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Research article

Bioactivity assessment of exopolysaccharides produced by *Pleurotus pulmonarius* in submerged culture with different agro-waste residues

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ABSTRACT

Pleurotus spp. are white-rot fungi that utilize different agro-wastes to produce useful biologically active compounds. In this study, exopolysaccharides (EPS) were produced by *Pleurotus pulmonarius* in submerged culture supplemented with different agro-wastes. Functional groups in EPS were revealed using Fourier Transform-Infrared (FT-IR) spectroscopy. Antimicrobial activity of EPS was tested against microorganisms using agar well diffusion. Scavenging potentials of EPS was tested against 1, 1- diphenyl-2-picryhydrazyl (DPPH), hydroxyl (OH), iron (Fe^{2+}) and nitric oxide (NO) radicals. *In vitro* prebiotic activity of EPS was carried out. The highest yield (5.60 g/L) of EPS was produced by *P. pulmonarius* in submerged culture supplemented with groundnut shell (20.0 g/L). The functional groups in EPS were hydroxyl (-OH), methyl (-CH_3), ketone (-RCOH) and carbonyl group (-C=O). EPS displayed zones of inhibition (5.00–14.00 mm) against tested microorganisms. Scavenging activity of EPS ranged from 65.70-81.80% against DPPH. EPS supported the growth of *Lactobacillus delbrueckii* and *Streptococcus thermophiles* with values ranged from 3.04×10^4–3.40×10^4 cfu/ml and 2.50×10^4–2.81×10^4 cfu/ml, respectively. Submerged culture of *P. pulmonarius* with addition of agro-wastes enhanced yield of EPS. The EPS exhibited bio-functional properties like antimicrobial, antioxidant and prebiotic activities. Hence, agrowastes can be recycled in submerged fermentation with fungi to produce promising biomaterials for biopharmaceutical applications.

1. Introduction

Agro-wastes are often disposed indiscriminately and constitute a great nuisance to the environment (Sadh et al., 2018a). This has resulted to climatic variability with serious risk to human and ecological health (Anbus et al., 2017; and Ferronato and Torretta, 2019). Agro-waste residues such as peels, seeds, stones from fruits and vegetables contain indispensable chemical constituents, which can be used for the production of useful products (Sagar et al., 2018). The cultivation of microbial cells on agro-wastes (substrates) to synthesis biologically active compounds using different biotechnological innovations is gaining an interest and is of progressive boons (Sadh et al., 2018a).

Fungal mycelia have the ability to utilize complex organic compounds in agro-residues through fermentation processes and produce biomolecules, which are useful as nutritional supplements and serves as complementary medicine to prevent degenerative diseases (Gupta et al., 2019; Hyde et al., 2019). The recovery of by-products from biologically treated agro-wastes will therefore, improve utilization of agro-wastes and then minimize the problem of environmental pollution (Sadh et al., 2018b).

Agricultural wastes (lignocellulosic biomass) composed of cellulose, hemicelluloses and lignin, which can be enzymatically converted by microbes into various values added biotechnological products (Baig 2020). Plant biomass waste is therefore, a desirable alternative raw material to produce functional products since it is readily available and bio-renewable. Fungi such as *Pleurotus* spp., *Polyporus ostriformi*, *Phanerochaete chrysosporium* and many more have colonizing potentials on ligno-cellulosic materials (Singh et al., 2012). *P. pulmonarius* is an economically important edible and medicinal macrofungus that traditionally grown on different substrates namely; corn cob, corn straw, peanut straw, soybean straw, rice straw, rape straw, wheat bran and cottonseed hulls (Wu et al., 2019). Information gathered by Baeva et al. (2019) revealed that, species of genus *Pleurotus* capable of utilizing different agrowastes to produce polysaccharides. Fungal

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polysaccharides, especially EPS possess therapeutic properties like antimicrobial, antioxidant, and anticancer (Osemwegie et al., 2020). This has made EPS to receive greater interest for extensive applications in food, pharmaceutical, and cosmeceutical industries (Ozcan and Oner, 2015). Considering a great interest and uses of EPS for different biological purposes, submerged culture of fungi with agrowastes will be a new approach and an eco-friendly strategy to produce EPS. This study therefore, produces EPS by *P. pulmonarius* in submerged fermentation with some agro-wastes namely; peels of plantain, pineapple, mango, groundnut shell, coconut coir and walnut husk. The antimicrobial, antioxidant and prebiotic activities of EPS produced by *P. pulmonarius* were also investigated.

2. Materials and methods

2.1. Chemical reagents

Fructooligosaccharides (FOS), K_2HPO_4, $CaCO_3$, and DPPH were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Butanol, ethanol, trichloro acetic acid, $FeSO_4$, butylated hydroxytoluene (BHT) were products of Sigma-Aldrich (Steinheim, Germany). Hydrogen peroxide (H_2O_2), deoxyribose, thiobarbituric acid, $MgSO_4$ were from Amresco (Ohio, USA). Chloroform and methanol were from Univar (Downers Grove, IL, USA).

