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Research Article

BIOTECHNOLOGICAL APPLICATIONS OF EXOPOLYSACCHARIDE PRODUCED BY THE WOOD ROT FUNGI

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ABSTRACT

In the present study, the diverse localities of the Chandigarh region were explored for the collection of wood rot fungi producing biotechnologically important exopolysaccharide. A total of 51 different wood rot specimens were collected of which, 24 specimens belonging to 5 different families and 5 different genera were successfully isolated. The isolates were further screened for their exopolysaccharide producing ability and the maximum yield of 2.155g/l was obtained from the isolate WRE7 (*Ganoderma* sp.). The polysaccharide obtained was evaluated for its bio-emulsifying, bio-flocculating and anti-oxidant potential. The emulsifying activity was determined by using different vegetable oils and hydrocarbons and the results revealed that the polysaccharide was able to form stable emulsions with a maximum emulsifying activity of 57.14%. The test polysaccharide was also found capable of flocculating the suspended clay particles and a maximum flocculating activity of 61.3% was obtained at a concentration of 1000µg/ml. The extracted polysaccharide also exhibited strong antioxidant activity. It was found capable of neutralizing the DPPH radicals and scavenging the superoxide radicals and hydroxyl radicals with maximum activity of 72.6%, 60.12%, and 75.52%, respectively at 1000µg/ml.

Keywords: Wood rot fungi, Exopolysaccharide, Bio-emulsifier, Bio-flocculant, Antioxidant.

1. INTRODUCTION

The total carbon present in terrestrial ecosystems is estimated to be 2000 billion [1], of which 550 billion tons is fixed in vegetation [2]. In forest ecosystems, most plant biomass is stored in the form of dead wood which consist mainly of the lignocellulose complex which is composed of the polymeric polysaccharides such as cellulose (40-50%), hemicellulose (15-30%), lignin hetero-polymers (15-30%) [3] and other macromolecules such as lipids, waxes, proteins and phenolic compounds. The most efficient agents of the decay of the lignocellulose complex are saprotrophic fungi, which therefore play pivotal roles in the cycling of carbon [4] and nutrients in the forest ecosystem. A wood decay fungi is any species of fungus that digests moist wood, causing it to rot. Wood decay fungi or lignicolous fungi include Ascomycetes, Basidiomycetes and a large number of classes and orders within each of these groups. Wood decay is generally classified into two main groups, white rot and brown rot, based on the wood residue left behind following fungal digestion. Two other types include "dry rot", which is a form of brown rot caused by water conducting decay fungi, and "soft rot", referring to decay caused by certain ascomycetes and asexual fungi. Each of these fungi produces different enzymes which are capable of degrading different plant materials and can colonize different environmental niches. Besides enzyme complexes these fungi are also known to produce a variety of value added exopolysaccharides.

There is a high demand from the consumers for naturally derived products as the synthetic derivatives are often associated with potential health and environmental hazards. This rise in demand has led to an increased attention towards the exopolysaccharide (EPS) production by a variety of fungi as these polysaccharides can act as an alternative to existing synthetic derivatives. The fungal kingdom is known to produce high value exopolysaccharides which possess various biological and chemical properties. Polysaccharides are the most active component produced by fungi, which act as a functional food and are responsible for varied biological activities such as anti-oxidant, radical scavenging, detoxificating etc. [5,6]. Besides they are also considered to be a good source of bioemulsifiers, bioflocculants, gelling agents,

viscosifiers etc. Thus, the present study deals with the evaluation of biotechnological applications of exopolysaccharide produced by wood rotting fungi.

2. MATERIAL AND METHODS

2.1. Collection and Isolation

The diverse localities of Chandigarh region (including Kansal forest, Jainti Devi Ki Rao, Haripur forest, Patiali Rao, Sukhna Lake forest, Morni Hills, Dhanas forest, and other bio-diverse regions of Chandigarh) were surveyed to collect the wood rot fungi. The fruiting bodies were collected in butter paper and were brought to the laboratory in sealed bags for further macroscopic and microscopic studies. The samples were marked with information such as collection number, procurement location, and date of collection. Besides, information regarding temperature, humidity, substrate/host, was also noted.

