Exopolysaccharide Producing Potential of Indigenous White Rot Fungi From Foot Hill Forests of Lower Shivalik Ranges of Chandigarh

Deepika Malik, *Deepak K. Rahi & Vijay Prabha
Department of Microbiology, Panjab University, Chandigarh-160014, India
**Corresponding Author: Deepak K Rahi*

ABSTRACT White Rot Fungi Are Known To Produce Highly Value Added Exopolysaccharides (EPS) On Virtues Of Their Structural And Functional nature. However, Till Date Only Hands Of Fungi Have Been Explored For Their Potential To Produce EPS, Especially The Wood Rot Group Have Not Been Given A Deserved Attention Despite Their Tremendous Diversity In Various Habitats Of India. Foot Hill Forests Of Lower Shivalik Ranges Of Himalaya Of Chandigarh Capital Region Are An Important Biodiverse Region Harbouring A Great Diversity Of White Rot Fungi. In Present Investigation A Total Of Sixteen Important Fungal Isolates Of White Rot Group Have Been Reported From These Forests. All Of Them Have Been Identified Tentatively Upto Genus Level On The Basis Of Morphological And Microscopical Characteristics And Isolated Into Pure Culture. They Have Been Found To Belong To 6 Different Families Viz., Ganodermataceae, Polyporaceae, Schizophyllaceae, Phanerochaetaceae, Fomitopsidaceae And Hymenochaetaceae. Further Studies On Screening Of Their Potential For Exopolysaccharide Production Resulted In Twelve Positive Cultures Out Of Sixteen With The Isolate RDM9,Giving The Maximum Yield Of EPS (7.9 g/L) Which Was Later Identified As *Pleurotus pulmonarius* by 18s RNA Sequencing.

Keywords: Indigenous; White Rot Fungi; *Pleurotus pulmonarius*; 18s RNA Sequencing; Exopolysaccharides Production

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Date of Submission: 19-02-2018 Date of acceptance: 06-03-2018

I. INTRODUCTION

Exopolysaccharides Are Natural Macromolecules Composed Of Several Monosaccharide Units And Are Synthesized At Different Stages Of Life Cycle Of Every Living Organism For Different Purposes. Presence Of Different Sequences Of Monomeric Units, Gylcosidic Linkages And Different Types Of Branching Patterns Impart These Polysaccharides With Great Structural And Functional Properties Which In Turn Are Employed In Various Applications. Exopolysaccharides Produced By Fungi Are Regarded As Value Added Biological Macromolecules For The Last Several Years And Find Many Applications In Industries, Pharmaceuticals, Food Etc. Although Fungal EPSs Are Highly Relevant, To Date Information Concerning Fungal Production Is Scarce And An Extensive Search For New Fungal Species That Can Produce Novel EPSs Is Still Required. The Diversity Of Climatic Conditions Present In India Made It A Natural Habitat Of Large Number Of Diverse Fungal Flora. Foot Hill Forests Of Lower Shivalik Ranges Of Himalaya Of Chandigarh Capital Region Occupy An Important Place In Harbouring Diverse Fungal Groups including White Rots. A Tremendous Diversity Of These Fungi Belonging To Various Genera And Species Are Found In Various Habitats Of These Forests. Besides, White Rot Fungi Have Been Reported To Produce Exopolysaccharides With Different Types And Properties. Therefore, In Present Communication The Exopolysaccharide Producing Potential Of White Rot Fungi Of These Forests Ranges Have Been Screened And Studied.

II. MATERIALS & METHODS

2.1. Collection And Isolation Of Fungal Cultures

The Fungal Samples (Fructifications) Belonging To Different White Rot Fungi Were Collected As Per Atri et al. [1] From Biodiverse Localities Of Foot Hill Forests Of Lower Shivalik Ranges Of Himalaya Of Chandigarh Capital Region. All The Important Characteristics Of Taxonomical Relevance Like Habit, Habitat And Morphological Details Of Fructifications (Shape, Size, Colour, Surface Details, Margin, Attachments Of Pileus, Gills, Stipe) Were Noted At The Spot Itself And The Samples Were Brought To The Laboratory For Microscopic Studies And Spore Prints Were Taken As Per Singer [2]. The Spore Prints Were Obtained By Removing The Pileus From The Stipe And Placing Over Half Black And White Paper Card, With Gills Facing The Card And Covered With A Large Petridish. The Pileus Was Kept Moist By Placing Wet Cotton Inside The Petri Plate. The Preparation Of Spore Print Usually Takes 6 Hours. The Paper With Spore Print Was Preserved