2.2. Source of agro-wastes

Peels of plantain, pineapple, mango, groundnut shell, coconut coir and walnut husk were obtained from fruit market in Odeomu, Nigeria. The agrowastes were air-dried at 29 °C for 21 days and pulverized using a mill machine (5657 HAAN 1 TYPE ZM1, Retsch GmbH, Haan, Germany).

2.3. Collection of microorganisms

P. pulmonarius was collected from Federal Institute of Industrial Research Oshodi (FIIRO) Lagos, Nigeria. The fungus was sub-cultured on Potato Dextrose Agar to maintain a pure strain of the fungus. Indicator bacteria and fungi were obtained from Nigeria Institute of Medical Research (NIMR), Lagos, Nigeria. The indicator microorganisms include: *Shigella dysenteriae, Escherichia coli* O 157:H7, *Salmonella typhi*, *Vibrio cholerae,* Methicillin resistant *Staphylococcus aureus* (MRSA), *Bacillus subtilis, Candida albicans,* and *Candida tropicalis* (ATCC 66029). All the tested microorganisms were aseptically sub-cultured into appropriate media and incubated at 37 °C for 24 h and 26 °C for 48 h for bacteria and fungi, respectively. Two probiotic strains; *L. delbrueckii* and *S. thermophiles* used in this study were isolated from yoghurt starter culture.

2.4. Production and extraction of EPS

EPS was produced by *P. pulmonarius* in submerged fermentation with agro-wastes using methods of Smirderle et al. (2012) and Silveira et al. (2015) with slight modification. Briefly, 5.0 g/L glucose, 1.0 g/L yeast extract, 1.0 g/L peptone, 0.1 g/L $MgSO_4$, 0.1 g/L K_2HPO_4 and 0.1 g/L $CaCO_3$ and agro-waste of 4.0 g/L, 12.0 g/L and 20.0 g/L in different Erlenmeyer flask. The flasks containing the mixture were autoclaved at 121 °C for 15 min. Thereafter, the flasks were allowed to cool before inoculation of 7 days-old mycelium of *P. pulmonarius*. The flasks were incubated at 26 °C and maintained at 120 rpm for 18 days in thermostat incubator with shaker (IN-SK100, Tianjin, China). After submerged fermentation, EPS was assayed using the methods of Svagelj et al. (2008) and Diamantopoulou et al. (2014). Briefly, the concentrated culture filtrates were mixed with four volume of ethanol 95% v/v, stirred thoroughly and allow to stand for 24 h at 4 °C. The precipitated EPS was treated with 1:4 of *n*-butanol: chloroform (1:5 v/v) to remove protein. The EPS content was determined by phenol-sulphuric acid methods and glucose was used as standard (Dubois et al., 1956). The solvent extract was purified by ion exchange chromatography at flow rate of 24 ml/h and elution was performed with distilled water. The samples were dialyzed exhaustively against distilled water to remove unwanted impurities for 24 h. The EPS obtained was freeze-dried (FD-10-MR, Xiangtan Xiangyi instrument Ltd, China) at -65 °C. EPS produced by *P. pulmonarius* in submerged culture without agrowaste, EPS produced by *P. pulmonarius* in submerged culture with pineapple peel, EPS produced by *P. pulmonarius* in submerged culture with groundnut shell and EPS produced by *P. pulmonarius* in submerged culture with coconut husk were selected for further studies.

2.5. FTIR spectroscopic analysis of EPS

Structural analysis of EPS was determined using Fourier transform-infrared (FT-IR) spectroscopy (8400S, Shimadzu Scientific Instruments Inc.). EPS (1.0 μl) was placed on fused KBr disc for detecting the functional groups. This was carefully placed on cell holder, clamped loosely and fixed on the infrared (IR) beam. The running was done at 400 to 4000 per cm wave number.

2.6. Antimicrobial activity of EPS against microorganisms

The antimicrobial activity of EPS was tested against strains of microorganism using agar well diffusion method (Cheesbrough 2000). Microorganisms (bacteria and fungi) were cultivated on their respective broth and incubated at 37 °C for 24 h and 28 °C for 48 h for bacteria and fungi, respectively. The inoculum size was a to 0.5 McFarland turbidity standard at 600 nm using visible spectrophotometer (UNICO S-1100 RS). A sterile swab stick moistened with bacterial or fungal inoculum was spread on Mueller Hinton agar. Subsequently, wells of 4 mm diameter were bored into the agar medium and filled with 50 μl of EPS (1.0 mg/ml). Antibiotics namely; amoxicillin and ketoconazole were used as positive control, while sterile distilled water was served as negative control. The plates were incubated at 37 °C for 24 h and 25 °C for 72 h for bacteria and fungi, respectively. After incubation, zones of inhibition were measured and recorded in millimeters (mm). Minimum inhibitory concentration (MIC) of EPS was determined by varying concentrations of 0.25 mg/ml to 1.0 mg/ml. The lowest concentration of EPS that shown no visible growth were regarded as MIC.