The isolation of the collected samples was done as per Stametes and Chilton [7] by tissue culture method. The surface of the collected fungal sample was wiped with cotton soaked in alcohol and any dirt or damaged tissues were removed. Upper layer of stipe or the cap was removed using a hot scalpel to expose the interior hyphae. A small fragment of tissue was removed and transferred to the center of Petri dish containing potato dextrose agar. The plates were then incubated at 28°C for 4-5 days. The successfully isolated cultures were routinely sub cultured into potato dextrose agar slants and plates. For long term preservation 5mm discs of pure cultures were placed in cryogenic tubes containing 10% glycerol solution. The tubes were then stored at 4°C in a freezer.

2.2. Tentative identification

Tentative identification of all the isolates was done as per the method of Hawksworth, Singer and Atri et al., [8,9,10] by studying detailed morphological characteristics like Pileus (shape, color, surface, margin), Gills (arrangement, shape, texture, color, margin), Veil (type, color, position, texture, size), Annulus (type, color, position, texture, size), Stipe (shape, size, color), Volva (type, nature, shape, color, texture), Odour (pleasant/ unpleasant). Microscopic studies were also done to determine the type of hyphae and spores.

2.3. Screening of exopolysaccharide producing efficiency

Successfully isolated wood rot fungi were screened for their exopolysaccharide producing efficiency as per Kim et al., [11]. The isolated pure cultures were cultivated at 28°C and 120 rpm for 10 days in a 250 ml conical flask containing100 ml of potato dextrose broth. After the completion of fermentation, the biomass was separated by filtration and the resultant filtrate was centrifuged at 10,000 rpm for 20 minutes at 4°C to remove any remnant mycelial mass. To the resultant supernatant 5% TCA was added and was left for 24hrs at 4°C for protein precipitation. The precipitated proteins were separated by centrifuging the supernatant at 10,000 rpm for 20 minutes at 4°C. For the precipitation of the EPS, equal volume of chilled ethanol was added and was kept overnight at 4°C. The precipitated exopolysaccharide was recovered by centrifugation at 10,000 rpm for 20 minutes at 4°C. The extracted EPS was dried in an oven at 60°C till constant weight was achieved and was expressed in g/l.

2.4. Evaluation of Biotechnological applications of exopolysaccharide

2.4.1. Evaluation of Bio-emulsifying activity

The emulsifying activity of the test EPS was determined as per the modified method of Cooper and Goldenberg [12]. In this method, 1ml each of different vegetable oils (olive, sesame, sunflower, coconut, mustard and groundnut) and hydrocarbons (petroleum, diesel, liquid paraffin and xylene) were added to 1ml of EPS suspension (1mg/ml). The mixture was vortexed at high speed for 2 minutes. Guargum was used as the standard emulsifier. The emulsification activity (EA%) was determined after an hour of incubation whereas the emulsification index (E%) was determined after 24, 48, 72 and 96 hours of incubation. EA% and E% were determined as per the following formula:

E%= height of emulsion layer/total height of the emulsion x 100

2.4.2. Evaluation of Bio-flocculating properties

The flocculating activity was determined as per the modified method of Shih et al., [14]. In this method, 10 ml of kaolin clay suspension (5mg/ml) was mixed with 0.5ml of the test EPS (1mg/ml) and 0.5ml of $CaCl_2$ (1%). The mixture was vortexed at high speed for 2 minutes and was allowed to stand for 5 minutes. The clear upper layer was removed and the absorbance was taken at 550nm. Sodium alginate and Guargum were used as the standard and distilled water served as the blank.