And The Colour Of The Spores Was Compared With The Standard Colour Catalogue As Per Kornerup And Wanscher [3]. For Microscopic Observation Of Different Tissues, The Dried Fructifications Were First Rehydrated In 5% KOH. The Exosporial Ornamentations Were Observed By Staining The Basidiospores With Melzer's Reagent As Per Singer [2]. Later On The Collected Fungal Samples Were Identified Tentatively Up To Genus Level By Studying And Correlating The Characteristics Of Taxonomical Importance With Respective Keys And Monographs [4,5,6,7,8,9,10,11,12,13,14,15,16,17,18]. The Samples Were Isolated Into Pure Cultures On Mushroom Complete Medium (MCM) And Preserved At 4°C Under Mineral Oil.

2.2. Screening Of Exopolysaccharide Producing Potential

The Successfully Isolated Cultures Of Different White Rot Fungi Were Screened For Their EPS Producing Efficiency As Per Maziero et al. [19]. For This, The Pure Cultures Of Different Fungal Organisms Were Grown In 100ml Sterilized Mushroom Complete Medium Broth At 28°C, With pH 5.5, For 7 And 14 Days Separately Under Shaking (At 150 rpm). After Respective Incubation, The Mycelial Biomass Was Separated By Centrifuging The Fermentation Broth At 10,000 rpm For 20 Minutes At 4°C And Subjected To Mycelial Dry Weight Determination By Drying At 60ºC Till The Constant Weight Was Obtained. The Resulting Culture Filtrate Was Mixed With 5% TCA (Tricarboxylic Acid) To Precipitate Out The Protein And Left Overnight At 4ºC. Next Day The Filtrate Was Centrifuged At 10,000 rpm For 20 Minutes At 4°C To Remove The Precipitated Protein And Again Left Overnight At 4°C After Adding With 1:4 (Filtrate: Ethanol) v/v Ethanol For Precipitation Of The Exopolysaccharides. Next Day, The Precipitated Exopolysaccharide Was Separated By Centrifugation Similarly As Mentioned Above. The Supernatant Fluid Was Discarded And The Pellet Of Precipitated Crude EPS Was Weighed, Lyophilized And Expressed In g/L.

2.3. Identification Of The Selected Isolate

The Fungal Isolate Giving The Maximum EPS Yield Was Selected For Detailed Taxonomic Studies (Including Cultural, Macro- & Microscopic And Molecular Studies) For Its Identification Up To Species Level. The Molecular Analysis For Confirming The Species Of The Selected Fungus Was Done With The Help Of Chromus Biotech, Bengaluru, India.

III. RESULTS

3.1. Collection And Isolation Of Mushroom Culture

The Survey Of Various Biodiverse Regions Of Foot Hill Forests Of Lower Shivalik Ranges Of Himalaya Of Chandigarh Capital Region resulted In Collection Of A Total Of Sixteen White Rot Fungal Species Belonging To 10 Different Genera (Table 1). All Of Them Were Obtained Into Pure Culture (Fig. 1) And Identified Tentatively Up To Genus Level. They Were Found To belong To 6 Different Families Viz., Ganodermataceae, Polyporaceae, Schizophyllaceae, Phanerochaetaceae, Fomitopsidaceae And Hymenochaetaceae on The Basis Of Cultural, Macroscopic And Microscopic Studies (Table 2).

Table 1: List Of White Rot Fungal Species Collected From Various Biodiverse Regions Of Chandigarh, India.