2.7. DPPH scavenging activity of EPS

The free radical scavenging activity of EPS on DPPH was determined using the method of Gyamfi et al. (1999). EPS was mixed with 1.0 ml of 0.4 mM DPPH in methanol (5.0 ml). The mixture was incubated at 27 °C for 30 min in dark. The control contained only DPPH solution in methanol instead of sample while methanol served as the blank. Absorbance was noted at 517 nm by using a UV-visible spectrophotometer. The capacity of free radical scavenging was calculated as:

$$\text{Scavenging activity } (\%) = \{(\text{A control} - \text{A sample}) / \text{A control}\} \times 100\%$$

2.8. Hydroxyl radical scavenging assay

Scavenging potential of EPS against hydroxyl radical was assessed using the method of Halliwell et al. (1987). The reaction mixture containing an aliquot of EPS (100 μl), 120 μl, 20 mM deoxyribose, 400 μl, 0.1 M phosphate buffer, 40 μl, 20mM hydrogen peroxide, 40 μl, 500 μM $FeSO_4$ and the volume was made up to 800 μl with distilled water. The reaction mixture was incubated at 27 °C for 30min. Thereafter, 0.5 ml of trichloro acetic acid (2.8%) was added, followed by 0.4 ml thiobarbituric acid (0.6%). Mixture was heated for 20 min and cooled. Absorbance of blank (Ab) and absorbance of the sample (As) was measured at 532 nm in spectrophotometer.

Scavenging activity (%) = {(Ab – As) / Ab} × 100

2.9. Iron chelation activity of EPS

The ability of EPS to chelate iron (II) sulphate ($FeSO_4$) was determined using methods described by Puntel et al. (2005) with little modification. Briefly, 150 mM $FeSO_4$ was added to a reaction mixture containing 168 µl of 0.1M Tris-HCl pH 7.4, 218 µl saline (0.9% NaCl) and EPS (100 µl) of different concentrations (100–200 µg/ml). The reaction mixture was incubated at 27 °C for 5min, before the addition of 13 µl of 0.25% 1, 10-phenantroline (w/v) and the absorbance was subsequently measured at 510 nm in the spectrophotometer. Chelating activity of EPS on iron radical was calculated using this equation:

Chelating effect (%) = {(A control –A sample) / A control}x 100%

2.10. Nitric oxide radical scavenging activity of EPS

Nitric oxide scavenging activity of EPS was carried out using the method described by Jagetia and Baliga (2004). A volume of 1.0 ml of sodium nitroprusside (10 mM) prepared in 0.5 mM phosphate buffer saline (pH 7.4) was mixed with EPS (100 µl) and vortexed. The mixture was incubated at 25 °C for 150 min. Thereafter, 1.0 mL of previous solution was mixed with 1.0 ml of Griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthylethylenediamine hydrochloride) and incubated at 26 ± 2 °C for 30 min. The absorbance was recorded at 546 nm. Scavenging activity was calculated using the following formula:

NO Scavenging activity (%) = {(A control –A sample) / A control}x 100%

2.11. Influence of EPS on probiotic strains

Probiotic growth stimulation of EPS was determined using the method described by Sawangwan et al. (2018) with modifications. Briefly, de Man, Rogosa and Sharpe (MRS) broth (10.0 ml) was prepared in different test tubes, sterilized at 121 °C for 15 min and was allowed to cool. The MRS broth was supplemented with sterile (0.22 µm milipore) 1% v/v of EPS, a negative control contained MRS broth, positive control contained MRS broth with 1% w/v of glucose or commercial prebiotic; fructose oligosaccharides (FOS). Each tube was inoculated with 100 µl of 10^6 colony forming units (CFU) *L. delbrueckii* and *S. thermophiles*. The tubes were incubated at 37 °C for 48 h under anaerobic conditions. After incubation, growth rate of probiotics was quantified by measuring their optical cell density using spectrophotometer at 620 nm. To determine load of *L. delbrueckii* and *S. thermophiles*, 100 µl was serially diluted in sterile distilled water up to 10^{-4}, where 100 µl was transferred to MRS agar plates. Petri dishes were incubated at 37 °C for 48 h. Thereafter, colonies of *L. delbrueckii* and *S. thermophiles* were counted using colony counter (TT-20, Techmel and Techmel, USA) and reported as colony forming unit per milliliter (CFU/ml).

2.12. Statistical analysis

All experimental studies were performed in replicate (n = 3). Data were subjected to one-way analysis of variance (ANOVA) and results were presented as mean ± standard deviation (SD). Tests of significant differences were determined by Duncan's Multiple Range Test at $P < 0.05$. The statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) version 23.

