min at 25°C (using Megafuge 1.0R Heraeus). The mycelia biomass was separated from the fermented broth using Whatman filter paper, washed with MilliQ water and dried at 60°C for 48 h. Absolute ethanol was added to the supernatant (4:1), shaken vigorously and left to precipitate overnight at 4°C. The precipitate was separated by centrifuging at 10,000 g for 30 min at 4°C (using Beckman Coulter Avanti J.E centrifuge). The crude EPS obtained was re-suspnded in MilliQ water, frozen at -80^{o}C and lyophilized (using Labconco Vacutec).

2.3. Extraction of Mycelial Intracelullar Polysaccharide (IPS)

The mycelial biomass of Pw, Pt60 mutant, 4H and 5H (hybrids) were subjected to hot water extraction at 118^{o}C for 30 minutes. Residue obtained was further suspended in 2% Na_2CO_3, incubated at 90°C for 2 h; followed by 1% ammonium oxalate (90°C, 6h) and 5% sodium hydroxide (25°C, 12h) in that order. All the supernatants were pooled together, frozen at – 80^{o}C and lyophilized.

2.4. Determination of Antimicrobial Activity of the Exopolysaccharide

In vitro antimicrobial susceptibility studies was performed using isolates obtained from the Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, Eastern Cape, South Africa. The organisms included; *Bacillus subtilis*, *Staphylococcus aureus*, *Mycobacterium aurum*, *Escherichia coli*, *Salmonella soneii*, *Pseudomonas aeruginosa*, *Fusarium sp* and *Phytophtora sp.* Antimicrobial activities of the exopolysaccharide was screened by the well diffusion and micro-dilution method.

2.5. Agar Well Method

Test microorganisms were activated in Nutrient Broth (37°C, 24 h) and spread plated unto nutrient agar for bacteria cultures while PDA (28°C, 48 h) for fungi. The crude EPS and IPS were filter sterilized using syringe filter with pore size 0.45 µm diameter. Aliquots of 50 µl standard antibacterial agents (Chloramphenicol, Vancomycin

and Ampicillin) as well as the crude extracts (100 mg/ml) were separately added to each 5 mm diameter wells cut in the agar gel. The plates were incubated at 37°C for 24 h for bacteria, and at 28°C for 48 h for fungi. Antimicrobial activity was determined by measuring the radius of the clear inhibition zone around each well. The crude extract showing inhibition potential was selected for further analysis using microdilution method.

2.6. Micro-dilution Method

The polysaccharide extract solution IPS PW, Pt60 mutant, 4H and 5H(hybrids) was prepared by suspending 50 mg/ml in MilliQ water, filter sterilized using 0.45 µm filter, and serially diluted in Nutrient broth to give different concentrations (50, 25, 12.5, 6.25, 3.125 mg/ml). Fifty microlitres of prepared extract were introduced into microplate wells in triplicates plus 50 µl of *E. coli* culture. A series of controls containing corresponding extract only were set-up and a positive control of *E. coli* suspension alone was introduced into another set of wells. A 100 µl media was dispensed in the four-angles of the outer wells to provide and maintain humidity. The microplate was incubated at 37°C for 24 h and the absorbance read at 750nm.

The percentage inhibition was calculated from the formular;

$$Inhibition(\%) = \frac{[AB - (ABE - AE)]}{AB} \times 100$$

Where AB was the absorbance of bacterial growth control only,
ABE was the absorbance of bacterial growth in polysaccharide extract,
AE was the absorbance of crude polysaccharide extract only.

2.7. Antioxidant Assay

The qualitative antioxidant assay was determined by detecting the DPPH radical scavenging activity and hydroxyl radical scavenging ability of both crude EPS and IPS fractions.