Isolate No.	Genera	Location
RDM1	*Fomitopsis* sp.	Zakir Hussain Rose Garden, Sector 16, Chandigarh
RDM2	*Ganoderma* sp.	Kasauli forest, Chandigarh
RDM3	*Ganoderma* sp.	Leisure valley, Sector 10, Chandigarh
RDM4	*Daedaleopsis* sp.	Kasauli forest, Chandigarh
RDM5	*Pleurotus* sp.	Dhanas forest, Chandigarh
RDM6	*Pleurotus* sp.	Saketari forest, Chandigarh
RDM7	*Ganoderma* sp.	Botanical Garden, Panjab University, Sector 14, Chandigarh
RDM	*Ganoderma* sp.	Saketri forest, Chandigarh
RDM9	*Pleurotus* sp.	Morni hill region of lower Shivalik ranges, Chandigarh
RDM10	*Lentinus* sp.	Kansal forest, Chandigarh
RDM11	*Schyzophyllum* sp.	Sukhna lake forest, North-East Of Sukhna Lake, Chandigarh
RDM12	*Phanerochaete* sp.	Kaimbwala forest, Chandigarh
RDM13	*Lentinus* sp.	Saketri forest, Chandigarh
RDM14	*Trametes* sp.	Zakir Hussain Rose Garden, Sector 16, Chandigarh
RDM15	*Polyporus* sp.	Kansal forest, Chandigarh
RDM16	*Hymenochaete* sp.	Kaimbwala forest, Chandigarh

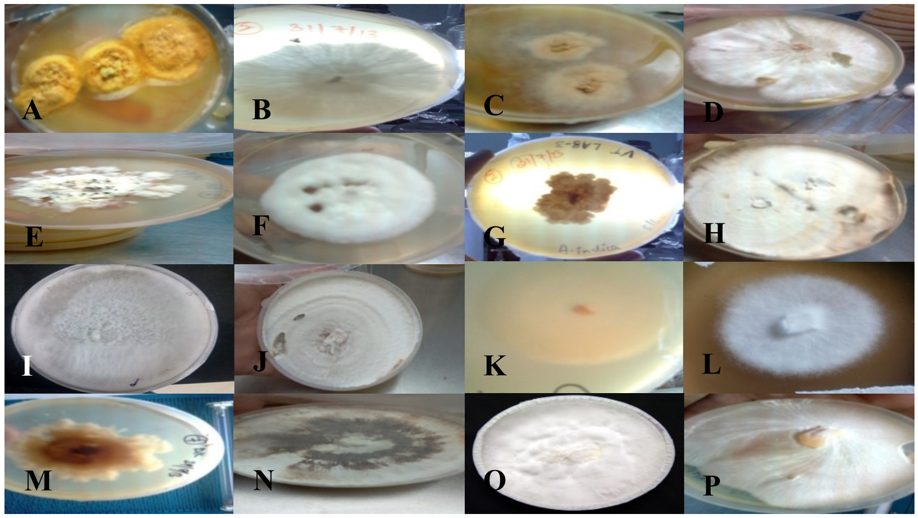

Figure 1: Pure Culture Of Sixteen Different White Rot Fungi On Mushroom Complete Medium (MCM) Plate.

(A): RDM1: *Fomitopsis* sp.; **(B):** RDM2: *Ganoderma* sp.; **(C):** RDM3: *Ganoderma* sp.; **(D):** RDM4: *Daedaleopsis* sp.; **(E):** RDM5: *Pleurotus* sp.; **(F):** RDM6: *Pleurotus* sp.; **(G):** RDM7: *Ganoderma* sp.; **(H):** RDM8: *Ganoderma* sp.; **(I):** RDM9: *Pleurotus pulmonarius*; **(J):** RDM10: *Lentinus* sp.; **(K):** RDM11: *Schyzophyllum* sp.; **(L):** RDM12: *Phanerochaete* sp.; **(M):** RDM13: *Lentinus* sp.; **(N):** RDM14: *Trametes* sp.; **(O):** RDM15: *Polyporus* sp.; **(P):** RDM16: *Hymenochaete* sp.

Table 2: The Generic Characteristics Of 16 Different White Rot Fungi Collected From Biodiverse Locations Of Foot Hill Forests Of Lower Shivalik Ranges Of Himalaya Of Chandigarh Capital Region.