3. Results and discussion

Agro-industrial wastes contain nutrients and bioactive compounds that can be biologically converted into value added products and this therefore, remain circular strategies, implementing policy regulation and most cost-effective methods to utilize lignocellulose biomass as eco-friendly alternative to increase biobased products (Chhetri et al., 2020). In this study, peels of plantain, pineapple, mango, shell of nut-foods namely; groundnut, walnut and coconut were successfully utilized by *P. pulmonarius* to enhance production of EPS. Figure 1 shows the yield of EPS produced by *P. pulmonarius* in a submerged culture supplemented with agro wastes. *P. pulmonarius* produced EPS of 1.20 g/L in a submerged culture without agrowaste. When 4.0 mg/L of agrowaste was supplemented into submerged culture, EPS produced by *P. pulmonarius* was within 2.0–3.8 g/L. When 12.0 mg/L and 20.0 g/L of agrowastes were added into submerged culture, EPS produced by *P. pulmonarius* ranged from 2.0 to 2.7 g/L and 2.0–5.6 g/L, respectively. *P. pulmonarius* produced more EPS when agrowastes was supplemented into submerged culture. The higher proportion of EPS from culture media containing agro-wastes could be attributed to colonizing potential of *Pleurotus* spp. on various agro-industrial residues (Lavelli et al., 2018). *Pleurotus* spp. possesses ability to grow on varieties of lignocellulosic biomass, utilizing them as substrates to degrade both natural and anthropogenic aromatic compounds, which occurs by virtue of nonspecific oxidative enzymatic system that consist mainly laccases, phenol oxidases, manganese peroxidases and versatile peroxidases (Hofrichter et al., 2010; Sekan et al., 2019). The degrading enzymes in *Pleurotus* spp. mycelia enable them to store some metabolic products such as sterols, triterpenes, phenolic compounds and largely; polysaccharides (Liu et al., 2010; Vamanu, 2012).

Supplementation of agrowastes into cultivating media of fungi during fermentation processes is a mechanism to produce useful bioavailable metabolites for medicinal purposes. Bioproduction of multiple secondary metabolites by higher fungi have increased for industrial applications through optima fermentation conditions, application of metabolic engineering techniques, and fungal genetic manipulation (Takahashi et al., 2020). Solid state fermentation and submerged cultivation of Basidiomycota are much faster biotechnological processing as a promising tool for industrial production of exopolysaccharides. Submerged cultivation of fungal mycelium is still attractive alternative to produce high-value metabolites; exopolysaccharides (EPS), which are often synthesize in the cell membrane through the action of enzymes (Jaros et al., 2018). *Trametes versicolor, Lentinula edodes*, and *Pleurotus ostreatus* grown on twelve formulations of different inexpensive lignocellulosic biomass such as oak sawdust, coconut fiber (hairs), coffee husks, and corn bran plus soybean oil produced better yield of polysaccharides (Sánchez and Montoya, 2020). Agro-wastes are media that can be utilized by microorganisms to produce natural metabolites with antioxidant and antimicrobial activities (Sadh et al., 2018a). Hence, the utilization of agro-wastes to produce biologically active compounds by submerged fermentation with microbial technology is a major way to proffer solution to poor management and disposal of agro wastes. Shells of

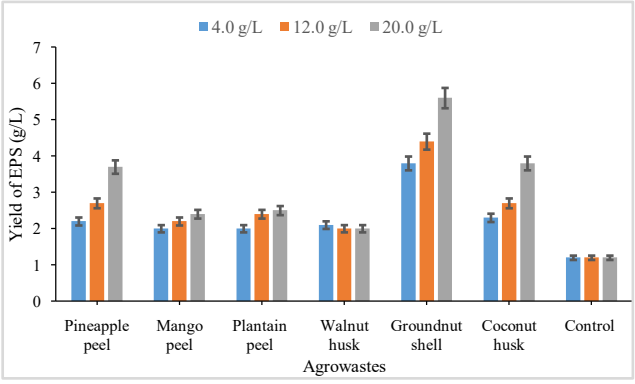

Figure 1. Yield of EPS (g/L) produced by *P. pulmonarius* in submerged culture with agro-wastes. Control is without any agrowastes. Error bar: SD.

Table 1. Functional groups in EPS produced by *P. pulmonarius* in submerged culture with and without agro-wastes.