The flocculating activity was determined as per the following formula:

F.A.=[A-B[/[A] X100
A= O.D. of blank; B = O.D. of test (sample)

2.4.3. *Determination of antioxidant activity*

2.4.3.1. Free radical scavenging assay by DPPH method

The DPPH radical scavenging of the EPS was determined according to Kao and Chen [14]. The reaction mixture consisted of 1ml of the EPS (200, 400, 600, 800, and 1000 μg/ml) and 2ml of ethanolic DPPH (0.05mM). The contents were mixed vigorously and were incubated for 30 minutes at room temperature in dark. After incubation the mixture was centrifuged at 8000rpm for 10 minutes. The upper layer was separated and the absorbance was measured at 517nm.

% DPPH scavenging activity = $[(A_0 – A_1) / A_0]$ x 100.

Where A_0 and A_1 is the absorbance of control and sample respectively. Distilled water served as blank and ascorbic acid as positive control

2.4.3.2. Superoxide radical scavenging activity

The superoxide radical scavenging activity was determined as per the method of Li and Shah [15]. 0.5 ml of 50 mM phosphate buffer was mixed with 0.4 ml of the EPS sample in different concentrations (200, 400, 600, 800, 1000 μg/ml). The mixture was stored for 20 min at 25°C to which 0.1 ml of 3mM pyrogallol preheated to 25°C was added. The mixture was mixed and the absorbance was measured at 325 nm every 30s for 5 min. The Superoxide scavenging activity was calculated as per the formula:

% Superoxide scavenging activity: $[(A_0 - A_1) / A_0]$ x 100

Where, A_1 is the difference of absorbance values per 30 s for different concentrations of samples. A_0 is the difference of absorbance values per 30 s without samples.

2.4.3.3. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the polysaccharide was determined as per the method by Thomas et al., [18]. To 1 ml of polysaccharide solution (200, 400, 600, 800, 1000 μ/ml) 0.9 ml of EDTA-$FeSO_4$ (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml H_2O_2 (8.8 mM) and 0.5 ml of salicylic acid (9 mM) was added and was incubated at 37°C for 60 min. Following incubation the absorbance was measured at 510 nm and the hydroxyl radical scavenging activity was calculated as follows:

Scavenging ability (%) = $[1-(A_1-A_2)/A_3]$ ×100

A1: Absorbance of sample; A2: Absorbance of control; A3: Absorbance of blank

Distilled water was used as blank and in the control group the test EPS and H_2O_2 were replaced by distilled water and sodium phosphate buffer, respectively.

3. RESULTS AN DISCUSSION

3.1. Collection and Isolation

A total of 51 different samples were collected from different areas of which 24 (WRE1, WRE6, WRE7, WRE8, WRE9, WRE10, WRE11, WRE13, WRE14, WRE15, WRE16, WRE21, WRE22, WRE24, WRE25, WRE28, WRE30, WRE34, WRE35, WRE36, WRE42, WRE49, WRE50 and WRE51) were successfully isolated on artificial medium (Potato Dextrose Agar) in the laboratory and preserved at 4°C in cryogenic tubes containing 10% glycerol (fig. 1).

3.2. Tentative identification

The collected fungal species were tentatively identified up-to the genus level on the basis of their macroscopic and microscopic characteristics. It was found that 23 fungal species belonged to the genus *Ganoderma*, 15 to genus *Fomitopsis*, 6 to genus *Tricholoma*, 3 to genus *Schizophyllum*, and 4 to genus *Pleurotus* (fig. 2, 3; table 1).