Family	Genera	Characteristics
Ganodermataceae	*Ganoderma* sp.	Rapid And Permanent Browning Of The Pore Artist’s Bracket; Brown Surface When Scratched; The Bruising Is Permanent, So It Is Commonly Known As Coloured Spores With Size 6–8µm × 5-6µm; Basidiocarp: 12 × 7× 3cm, Woody To Corky; Laterally Stipitate With 2–3 Cm In Length; Pileus: Reniform, Upper Surface Laccate, Dark Reddish To Brown, Yellowish Towards Margin, Brittle, Soft, Margin: Blunt, Rounded, White; Pore Surface: Creamish To Milky Coffee, 4-6 Per Mm, Round; Context: 9mm Wide And Brown; Cutis Type: Thick Walled Colony White, Round, Radial, With Regular Margin And No Elevation; Hyphal System: Trimitic, Generative Hyphae, Thin Walled, With Clamp Connection; Basidiospore: Reddish Brown Ovalwith Size 6.5-7 µm × 5µm.
	Daedaleopsis sp.	Fruit Bodies Turn Pinkish Red When Bruised And With Age The Whole Bracket Turns To Dark Brown; Presence Of More Frequently Angular, Radially Elongated Or Labyrinthi Form Pores, Rigid, Corky, Thin, Applanate Pileus With Glabrous And Rugose Surface; Colony White, Round , Radial, Irregular Edges And Show No Elevation; Hyphal System: Trimitic, Generative Hyphae Present With Clamp Connection; Basidiospore Cylindrical, Cystidia Thick-Walled, 7-9 µm In Length And Nearly 2 µm Wide.
Polyporaceae	*Pleurotus* sp.	Habit Pleurotoid; Pigment Absent; Hymenophore Lamellate; Hyphae Thin Or Thick Walled; Veil Present; Spore Print Pure White Or Cream, Rarely Pink; Spores Hyaline, Smooth, Always Cylindrical; Basidia Normal; Metuloids Often Present; Cheilocystidia Usually Present; Subhymenium Well Differentiated And Broad; Stipe Present; Carpophores Sessile. Hyphal System Mono-, Di-, Or Amphimitic; Grows On Wood, More Rarely On Other Plant Tissues, On Dead And On Living Hosts.
	Lentinus sp.	Pigment Present, But Only In The Scales Of The Pileus, And/Or Appearing In The Carpophores In Age On Drying (Yellow); Hymenophore Usually Lamellate, Hyphae Parallel Or Interwoven But Always Distinctly Axillary Arranged; Spores Hyaline, Smooth, Ellipsoid-Oblong To Cylindric; Cystidia

		Absent Or Present With Thick Walls. Stipe Always Present.
	Polyporus sp.	Carpophores (Stem Of A Fruiting Body) Pleurotoid (Gilled Fungi With Laterally-Attached Fruiting Bodies) Or With Central Stipe, True Stipe Often Reduced And Pileus Directly Laterally Attached; Stipe Solid. Spores Mostly Cylindrical, Smooth And Thin-Walled; Basidia Relatively Broad, Clavate; Hyphae Are Thick Walled; Cystidia Absent; Mycelium With Clamp Connections.
	Trametes sp.	Small, Thin, Leathery; Stalk Absent; Usually Occurring In Overlapping Shelves Or Semi-Circular Rosettes; Many Multi-Coloured Zones, Alternating Hairy And Smooth, And Has White (Rarely Yellow) Pores. Caps Are 1" To 4" Wide.
Schizophyllaceae	*Schizophyllum* sp.	Gills On Its Underside; 1-5 Cm Wide; Fan-Shaped Or Irregular To Shell-Shaped; Upper Surface Covered With Small Hairs, Dry, White To Greyish Or Tan; Under Surface Composed Of Gill-Like Folds That Are Split Down The Middle, Whitish To Greyish; Without A Stem; Flesh Tough, Leathery, Pallid; Spore Print Is White; Microscopic Features: Spores 3-4 X 1-1.5 µ; Cylindrical To Elliptical; Smooth. Cystidia Absent. Pileipellis A Cutis Of Elements 3-6 µ Wide. Clamp Connections Present.
Phanerochaetaceae	*Phanerochaete* sp.	Corticioid, Lignicolous, White Rot Saprotrophs; Variably Smooth, Tuberculate, Or Spiny Spore-Bearing Surfaces; Inamyloid, Smooth, Thin-Walled Spores, And Monomitic Hyphal Construction.
Fomitopsidaceae	*Fomitopsis* sp.	Fruiting Bodies Are Generally Dark Coloured To Reddish Brown Or Black, With A Convex To Hoof Shaped (Cap) Supported By A Dark Stipe. The Pileus Can Be 2–6 Inches In Diameter, Texture Is Hard, Tough, Pore Surface White To Brown; Spores Brown.
Hymenochaetaceae	*Hymenochaete* sp.	Simple Septa In The Generative Hyphae; Xanthochroic Reaction, Yellow To Deep Brown Basidiomata (Sporocarp Of A Basidiomycete), Frequent Occurrence Of Setae, And They Cause White Rot.