Sample	Wave number (cm^{-1})	Functional groups	Name of group
EPS-A	3448.84	-OH $_{stretch}$	Hydroxyl
	2929.97	-CH_3 $_{stretch}$	Methyl
	2360.95	-RCOH $_{stretch}$	Aldehyde
	1639.55	-C=O $_{stretch}$	Ketone
EPS-B	3404.47	-OH $_{stretch}$	Hydroxyl
	2937.68	-CH_3 $_{stretch}$	Methyl
	2372.52	-RCOH $_{stretch}$	Aldehyde
	1724.42	-C=O $_{stretch}$	Ketone
EPS-C	3423.76	-OH $_{stretch}$	Hydroxyl
	2928.04	CH $_{stretch}$	Methyl
	2359.02	-RCOH $_{stretch}$	Aldehyde
	1641.48	-C=O $_{stretch}$	Ketone
	1078.24	C–O–C $_{stretch}$	Epoxy
EPS-D	3404.47	-OH $_{stretch}$	Hydroxyl
	2933.83	CH $_{stretch}$	Methyl
	2362.88	-RCOH $_{stretch}$	Aldehyde
	1637.62	-C=O $_{stretch}$	Ketone
	1076.32	C–O–C $_{stretch}$	Epoxy

EPS-A: EPS produced by *P. pulmonarius* in submerged culture without agro-wastes.
EPS-B: EPS produced by *P. pulmonarius* in submerged culture with groundnut shell.
EPS-C: EPS produced by *P. pulmonarius* in submerged culture with coconut husk.
EPS-D: EPS produced by *P. pulmonarius* in submerged culture with pineapple peel.

groundnut, coconut, walnut and dried fruit peels contain cellulose, hemicellulose and lignin, which enable fungi to grow and produce better metabolic products. Groundnut shell was utilized for the production of various bio-products like biodiesel, bioethanol, and enzyme (Duc et al., 2019). Shells of coconut and walnut, fruit peels, seeds and other agro-wastes are renewable sources for production of biopolymers (Maraveas, 2020).

The varying amount of EPS could base on quantity of agrowastes, different type of agro-wastes used, their chemical composition and choice of fungi. It has been reported that optimal submerged culture conditions for maximum mycelial growth and EPS production depend strongly on type of substrates and fungal species (Park et al., 2002a, 2002b; Mahapatra and Banerjee 2013). Other environmental parameters or physical conditions on the fungal mycelia formation during fermentation with culture medium composition could be associated with yield of EPS. The morphological nature, physicochemical parameters and various physiological activities on fungal mycelia in submerged cultures were strongly depended on the quantity of metabolites (Znidarsic and Pavko, 2001; Kirsch et al., 2016). The findings of Kim et al. (2006) revealed that, characteristics of mycelia in submerged fermentation were influenced by culture conditions such as composition and initial pH of fermentation medium, age and size of inoculum, aeration rate, agitation speed and thus, affect yield of EPS. The basic composition of agro-wastes residues like nitrogen, carbon and minerals could also affect the production of EPS. Findings of Lee et al. (2004), Smiderle et al. (2012) and Thai and Keawsompong (2019) associated the production of EPS and cell mass to different carbon and nitrogen sources. Mahapatra and Banerjee (2013) suggested that, most of the EPS producing fungi are aerobic or facultative anaerobic, and oxygen limitation did not support EPS production and thus, revealed that fungi needed long incubation time for maximum EPS production.

Figure S1(supplementary file 1) shows FT-IR spectra of EPS produced by *P. pulmonarius* in a submerged culture without agro-wastes, EPS produced by *P. pulmonarius* in submerged culture supplemented with groundnut shell, EPS produced by *P. pulmonarius* in submerged culture supplemented with coconut shell and EPS produced by *P. pulmonarius* in submerged culture supplemented with pineapple peel. The wave number (cm^{-1}) interpreted for functional groups in EPS were shown in Table 1. A peak of 3448.84 cm^{-1} and 3404.47 cm^{-1} indicated hydroxyl group (-OH). A peak around 2929.97cm^{-1} and 2937.68 cm^{-1} suggested the presence of methyl (–CH_3). Bands at 1639.55 cm^{-1} and 1724.42 cm^{-1} were assigned to carbonyl (-C=O). The –C=O group (carbonyl), –CH_3 group (methyl) and –OH group (hydroxyl) were functional groups in EPS produced by *Pleurotus* spp. The result is in agreement with a previous study by Shen et al. (2013) who conducted FT-IR analysis on EPS produced by *P. pulmonarius*. Findings of Patel et al. (2014) indicated sharp peak in FT-IR spectra of functional groups in fungal EPS, which ranged from 1400-1500 cm^{-1} and 1631.45 cm^{-1}. The band at 1,162 cm^{-1} corresponds to 1,4-glycosidic linkage in polysaccharides that gives absorption bands in the range of 1,175–1,140 cm^{-1} (Nikonenko et al., 2000). The different functional groups in EPS occurred as a result of varying contents of monosaccharide composition. Fungal EPSs contain sugars of different monosaccharide units such as glucose, mannose, galactose, xylose, arabinose, fucose and rhamnose (Osińska-Jaroszuk et al., 2015).