Table 1: The generic characteristics and tentative genera of wood rot fungi collected from bio diverse regions of Chandigarh

Family	Genera	Characteristics	Isolates
Ganodermataceae	*Ganoderma* spp.	Basidiocarp: laccate, brown, woody to corky, dimidiate, 4-6cm in diameter; Upper surface ranges from white to yellow to pale brown and dark brown; Margin: Rolled; Pore surface: white, turns brown on bruising, 3-4 pores per mm, spherical to ovoid; Colony: White, round with regular margin and no elevation; Hyphal system: generative hyphae; Basidiospore: Ellipsoid, double walled, chambered looking, 9-12 μm in length.	WRE1, WRE2, WRE3, WRE4, WRE5,WRE7, WRE9, WRE10, WRE11,WRE16, WRE19, WRE20,WRE21, WRE24, WRE25, WRE26, WRE27, WRE28, WRE33, WRE36, WRE40, WRE41 and WRE48

Polyporaceae	*Pleurotus* sp.	Basidiocarp: dimidiate, fleshy, 14-15cm in size; Upper surface irregular, white; Margin: Wavy; Gills: white, decurrently arranged, 3-4 per cm, thick texture and fimbriate margin. Hyphal system: mono/dimitic, thin walled; Colony: White, round with regular margin.	WRE30, WRE37, WRE42 and WRE46
Schizophylaceae	*Schizophyllum* spp.	Basidiocarp: fan shaped, white, hairy, 2-3cm in size; Margin: Wavy; Gills: grey, radiating from the center, 1-2 per cm, entire margin; Basidiospore: ellipsoid.	WRE18, WRE50 and WRE51
Fomitopsidaceae	*Fomitopsis* spp.	Basidiocarp: white, fleshy with irregular surface and no defined shape. Margin: Wavy; Pore surface: White, 2-3 pores per mm, circular; Hyphal system: Trimitic, clamped hyphae.	WRE6, WRE8, WRE14, WRE15, WRE17, WRE22, WRE23, WRE29, WRE32, WRE34, WRE38,WRE39, WRE43, WRE44 and WRE47
Tricholomataceae	*Tricholoma* spp.	Pileus: Convex, Fleshy, 2-3cm in diameter; Upper surface fibrillose scaly, cream colored; Margin: Rolled; Stipe: Cylindrical, fleshy, 8-9 cm in length, not easily separable, attached centrally; Gills: simple, 2-3 per mm, cream colored, soft, attached to the pileus with wavy margin. Colony: White, round with regular margin and no elevation.	WRE12, WRE13, WRE31, WRE35, WRE45 and WRE49

Fig. 1: The wood rot fungal specimens (WRE1-WRE51) collected from different regions of Chandigarh