2.7.1. Measurement of Scavenging Effect on DPPH Radical

The DPPH free radical scavenging activity was determined according to Muruke, 2014, with modification. The principle of the method is on the reduction of methanolic solution of coloured free radical DPPH by free radical scavenger. A series of dilutions (up to 10^4) of the crude EPS and IPS fractions were prepared by dissolving 1 mg/ml in 1 ml of methanol. DPPH solution was prepared by dissolving 0.04 mg/ml DPPH in 50 % ethanol in microcentrifuge tubes. Next, 100 µl of DPPH solution was added to 100 µl extract solution in microtitre plate. The mixture was incubated in the dark for 30 minutes and the absorbance read at 515 nm. The percentage of DPPH radical scavenging activity of the EPS, IPS fractions and the positive control (Ascorbic acid in the same concentration as the extracts) was determined at these five concentrations in triplicates and was calculated as:

$$Scavenging\ activity\ on\ DPPH(\%) = \frac{[A0 - (A1 - A2)]}{AO} \times 100$$

Where A0 was the absorbance of the control solution containing DPPH only,
A1was the absorbance in the presence of EPS or IPS in DPPH solution,
A2 was the absorbance of the sample extract solution without DPPH.

2.7.2 Measurement of Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity of both IPS and EPS was determined by following the method of Deng et al., [5] with some modifications. Briefly, various concentrations (0.1, 0.3, 0.5, 0.7, 1.0 mg/ml) of all polysaccharide fraction and ascorbic acid (positive control) was dissolved in 60% methanol. The sample solution (50 µl) was mixed with 100 µl 0.15mM EDTA-Fe, 50 µl 2.0 mM Salicylic acid, 100 µl 6.0 mM H_2O_2 and 50 µl MilliQ water. The mixture was incubated for 30 min at 37^{o}C and the hydroxyl radical detected by monitoring absorbance at 510 nm. In the control, the sample was substituted with distilled water and H_2O_2 with sodium phosphate buffer. A blank was prepared by replacing sample and H_2O_2 with MilliQ water. The hydroxyl radical scavenging activity was expressed as:

$$Scavenging\ rate(\%) = \frac{A\,s510 - A\,control510}{A\,blank510 - A\,control510} \times 100$$

Where As was the absorbance of the sample solution,
Ac was the absorbance of the control solution,
Ab was the absorbance of the blank solution.

3. Results and Discussion

It is often desirable to improve upon existing varieties of mushroom via hybridization and mutagenesis. In this study, the mutation experiment was a success; the produced mutants were denoted Pt30, Pt60, Pt90, Pt120, Pt150 and Pt180. The sequences of these mutants had been characterized and submitted to the GenBank with molecular accession number KP325382, KP325383, KP325384, KP325385, KP325386, KP325387, KP325388 respectively. Hybridization experiment between *P. pulmonarius* and *P. sajor-caju* yield two successful hybrids, denoted as 4H and 5H respectively. EPS and IPS was successfully obtained from the culture broth and mycelial of all desired strains and were screened for biological activity. The growth vigour of the mutant mycelial and hybrid was improved compared to the wild type (data not provided).

3.1. Antimicrobial Activity of EPS and IPS

In the study of antibacterial activity, the agar well method showed that the EPS and IPS was resisted by most bacterial strains used, with only *E. coli* and *S. aureus* showing some sensitivity to only the IPS fractions (10 mm and 0.75 mm diameter respectively). Further study on this using the microdilution method revealed MIC_{50} of 15.75 mg/ml, 20.75 mg/ml, 27.0 mg/ml and 29.25 mg/ml by Pt60, Pw, 4H and 5H respectively (Figure 1) against *E.coli*. The MIC_{50} for the IPS was however higher than that obtainable for commercial antibiotics used as control,

meaning it is less effective. Chloramphenicol and Ampicillin had a MIC_{50} less than 3 µg/ml while Vancomycin had approximately 30 µg/ml. *S. aureus* was more resistant to IPS compared to *E. coli* as 80 mg/ml clears 99.6 %, 86.8 %, 85.2 % and 84.2 % by IPS 4H, Pt60, 5H and Pw strains in that order (Figure 2). It is obvious that the polysaccharide extracted from culture broth (EPS) had a very weak or no activity against the tested microorganisms. This could mean that EPS from *P. tuber-regium* wild, mutants and hybrid is probably not cytotoxic in its activity.