Zones of inhibition by EPS on pathogenic microorganisms were shown in Table 2. The zones of inhibition displayed by EPS-A, EPS-B, EPS-C and EPS-D against *Shigella dysenteriae* and *E. coli* O 157:H7 were not significantly different at $p < 0.05$. EPS inhibited the growth of Gram positive bacteria such as *Bacillus subtilis*, *Staphylococcus aureus,* yeast and other Gram-negative bacteria. Table 3 showed MIC of EPS against tested

Table 2. Zones of inhibition (mm) displayed by EPS against pathogenic microorganisms at 1.0 mg/ml.

Microorganisms	EPS-A	EPS-B	EPS-C	EPS-D	*KET/AMX
S. dysenteriae	13.00 ± 0.11^{b}	14.00 ± 1.21^{b}	13.60 ± 0.76^{b}	13.00 ± 0.22^{b}	17.00 ± 2.48^{a}
E.coli O 157:H7	10.00 ± 0.15^{b}	10.30 ± 0.15^{b}	11.00 ± 0.03^{b}	10.00 ± 0.55^{b}	16.00 ± 0.87^{a}
S. typhi	9.00 ± 0.40^{c}	12.00 ± 1.11^{b}	10.00 ± 0.34^{c}	12.20 ± 0.61^{b}	15.00 ± 1.39^{a}
V. Cholerae	NI	NI	5.00 ± 0.00^{b}	5.70 ± 0.03^{b}	11.00 ± 0.85^{a}
MRSA	7.00 ± 0.05^{d}	12.00 ± 1.10^{b}	7.00 ± 0.01^{d}	10.00 ± 0.54^{c}	14.30 ± 0.98^{a}
B. subtilis	10.00 ± 0.71^{b}	10.00 ± 0.33^{b}	5.00 ± 0.01^{c}	10.00 ± 0.03^{b}	16.00 ± 1.44^{a}
C. albicans	8.00 ± 0.00^{c}	10.20 ± 0.22^{b}	7.00 ± 0.04^{c}	8.00 ± 0.01^{c}	14.00 ± 0.09^{a}
C. tropicalis (ATCC 66029)	7.00 ± 0.03^{b}	8.00 ± 0.10^{b}	NI	5.00 ± 0.01^{c}	13.00 ± 0.46^{a}

Values are mean ± SD of replicate (n = 3). Values with different alphabets along each row are significantly different from each other when $p < 0.05$. NI: no inhibition at 1.0 mg/ml. MRSA: Methicillin resistant *Staphylococcus aureus*.
EPS-A: EPS produced by *P. pulmonarius* in submerged culture without agro-wastes.
EPS-B: EPS produced by *P. pulmonarius* in submerged culture with groundnut shell.
EPS-C: EPS produced by *P. pulmonarius* in submerged culture with coconut husk.
EPS-D: EPS produced by *P. pulmonarius* in submerged culture with pineapple peel.
AMX: Amoxicillin was used against bacteria and KET: *Ketoconazole was used against fungi.

Table 3. Minimum inhibitory concentration (mg/ml) of EPS against tested microorganisms.

Microorganisms	EPS-A	EPS-B	EPS-C	EPS-D
S. dysenteriae	0.25	0.25	0.25	0.25
E.coli O 157:H7	1.00	1.00	0.50	1.00
S. typhi	0.50	0.25	0.50	0.25
V. Cholerae	NI	NI	1.00	1.00
MRSA	0.50	0.25	1.00	0.50
B. subtilis	0.50	0.50	1.00	0.50
C. albicans	0.25	0.25	0.25	0.25
C. tropicalis (ATCC 66029)	0.50	0.25	NI	1.00

Keys NI: no inhibition at 1.0 mg/ml (MIC >1.0 mg/ml), MRSA: Methicillin resistant *Staphylococcus aureus*.
EPS-A: EPS produced by *P. pulmonarius* in submerged culture without agro-wastes.
EPS-B: EPS produced by *P. pulmonarius* in submerged culture with groundnut shell.
EPS-C: EPS produced by *P. pulmonarius* in submerged culture with coconut husk.
EPS-D: EPS produced by *P. pulmonarius* in submerged culture with pineapple peel.

microorganisms. The MIC ranged from 0.25-1.00 mg/ml against tested microorganisms. Saskiawan (2009) reported antimicrobial activity of EPS produced by *Pleurotus* spp. against *Escherichia coli, Bacillus subtilis* and *S. cerevisiae.* Findings of Osińska-Jaroszuk et al. (2015) revealed that EPS from Ascomycota and Basidiomycota displayed pronounced antibacterial properties against *Staphyloccoccus aureus* and *Vibrio fischeri*, *Bacillus subtilis* and *Micrococcus tetragenus*. Skalicka-Woźniak et al. (2012) associated the wide antimicrobial potentials of polysaccharides in four *Ganoderma lucidum* to their ability to chelate essential nutrients, which therefore, limit the growth of microorganisms. Polysaccharides (intra and extra-) produced by numerous microorganisms, most especially mushrooms are tagged with various biological and pharmacological activities such as antimicrobial, dietary supplements, immunostimulating and immunodulatory, anti-tumor, hypoglycemic and antioxidant (Ruthes et al., 2016; Wang et al., 2017). Therapeutic potentials of EPS are the main reasons for their medicinal and industrial uses. Hence, EPS produced by fungi in submerged culture with agro-wastes was enhanced and can be continually exploited for different bioactivities.