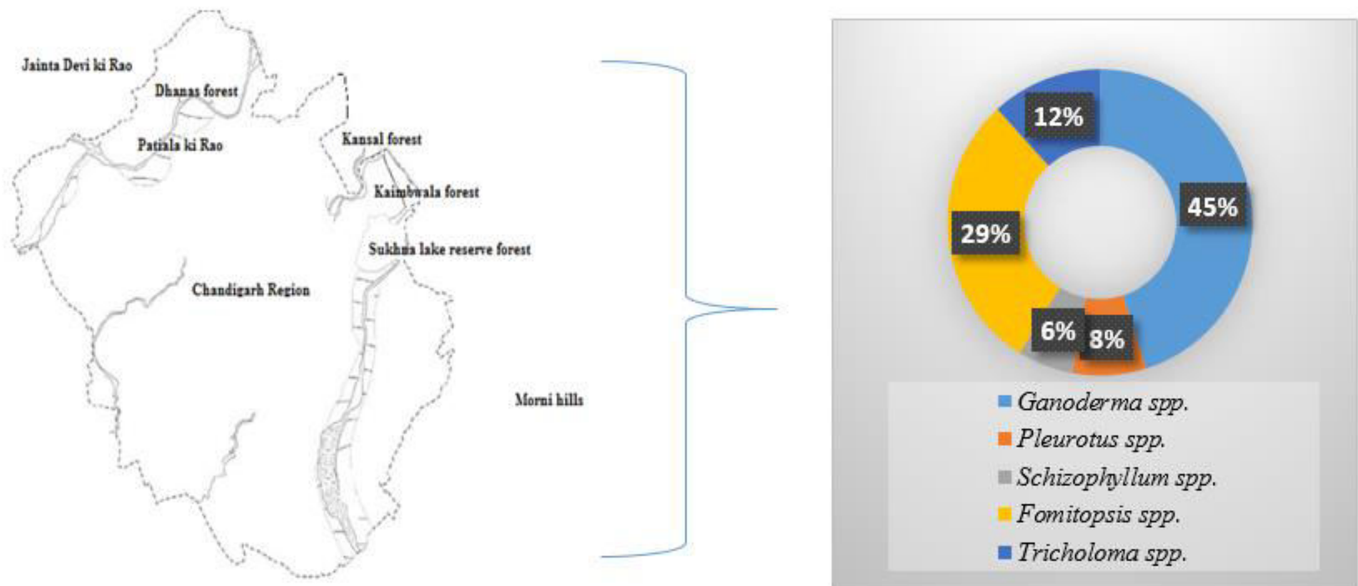
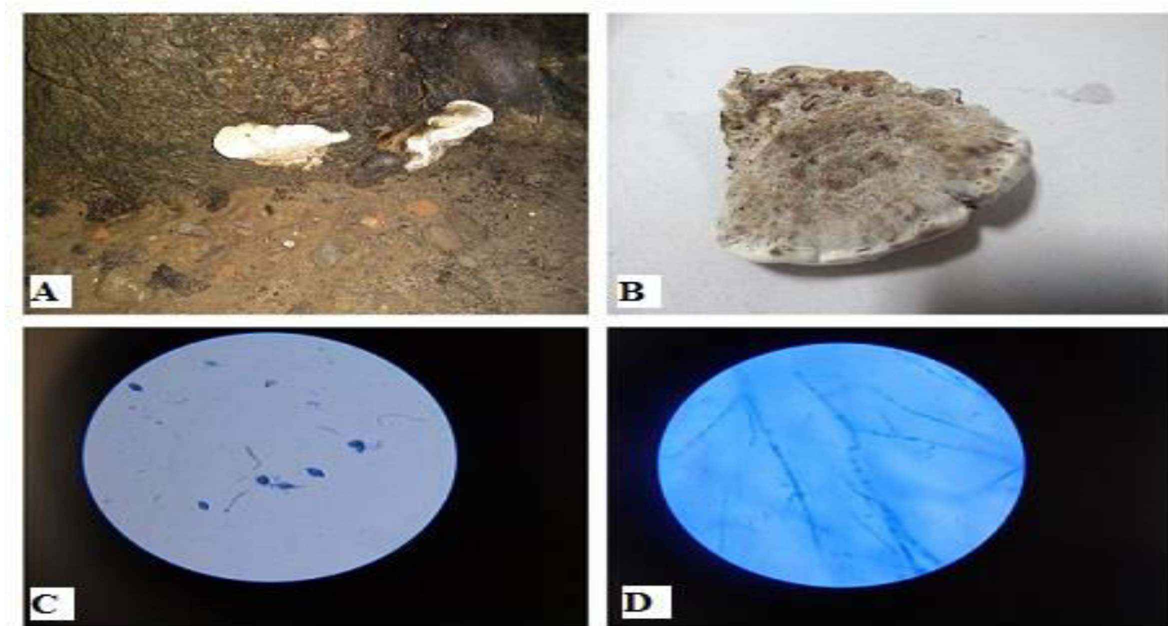


Fig. 2: Relative abundance of different wood rot species collected from the study area