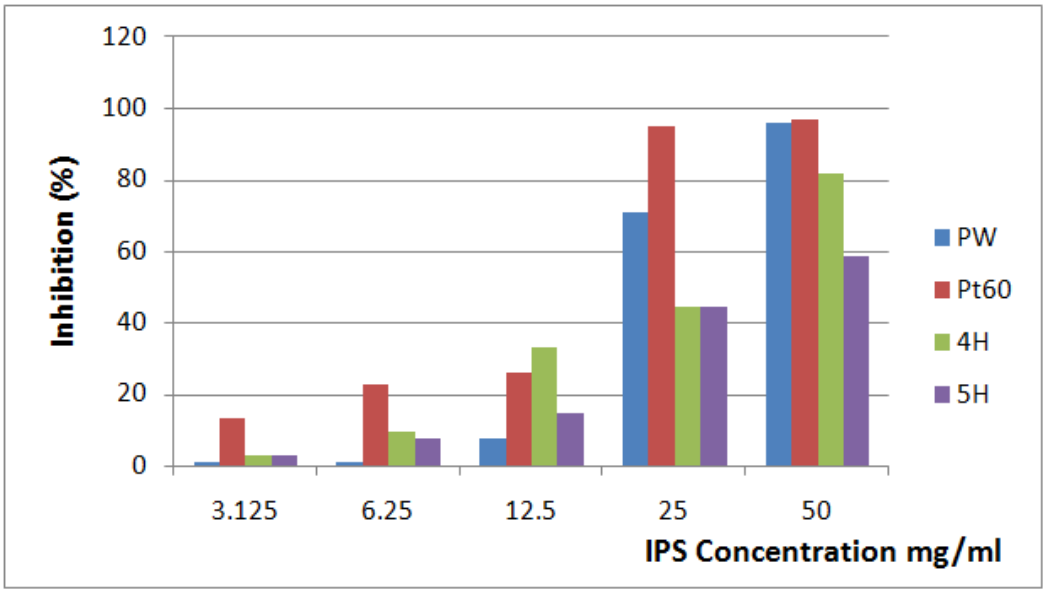

Figure 1. Inhibition by IPS (%) against *E. coli*

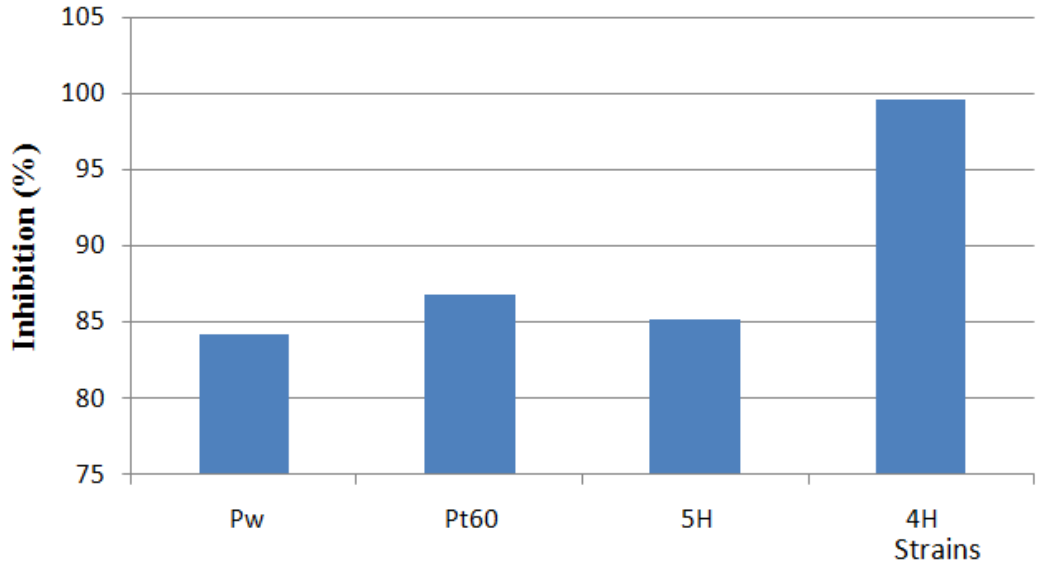

Figure 2. Inhibition by IPS (%) against *S. aureus* at 80 mg/ml

The mycelial extract (IPS) showed some inhibitory actions against few bacteria, but no inhibition against test fungi, this might be linked to the solvents used for extraction. It is well known that different biologically active components are solubilized to different extents in different solvent, meaning that the active fractions extracted by a solvent, may well vary widely from that of other solvents. The IPS from Pt60 showed an improved MIC50 of 15.75 mg/ml, which is lower compared to the wild (20.75 mg/ml). Mutagenesis has long been identified as a useful tool for strain improvement and has been applied in various improvement studies and to generate diversity in basidiomycetes [4,10,22]. However, the commercial antibiotics used in this study (Chloramphenicol, Ampicillin and Vancomycin) had better inhibitory actions than all extracts. This observation was also reported by Cilerdžic et al., [19], and concluded that *Ganoderma lucidum* extract showed promising activities.