Figure 2 shows antioxidant activity of EPS against DPPH, OH, Fe^{2+} and NO. The scavenging activity of EPS ranged from 67.80-81.80%, 60.60–81.20%, 70.40–84.70%, 78.40–88.50% against DPPH, OH^{-}, Fe^{2+} and NO, respectively. Zhang et al. (2017) revealed EPS as antioxidant agent that scavenged $OH^{-}$and DPPH radicals. The researchers revealed that EPS exhibited antioxidant activity of 100%, which is the same as scavenging activity of vitamin C. In the findings of Liu et al. (2015), EPS produced by submerged culture of *Inocutus hispidus* showed antioxidant activities of 70.7% on hydroxyl and 50% on 2,2-diphenyl-1-picrylhydrazyl radicals. EPS produced by *Gomphidius rutilus*, mutant *Cordyceps militaris* SU5-08 and *Pleurotus* spp. in submerged fermentation demonstrated pronounced antioxidant effects on superoxide anion, 1,1-diphenyl-2-picrylhydrazyl, hydroxyl radical, and reducing power (Gao et al., 2012; Lin et al., 2012; Sun et al., 2014; Bamigboye et al., 2016). EPS have different monosaccharide composition with functional groups such as –OH, $–CH_3$, –COOH, and –C=O that substantiate its free radical scavenging and metal chelating activities (Shen et al., 2013). Such functional groups in biomolecules or functional foods are stable enough to donate electrons to reduce the free radicals to a more stable form or react with the free radicals to dismiss the radical chain reaction (Leung et al., 2009; Lobo et al., 2010). Fungal polysaccharides (extra- and intra-cellular) are of great economic importance with wide variety of biological activities, that promote health benefits [with] relatively nontoxic property (Mantovani et al., 2008; Santa et al., 2015). Exopolysaccharides from different Basidiomycota are reported to have a large potential for human health maintenance as well as reducing the risk of various diseases. *Inonotus obliquus* polysaccharides suppressed cell viability, colony-formation, and triggered cell apoptosis and thus, can be used as a promising alternative or supplementary medicine for cancer therapy (Jiang et al., 2020). Huang et al. (2019) revealed that *Ganoderma lucidum* polysaccharides and *Polyporus umbellatus* polysaccharides significantly enhanced the phagocytic function of macrophages and the activity of NK cells. EPS, having displayed different bioactivities, can be combined with compounds of potential value to produce new drugs for better and effective treatments of organic diseases.

Table S1 (supplementary file 1) shows optical density at 620 nm for *L. delbrueckii* and *S. thermophiles* cultivated in MRS broth, MRS broth supplemented with EPS or commercial prebiotic after 48 h. Table 4 shows count (CFU/ml) of *L. delbrueckii* and *S. thermophiles* on MRS agar. Probiotic growth stimulation of EPS (A-D) was ranged within 3.04–3.40 $\times$ 10^4 CFU/ml for *L. delbrueckii* and 2.50 to 2.81 $\times$ 10^4 CFU/ml for *S. thermophiles* with varying optical density of 0.9910–1.0716 and 1.0216 to 1.0372, respectively. Sawangwan et al. (2018) revealed that *Lactobacillus acidophilus* grown in *Lentinus edodoes* extract and *Lactobacillus plantarum* cultured in *P. pulmonarius* extract have optical density (620 nm) of 1.9779 and 1.9702, respectively. This study indicated that EPS enhanced the growth of *L. delbrueckii* and *S. thermophiles*. Polysaccharides from *Pleurotus* spp. are supportive media that enhance the growth rate of probiotics (Synytsya et al., 2009). In the findings of Nowak et al. (2018), mushroom polysaccharides support the growth of *Lactobacillus* strains