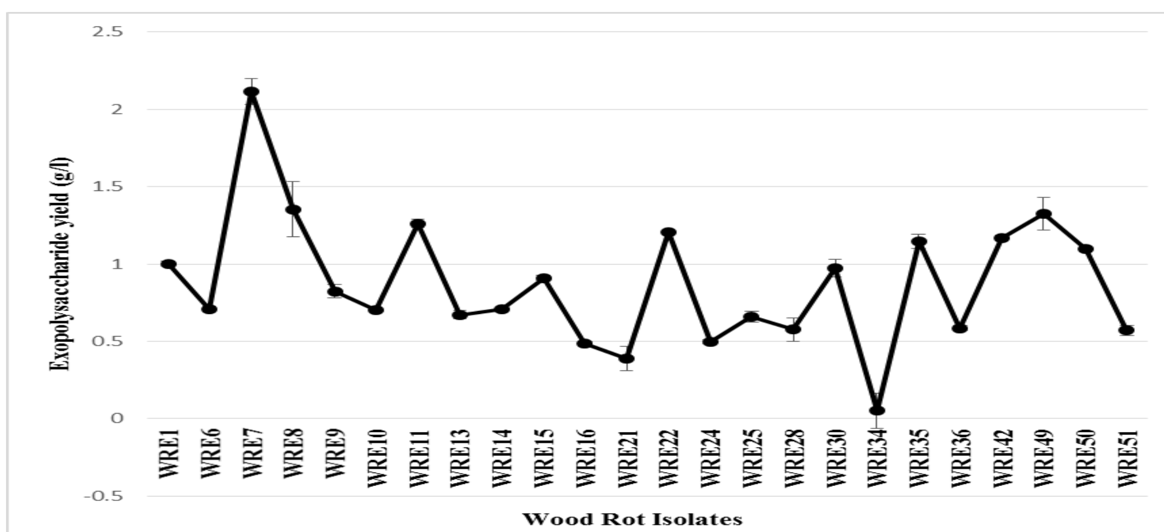


A) Fruiting bodies of WRE 7 in its natural habitat; B) Lower surface of the fruiting body bearing minute cylindrical pores; C) Ellipsoid, double walled, chamber like basidiospores; D) Generative, septate hyphae.

Fig. 3: Macroscopic and Microscopic characteristics of WRE7 (*Ganoderma* sp.) used in the present study

3.3. Screening of exopolysaccharide producing efficiency

The successfully isolated cultures were further screened for their ability to produce exopolysaccharide (EPS) under submerged conditions of fermentation and all the 24 successfully isolated cultures were found quiet capable of producing exopolysaccharide. EPS yield of 15 isolates was found to be less than 1g/l, 8 isolates gave the EPS yield in the range 1-2g/l and only one isolate gave the yield in the range 2-3 g/l. Maximum EPS was found to be produced by WRE7 (2.1555 g/l) and WRE34 was found to produce the minimum amount of EPS (0.051 g/l) (fig. 4). The polysaccharide produced by WRE7 (*Ganoderma* sp.) was thus further evaluated for its biotechnological potential.



Values are mean ±S.D. of three observations, Culture conditions: Medium: Potato Dextrose Broth; Temperature: 28±1°C; pH: 7; Inoculum size: two 5mm mycelial discs; Agitation speed: 120rpm; Incubation Period: 10 Days.

Fig. 4: The comparative yield of exopolysaccharide produced by different isolates.

3.4. Evaluation of biotechnological applications

3.4.1. Evaluation of bio-emulsifying activity

Bio-emulsifiers are microbial origin compounds which increase the availability of different hydrocarbons resulting in their degradation. Since the chemical emulsifiers are generally toxic and often non-biodegradable thus there is an increased demand from consumers for natural non-toxic emulsifiers. The exopolysaccharide produced by WRE 7 (*Ganoderma* sp.) was evaluated for its emulsifying activity. The results obtained revealed that the test emulsifier was capable of forming stable emulsions with different vegetable oils (olive, sesame, sunflower, coconut, mustard, and groundnut) and hydrocarbons (petroleum, diesel, xylene and liquid paraffin). The emulsification activity of the emulsifier was observed to lie in the range of 38.9 EA% to 57.14 EA%. It was found best compatible with mustard oil as observed by the highest EA% (57.14%) and % E 24-96 (51.7%, 48.6%, 45.3% and 42.6%). The emulsifying activity of Guar gum (standard) was observed to lie in the range 37.03 to 55.5% showing maximum emulsification activity with sesame seed oil (51.7 EA%) and petroleum (55.5 EA%). To the best of our knowledge this is the first report wherein the polysaccharide produced by *Ganoderma* sp. has been evaluated for its bio-emulsifying activity.