3.2. Antioxidant Activity of EPS and IPS

3.2.1. DPPH Scavenging Activity

DPPH is a stable free radical with maximum absorbance at 517 nm in ethanol. In the presence of an hydrogen donating antioxidant, the radical is scavenged and the absorbance reduced. In this study, IPS from Pt60 and Pw had a EC_{50} of less than 0.1 µg/ml while 4H and 5H had EC_{50} of 0.1 µg/ml and 1.0 µg/ml respectively as shown in Figure 3. Wu *et al.*, 2014 extracted the sclerotium of *P. tuber-regium* with alkaline and water and

obtained EC_{50} value of 0.91 g/l and 1.51 g/l respectively. This means the biotechnologically improved variety in this study seemed to have a higher scavenging activity than the corresponding wild fungus. The EPS fractions obtained from the hybrid seems to have a higher scavenging activity compared to other strains with a EC_{50} of less than 0.1 µg/ml followed by mutants and the wild. The control, ascorbic acid consistently had the highest DPPH scavenging effect with 0.0001 mg/ml having 87% activity (Figure 4).

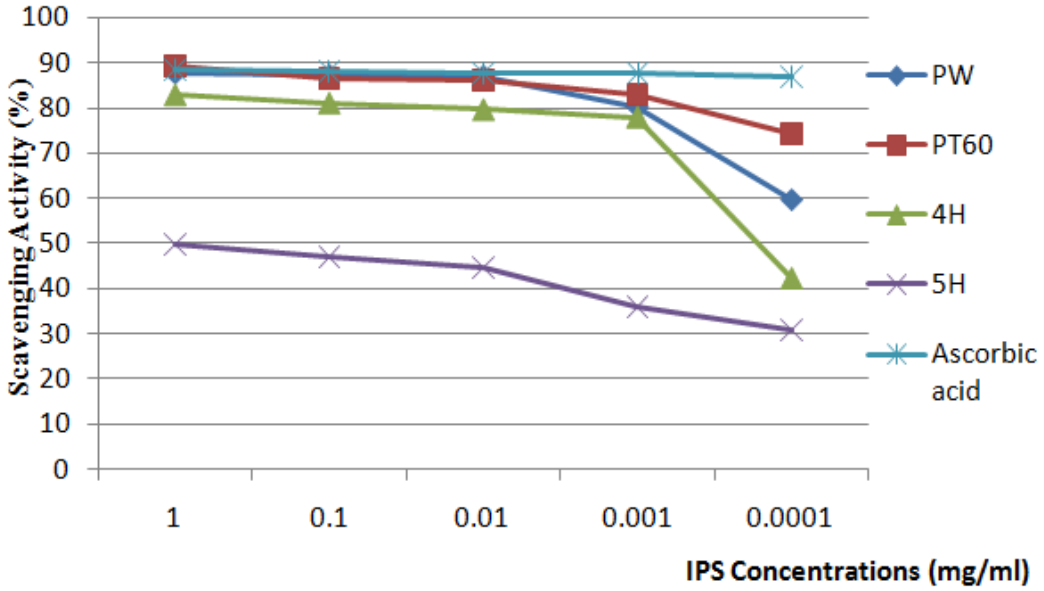

Figure 3. DPPH Scavenging activity of Intracellular Polysaccharide from Wild, Mutant and Hybrid strains of *P. tuber-regium*