3.2. Screening Of Exopolysaccharide Producing Potential

All The Sixteen Successfully Isolated Fungal Samples Were Screened For Their Potential To Produce Exopolysaccharides (EPS). Results Obtained Revealed That The 12 Cultures Were Capable Of Producing EPS After 7 Days Of Incubation (Fig. 2). However, The Species Of *Pleurotus pulmonarius* Was Found To Produce The Maximum EPS (7.9 g/L) With The Corresponding Biomass Of 4.29g/L, Followed By The Species Of RDM10 (4.8 g/L) And RDM6 (3.1 g/L) With The Corresponding Biomass Of 4.4 g/L And 2.99 g/L Respectively. The EPS Yield Of RDM16 Was Found To Be The Least (0.17 g/L) With The Corresponding Biomass Of 3.5 g/L. The Species Of RDM1, RDM3, RDM14 And RDM15 Produced The EPS In A Negligible Amount. The Study Revealed No Correlation Between The EPS Yield And Biomass Production. The Production Of EPS Was Observed After 3 To 4 Days Of Incubation With The Continuous Increase In Viscosity Of The Medium Till 7th Day (Fig.3). Thereafter, A Gradual Decrease In The Yield Was Observed With The Least On 14th Day As Evidenced In Fig.4.

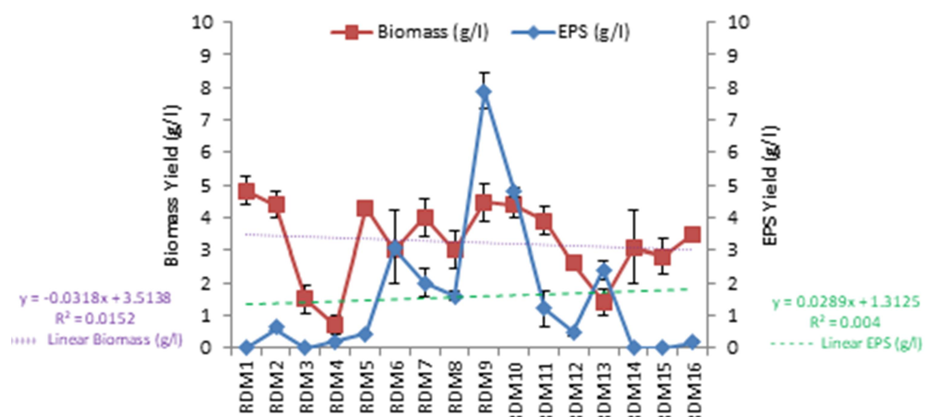

Figure 2: Production Of Exopolysaccharides By 16 Different White Rot Fungal sp. After 7days Of Incubation.

Fermentation Conditions: Medium: Mushroom Complete Medium Broth; Medium Quantity: 100ml/250 ml Flask; Temperature: 28±1°C; pH: 5.1 ± 0.2; Inoculum Size: Mycelial Disc (10mm); Agitation Speed: 150 rpm.
Values Are Mean ±S.D. Of Three Observations.