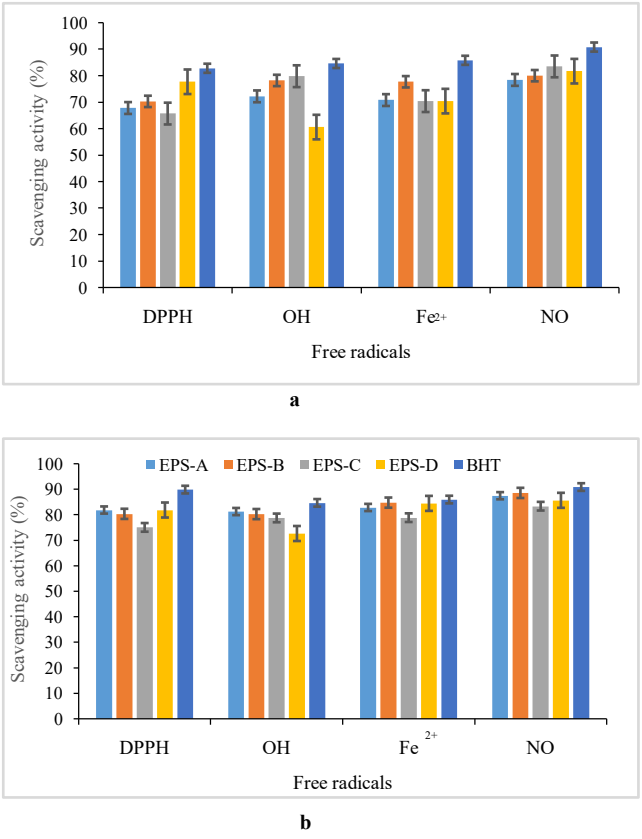

Figure 2. Scavenging activity of EPS against free radicals at (a) 100 μg/ml and (b) 200 μg/ml. EPS-A: EPS produced by *P. pulmonarius* in submerged culture without agro-wastes, EPS-B: EPS produced by *P. pulmonarius* in submerged culture with groundnut shell, EPS-C: EPS produced by *P. pulmonarius* in submerged culture with coconut husk, EPS-D: EPS produced by *P. pulmonarius* in submerged culture with pineapple peel,. BHT: butylated hydroxytoluene. Error bar: SD.

Table 4. Growth ($\times 10^4$ CFU/ml) of *L. delbrueckii* and *S. thermophiles* on MRS agar plates at 37 °C after 48 h. Values are mean ± SD of replicate (n = 3). Values with different alphabets along each column are significantly different from each other when $p < 0.05$.

Agar plates	*L. delbrueckii*	*S. thermophiles*
MRS	2.40 ± 0.03^{e}	1.85 ± 0.00^{e}
MRS + G	2.52 ± 0.01^{e}	2.07 ± 0.03^{d}
MRS + FOS	3.11 ± 0.03^{c}	2.48 ± 0.00^{bc}
MRS + EPS-A	3.20 ± 0.10^{b}	2.50 ± 0.01^{b}
MRS + EPS-B	3.04 ± 0.06^{d}	2.81 ± 0.03^{a}
MRS + EPS-C	3.26 ± 0.03^{b}	2.70 ± 0.04^{a}
MRS + EPS-D	3.40 ± 0.03^{a}	2.64 ± 0.02^{ab}

MRS: de man, rogosa and sharpe, G: glucose, FOS: fructose oligosaccharides.
EPS-A: EPS produced by *P. pulmonarius* in submerged culture without agro-wastes.
EPS-B = : EPS produced by *P. pulmonarius* in submerged culture with groundnut shell.
EPS-C: EPS produced by *P. pulmonarius* in submerged culture with coconut husk.
EPS-D: EPS produced by *P. pulmonarius* in submerged culture with pineapple peel.

than some commercially available prebiotics like inulin and fructooligosaccharides.

The variability observed in biofunctionality of EPS against microorganisms, free radicals and towards the growth of *L. delbrueckii* and *S. thermophiles* could due to the content and quantity of monosaccharides as well as their molecular weight. Biological activities and therapeutic effects of polysaccharides depend on specific glycosidic linkages, degree of branching, monosaccharide composition and molecular weight (Wang et al., 2017). EPSs are natural polymers of microbial products with novel biological properties (anticancer, antimicrobial and antioxidants) and thus, serve as valuable resources with multiple biotechnological applications in industries.

4. Conclusion

Fruit peels, coconut husk, groundnut shell and walnut shell supplemented into submerged culture of *P. pulmonarius* mycelia supported the production of EPS. EPS inhibited the growth of pathogenic microorganisms, displayed antioxidant activities against free radicals and sustained the growth of probiotics. Therefore, bioactivities of EPS make it a better candidate of natural products that is often used as a preservative agent in foods industries. Production of natural bioactive compounds in submerged culture of fungi with agro-wastes will therefore, reduce indiscriminate disposal of agro-wastes into the environment. Chemical constituents of agro-industrial residues can be utilized by fungal enzymes to produce biologically active compounds (secondary metabolites) in larger scale production.

Declarations

Author contribution statement

Clement Olusola Ogidi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Adaeze Mascot Ubaru, Temilayo Ladi-Lawal: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Oluwakemi Abike Thonda: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Oluwatoyin Modupe Aladejana: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

Olu Malomo: Conceived and designed the experiments; Wrote the paper.

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The authors declare no conflict of interest.

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