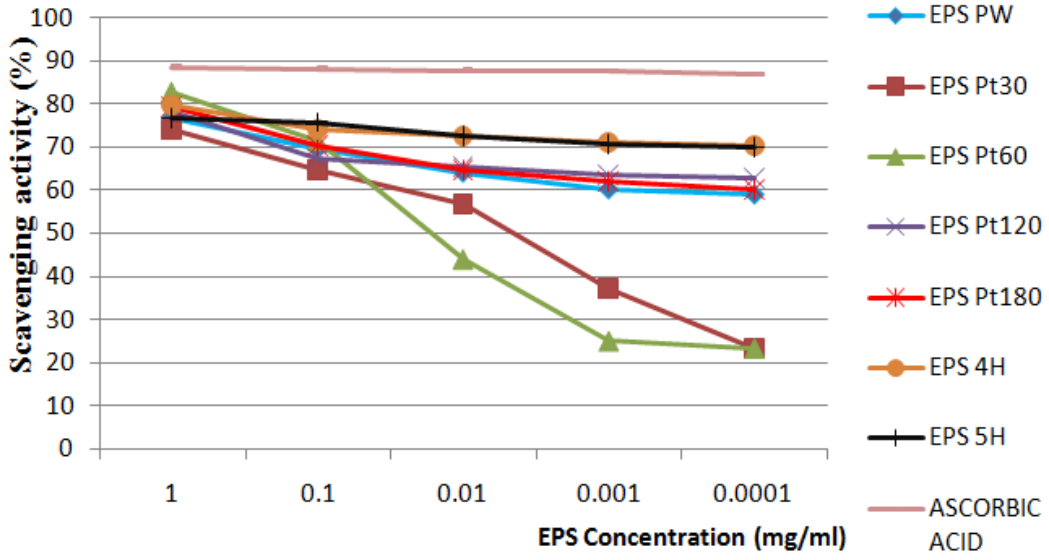

Figure 4. DPPH Scavenging activity of extracellular polysaccharide from wild, hybrids and mutants strains of *P. tuber-regium*

3.2.2. Hydroxyl radical scavenging activity

The highest hydroxyl scavenging activity for EPS fractions was noted to be one of the hybrids, 4H, with EC_{50} of 0.4 mg/ml. Figure 5 shows the overall strain activity as 4H>5H>Pt60>Pw, with a EC_{50} of 0.4, 0.5, 0.7, 0.9 mg/ml respectively. Deng et al., 2012 reported that the hydroxyl radical scavenging activity of a fungal purified polysaccharide reached 39.59 % at 1.0 mg/ml; a much higher concentration than that observed in this study. Hydroxyl radical is notably one of the most reactive oxygen species (ROS), inducing tremendous damage to adjacent biomolecules. In comparison with other ROS, it bears the shortest half life. It has been established that hydrogen peroxide molecules can produce oxidative injury in the biomolecules indirectly via the production of hydroxyl radicals in the iron-catalysed Haber-Weiss reaction. This hydroxyl radical can be effectively scavenged by antioxidants as shown in this study [20].

The EC_{50} observed for EPS fractions was lower than that of IPS, and does not seem to follow the same trend. Pt60 had the highest value of 56% at 1.0 mg/ml, closely followed by 5H, Pw and 4H (50%, 43% and 35% respectively) at the same concentration (Figure 6). Zheng *et al*., [11] reported fractions of EPS from *Boletus aereus* as having 78.3 % at 10 mg/ml; while He *et al*., (2012) reported 60.68 % for *Morchella crassipes*. The EC_{50} obtained for both EPS and IPS fractions of P. tuber-regium in this study is promisingly high. Previous reports on some extracts of *P. tuber-regium* established its antitumor potential, both in-vivo and in-vitro [14,23]. These polysaccharides were described as not having a

direct cytocidal activity, but rather via the activation of the host immune responses [23]. It is well known that antioxidants are excellent in the treatment of cancer, thus the extracts from these improved strains could be good immunomodulators with antitumor potentials.