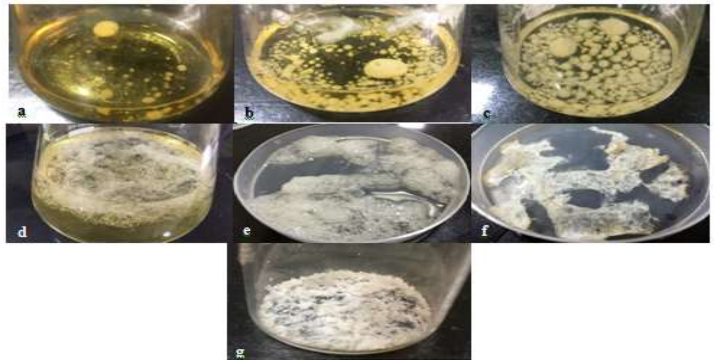

Figure 3: Fermentation For Production Of Exopolysaccharide (EPS) In Mushroom Complete Medium Broth By *Pleurotus pulmonarius* (The Test Fungus; RDM 9) For 7 Days (A) Flask Showing The Pelleted Growth Of The Test Fungus On 3rd Day; (B): Flask Showing The Increase In Pelleted Growth Of The Test Fungus On 5th Day; (C): Flask Showing The Thick Growth Of The Test Fungus And Viscous Consistency Of The Production Medium. Also Visible Is The Dense, Creamy White, Rough, Hairy, Star Shaped, Hard Pellets Of Fungal Growth; (D): Flask Showing The Crude Precipitation Of EPS Just After Addition Of Ethanol In The Ratio Of 1:4 (Filtrate: Ethanol = 1:4 v/v) At The End Of The Incubation; (E): Thick, Viscous, Creamish-White EPS On The Petriplate Separated By Centrifugation From The Fermentation Broth; (F): The Flakes Of Creamish-White, Lyophilized EPS; (G): The Powdered, Lyophilized EPS In A Glass Vial.

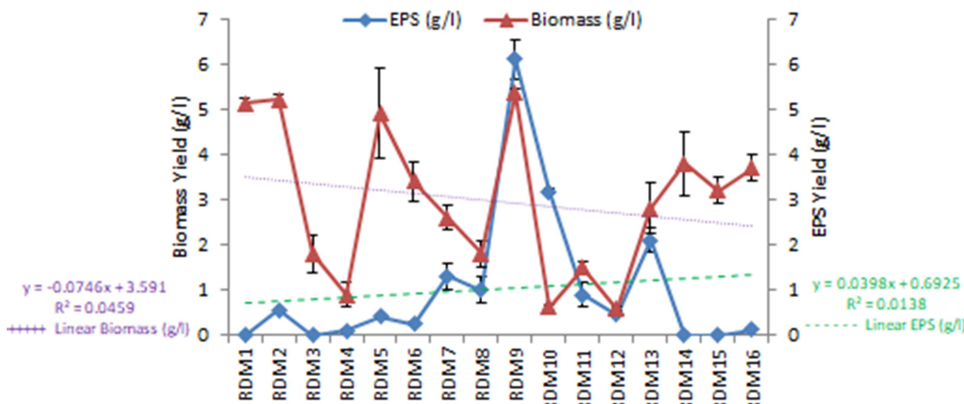

Figure 4: Production Of Exopolysaccharides By 16 Different White Rot Fungal sp. After 14 days Of Incubation.

Fermentation Conditions: Medium: Mushroom Complete Medium Broth; Medium Quantity: 100ml/250 ml Flask; Temperature: 28±1°C; pH: 5.1 ± 0.2; Inoculum Size Mycelial Disc (10mm); Agitation Speed: 150 rpm.
Values Are Mean ±S.D. Of Three Observations.

3.3 Identification Of The Selected Isolate

The White Rot Isolate Of *Pleurotus* (RDM9) Which Was Selected For Production Of EPS On The Basis Of Its High Yield Was Identified Up To Species Level By Studying Various Characteristics Including Cultural, Macro- And Microscopic (Table 3).