3.4.2. Evaluation of bio-flocculating activity

Flocculation is an essential parameter employed for the removal of suspended solids from a solution for which a variety of natural and synthetic flocculants are employed. Microorganisms, such as algae, bacteria, actinomyces and fungi, have been reported to produce bioflocculants composed of substances such as polysaccharides, proteins, glycoproteins or nucleic acids [17, 18]. Organic synthetic flocculants possess inherent drawbacks of being a source of carcinogenic monomers and are often non-biodegradable. Hence, bioflocculants could replace currently used organic, synthetic and toxic chemical reagents since they are biodegradable, nontoxic and free of secondary pollution risk. A limited number of studies are available on the bio-flocculant production by fungi [19]. The exopolysaccharide produced by WRE7 (*Ganoderma* sp.) was evaluated for its flocculating potential. The results obtained revealed that the polysaccharide was found capable of efficiently flocculating the suspended clay particles with a maximum flocculating activity of 61.3%. The flocculating activity however was found to be lower than the standard flocculants Sodium alginate (92.9%) and guar gum (96.2%). This is by far the first report wherein the bio-flocculating potential of *Ganoderma* sp. exopolysaccharide has been evaluated.

3.4.3. Evaluation of anti-oxidant activity

3.4.3.1. Free radical scavenging assay by DPPH method

The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517nm. When antioxidants react with DPPH, the stable free radical becomes paired off in the presence of a hydrogen donor and gets reduced to DPPHH as a result the

absorbance decreases from the DPPH-radical to the DPPH-H form resulting in decolorization of DPPH (purple to yellow). More the decolorization more is the reducing ability. The DPPH neutralizing activity of the exopolysaccharide was found to increase with an increase in the concentration of the exopolysaccharide. The test EPS was found to be capable of neutralizing the DPPH free radicals by 53.4%, 60.1%, 63.4%, 67.8% and 72.6% at a concentration of 200, 400, 600, 800 and 1000 µg/ml. The maximum DPPH neutralizing activity of 72.6% was obtained at a concentration of 1000 µg/ml (fig. 5).

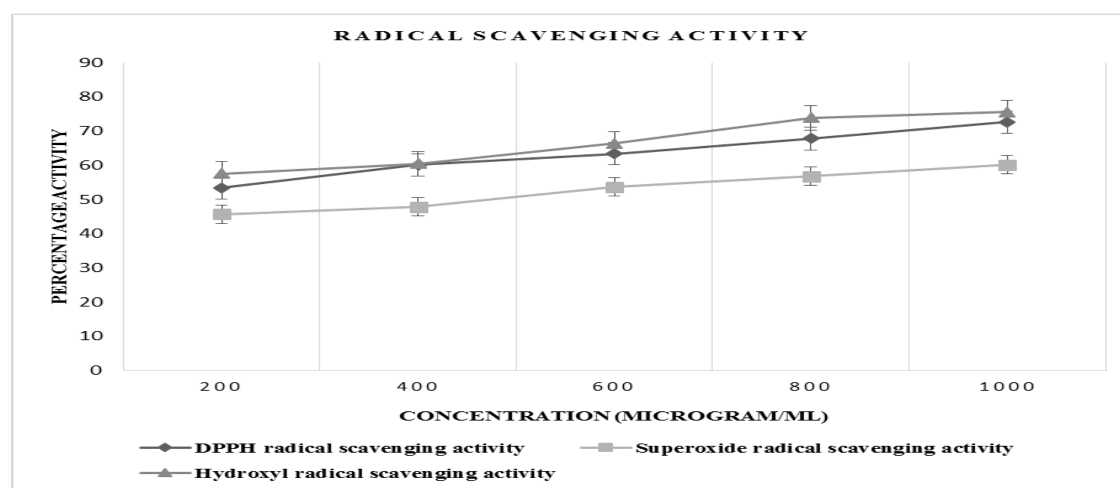
3.4.3.2. Superoxide radical scavenging activity