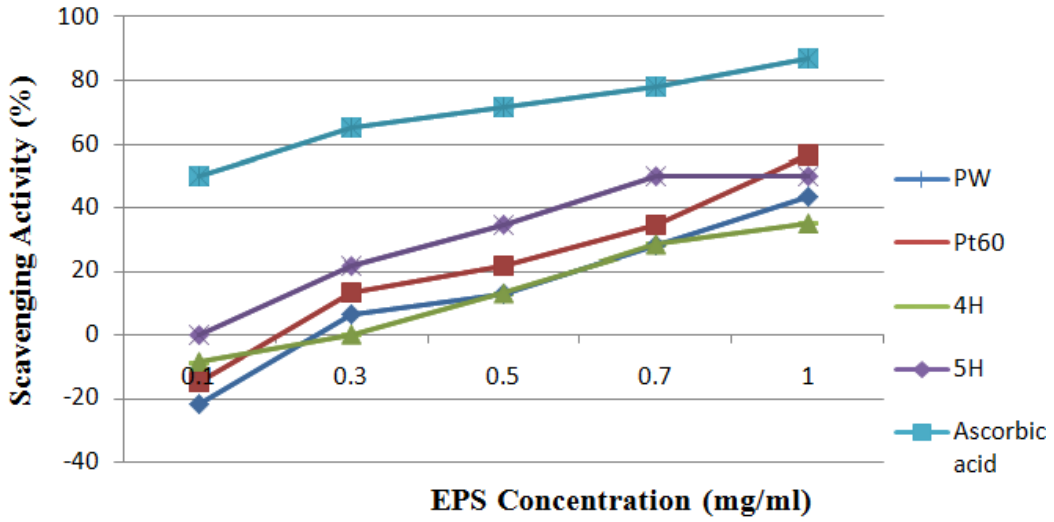

Figure 5. Hydroxyl Radical Scavenging Activity of EPS

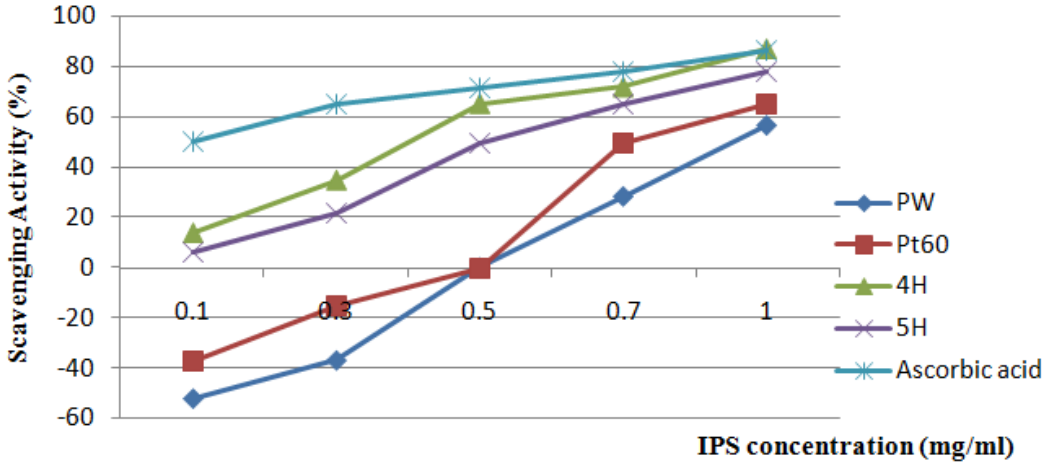

Figure 6. Hydroxyl Radical Scavenging Activity of IPS

The IPS fractions are noted to consistently have better activity than the EPS fractions except for 5H hybrid. The reason for this is not quite clear. Consumption of mushroom fruitbody has been encouraged over the years, with credence to their perceived medicinal potentials. However, there has been little concern as regards harnessing the mycelia of such edible and medicinal mushrooms. The mycelia of *P. tuber-regium* strains used in this study notably have a delightful aroma, with a baby cereal-like odour. The study by Wu et al., 2004 on the dietary fiber composition of *P. tuber-regium* mycelia showed it is a rich source of dietary fiber and polysaccharide sugars. They concluded that the mycelia had a potential of being used as food ingredient since its quality can be easily controlled and efficiently produced. A previous research in this group, produced *Lentinus* mycomeat [3] with good proximate compositions and great potential.

4. Conclusion

This research work was able to produce improved *P. tuber-regium* mutants and hybrids, with determined molecular sequence and further established its antimicrobial and excellent antioxidant activities. The biological activity of the mycelia extract in our findings, coupled to its appealing aroma showed it could be a useful inclusion in neutraceuticals or animal feed. Further work to characterize and purify the extracted fractions is ongoing.

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Highlights

- Novel P. tuber-regium hybrids and mutants were produced in this study.

- The scavenging activity of the intracellular polysaccharide fractions were found to be comparatively higher than those of the extracellular polysaccharide fractions.
- The nice aroma of these mycelia and notable biological activity make them suitable candidate for possible incorporation into neutraceutical formulations.

Abbreviations

EPS: Extracellular polysaccharide
IPS: Intracellular polysaccharide
DPPH: 1, 1-diphenyl–2picryhydrazyl
ROS: Reactive oxygen species

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