Table 3: Taxonomic Description Of *Pleurotus* sp. (RDM9)

<table>
<tr><th colspan="12">Habit and Habitat</th></tr>
<tr><td colspan="12">Lignicolous; found in shelf-like clusters on Ficus religiosa</td></tr>
<tr><th colspan="12">Cultural Characteristics (on PDA plate)</th></tr>
<tr><td colspan="12">White colony, round, radial, with irregular edges, becoming a thick elevated mycelial mat. Over incubated cultures become tough to cut from the plate.</td></tr>
<tr><th colspan="12">Macroscopic Characteristics</th></tr>
<tr><th colspan="5">Sporocarp/ Pileus</th><th colspan="3">Gills</th><th colspan="2">Stipe</th><th colspan="2">Texture</th></tr>
<tr><td colspan="5">Sporocarp white to cream, running down the stem; offset and fleshy; Pileus about 5-20 cm in diameter; 1.9-7.8 inches; convex, becoming flat or somewhat depressed; lung-shaped to semi-circular, or nearly circular; fleshy; greasy when young and fresh; fairly smooth; Light brown to creamish in colour, usually without dark brown colorations; the margin in rolled when young, later wavy and very finely lined.</td><td colspan="3">White to creamish, sub-decurrent to decurrent, thick, opaque, edges smooth, close or nearly distant.</td><td colspan="2">Sometimes absent or rudimentary, but often present; 1-7 cm long and up to 1.5 cm thick; eccentric or lateral or central; smooth.</td><td colspan="2">Thick and white.</td></tr>
<tr><th colspan="12">Microscopic Features</th></tr>
<tr><th colspan="6">Basidia/Basidiospores</th><th colspan="6">Hyphal system</th></tr>
<tr><td colspan="6">Basidia 20-30 x 6-8 μm; Spores 7-10 μm in length and 2.5-5 μm wide; smooth; cylindrical to long-elliptical, white to yellowish or grey coloured.</td><td colspan="6">Septate; thin- to moderately thick walled, generative hyphae present with clamp connections.</td></tr>
<tr><th colspan="12">Other Characteristics</th></tr>
<tr><th colspan="4">Chemical Reactions</th><th colspan="4">Spore Print</th><th colspan="4">Odour and Taste</th></tr>
<tr><td colspan="4">Appearance of orangish colour on addition on Potassium hydroxide (KOH) on cap surface.</td><td colspan="4">Whitish, greyish or lilac.</td><td colspan="4">Odour agreeable; taste mild.</td></tr>
</table>

IV. MOLECULAR IDENTIFICATION

The Molecular Studies Performed By 18S rRNA Sequencing Of The Selected Test Fungus *Pleurotus* sp. (RDM9) Revealed The Organism Belongs To *Pleurotus pulmonarius* (Fig. 5 & 6; Table 4).

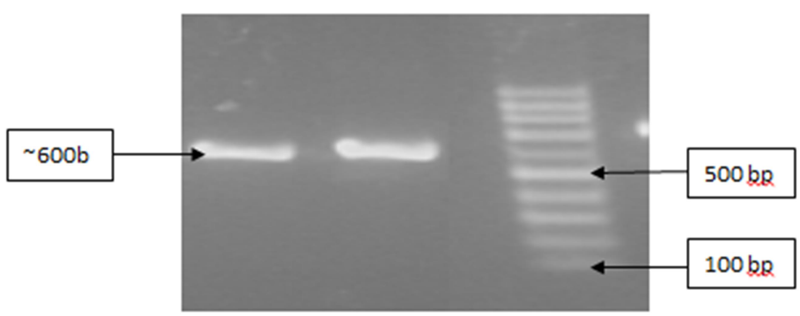

Figure 5: PCR Amplification Of ITS Region From The Fungal Sample RDM9. The Size Of PCR Amplified Product Is ~ 600bp.

Table 4: Aligned Sequence Data Of *Pleurotus pulmonarius.*

Sample RDM9 (639bp)
TAGTTGGCGGGAGGGACTGCGGAGGACATTAATGAATGCACTATGTAGTTGTTGCTGGCCTCTAGGGGCATGTGCACGCTTC ACGTAGTCTTTCAACCACCTGTGAACTTTTGATAGATCTGTGAAGTCGTCCTTCAAGTCGTCAGACTTGGTTTGCTGGGATTTA AACGTCTCGGTGTGACAACGCAGTCTATTTACTTAACACACCCCAAATGTATGTCTACGAATGTCATTTAATGGGCCTTGTGC CTATAAACCATAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTTGAG TGTCATTAAATTCTCAAACTCACATTTATTTGTGATGTTTGGATTGTTGGGGGTTGCTGGCTGTAACAAGTCGGCTCCTCTTAA ATGCATTAGCAGGACTTCTCATTGCCTCTGCGCATGATGTGATAATTATCACTCATCAATAGCACGCATGAATAGAGTCCAGC TCTCTAATCGTCCGCAAGGACAATTTGACAATTCGACCTCAAATCAGTAGGATGCAAG