Superoxide is an inorganic radical anion which oxidizes various cellular components such as lipids and proteins leading to cell damage. The superoxide radicals in the present study were generated via the pyrogallol oxidation. The radical scavenging activity of the test EPS was found to be concentration dependent which increased with an increase in the EPS concentration. The radical scavenging activity at concentrations 200, 400, 600, 800 and 1000 µg/ml was found to be 45.7%, 47.8%, 53.6%, 56.8%, and 60.12% (fig. 5).

3.4.3.3. Hydroxyl radical scavenging activity

Hydroxyl radicals are highly reactive species which react with carbohydrates, lipids, proteins and DNA leading to cell death and tissue damage [20, 21]. The hydroxyl radical scavenging activity of the test exopolysaccharide (EPS) was found to be concentration dependent. The hydroxyl radical scavenging activity of the test EPS at a concentration of 200, 400, 600, 800 and 1000 µg/ml was found to be 57.5%, 60.45%, 66.35%, 73.87% and 75.52%, respectively. The maximum activity of 76.14% was obtained at a concentration of 1000 µg/ml (fig. 5).

Kan et al., [24] in a study on the antioxidant activities of polysaccharide from the medicinal mushroom *G. lucidum* reported that the polysaccharide was capable of efficiently scavenging the DPPH radicals, Hydroxyl radicals, superoxide anion radicals and Ferrous ion (Fe^{2+}) chelating radicals with a maximum activity of 90.50% at a concentration of the dose of 4 mg/ml. Similar results were reported by Kang et al., [23] and Yan et al., [24] in a study conducted on the antioxidant activity of polysaccharide obtained from *Ganoderma lucidium*. In a study by Chen et al., [25] it was reported that the purified polysaccharide obtained from the fruiting bodies of *Ganoderma atrum* showed strong DPPH and superoxide radical scavenging activity.



Values are mean S.D. of three observations

Fig.5: Free radical scavenging activity of the exopolysaccharide produced by isolate WRE7

4. CONCLUSION

The aim of the study was to explore the bio-diverse regions of Chandigarh for the collection and screening of exopolysaccharide producing potential of wood rot fungi belonging to various families. A total of 51 fungal samples were collected of which 24 were successfully isolated into pure cultures and were tentatively identified. The isolated cultures were found to belong to 5 different families *i.e.* Ganodermataceae, Polyporaceae, Schizophylaceae, Fomitopsidaceae, and Tricholomataceae. The isolates were further screened for their ability to produce exopolysaccharide.

Remarkable yields were obtained by the isolates belonging to the genus *Ganoderma* in the range of 0.484 to 2.155 g/l with the maximum of 2.155g/l by isolate WRE7. The polysaccharide produced by the isolate WRE7 was found to possess remarkable emulsifying and antioxidant activity with activity at par with commercially used emulsifiers and antioxidant agents. However, the flocculating activity of the test EPS was found to be comparatively lower than the standard flocculants. Thus, it can be concluded that Chandigarh region harbors a range of diverse groups of wood rot fungi, attributing to its climatic conditions and vegetation and the exopolysaccharide produced by them can be considered as a potential alternative source of animal and plant derived gums for varied applications in food, chemical, and pharmaceutical industries.

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Conflict of interest

The authors declare that there is no Conflict of Interests Regarding the Publication of this paper.

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