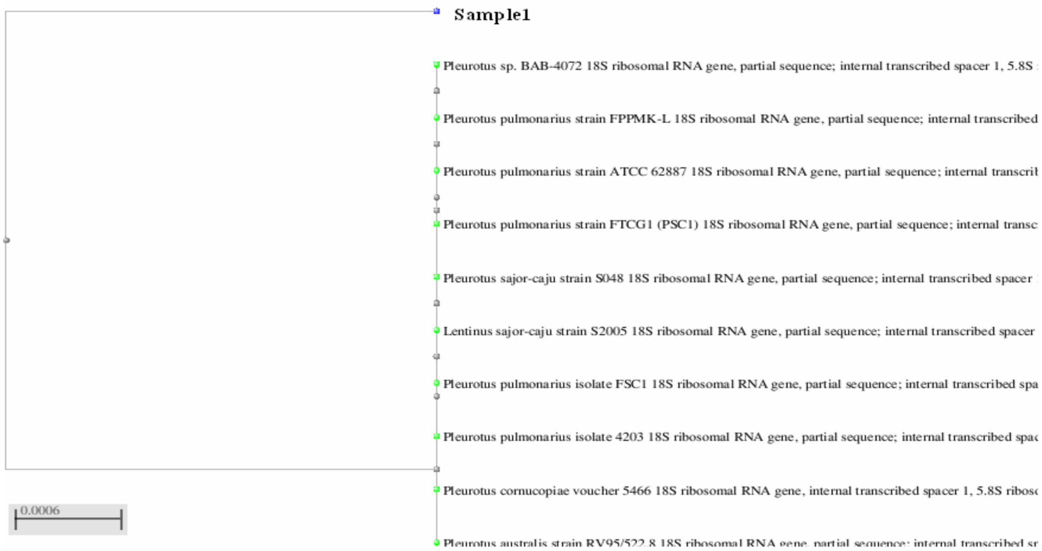

Figure 6: Tree Produced Using Weighbor, Jukes-Cantor Correction And Bootstrap.

V. CONCLUSION

The Fungal EPS Are An Important Metabolite Having Great Scope For Novel Properties And Applications, But The Information Concerning To Their Production By Different Groups Of Fungi Is Very Scarce And There Is Need To Explore And Screen More Fungal Groups For Their Ability To Produce Exopolysaccharides. Till Date The Exopolysaccharides Being Used In Various Industries Are Generally Plant And Bacterial Based Which Sometimes May Not Fulfill The Desired Expectations As Far As Properties, Yield And Economics Are Concerned. The White Rot Group Of Fungi Has An Enormous Potential To Fulfill Such Requirements If Explored Systematically. Besides, The Favorable Climatic Conditions Prevalent In The Country Have Made The Environmental Conditions Conducive For The Growth, Nurturing And Flourishment Of These Fungi. Therefore, The Need Is For Proper Identification, Screening And Characterization Of These Very Biomolecules For Their Development As Value Added Products.

ACKNOWLEDGEMENTS

Authors Are Thankful To UGC-BSR, Govt. Of India For Providing The Financial Assistance To Carry Out The Present Work.

Conflict Of Interest

The Authors Declare That There Is No Conflict Of Interests Regarding The Publication Of This Paper.

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IOSR Journal of Pharmacy (IOSR-PHR) is UGC approved Journal with Sl. No. 5012

Malik D, Rahi DK, Prabha V " Exopolysaccharide Producing Potential of Indigenous White Rot Fungi Rom Foot Hill Forests of Lower Shivalik Ranges of Chandigarh" IOSR Journal of Pharmacy (IOSRPHR), vol. 8, no. 3, 2018, pp. 01-07