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SOMNATH ROY AND BISHWANATH CHAKRABORTY

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Department of Botany,
University of Calcutta,
Kolkata 700 019, India

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Morphological and molecular characterization of four *Pleurotus* spp. and evaluation of their antioxidant properties

SOMNATH ROY[1] AND BISHWANATH CHAKRABORTY[2]*
[1] *Department of Botany, Malda College, Malda 732101, West Bengal*
[2] *Department of Biological Sciences, Aliah University, New Town, Kolkata700016*

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Four oyster mushroom species are cultivated in North Bengal for its good taste, texture and therapeutic values. Morphological and anatomical characteristics of four *Pleurotus* species (*P. djamor, P. sajor-caju, P. ostreatus* and *P floridanus*) have been elucidated. Malt extract agar showed highest mycelial growth rate followed by the potato dextrose agar, while water agar was lowest. All four *Pleurotus* species were identified using 18S rDNA sequencing as *P. djamor* (KT768094), *P. sajor-caju* (KT818506), *P. ostreatus* (KT768095) and *P floridanus* (KT826605) and subsequently all the sequence have been deposited in the NCBI Genbank. 18S r DNA sequence alignments of *P. djamor, P. sajor-caju, P. ostreatus* and *P floridanus* with other ex-type isolates have been presented and the conserved regions of the gene have been demonstrated in different colours. Phylogenetic placement of all four *Pleurotus* species with other 41 ex-type strains obtained from NCBI GenBank was compared using UPGMA method in MEGA11 software and result has been presented. Antioxidant activity such as DPPH scavenging activity, ferric reducing antioxidant power activity as well as flavonoid activity of four *Pleurotus* species were also evaluated. *P. djamor* and *P. ostreatus* showed higher DPPH scavenging activity in comparison to other two species. Highest amount of reducing power activity was observed in *P. djamor* and lowest in *P. ostreatus* and *P. sajor-caju. P. djamor* and *P. floridanus* also showed higher flavonoid content in comparison to other two species.

Keywords: *Pleurotus djamor, Pleurotus sajor-caju, Pleurotus ostreatus, Pleurotus floridanus,* antioxidant activity

INTRODUCTION

Mushroom cultivation is the bioconversion of organic waste materials into nutritional food with enriched therapeutic values from the ancient time (Manzi *et al*. 2001). Oyster mushroom, one of the widely cultivated mushrooms which is the second largest consumed edible mushroom in the world (Royse *et al*.,2017) and known for its good taste, texture and nutritional values. Several species of *Pleurotus* is being cultivated throughout the world such as *P. pulmonaris, P. ostreatus* (Roy *et al.,* 2015b), *P. djamor* (Roy *et al*. 2015a), *P. cystidiosus* (Ritota and Manzi , 2019) and *P. floridanus* (Andrew, 2023) . Effect of pruned tea leaves on the yield and nutritional quality of two species of *Pleurotus* in North Bengal have also been reported (Roy and Chakraborty, 2018). Application of molecular tools are more reliable to identify the species of *Pleurotus* along with their morphological characterization (Drug *et al*. 2012). PCR and non-PCR based molecular markers are the widely used molecular tools to characterize the mushroom (Fonseca *et al*. 2008), however direct sequence of DNA can be used for phylogenetic study. Comparison of the internal transcribed spacer (ITS) also can be used to analyse the molecular phylogeny as it is very easy to amplify with a very high degree of variations with the closely related fungal species (Schoch *et al*. 2011). In the present investigation attempts have been made to compare both morphological and molecular characters among four species of *Pleurotus* (*P. djamor*, *P. sajor-caju, P. ostreatus* and *P. floridanus*) cultivated in North Bengal and to evaluate their antioxidant properties.

*Correspondence: bncnbu@gmail.com

MATERIALS AND METHODS

Mycelial growth pattern

The pure culture of *Pleurotus ostreatus, Pleurotus sajor-caju, Pleurotus djamor* and *Pleurotus*

floridanus isolates were grown initially in potato dextrose agar medium for 7 days and agar block (4 mm) from advanced zone of hyphal mat were further allowed to grow in three different media like Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Water Agar and mycelial growth patterns were recorded following incubation at 27^{0}C .

Anatomical characters of fruit body

Transverse sections of the mushroom gills were stained with lacto phenol - cotton blue (1:1) and observed in the Leica DM3200 microscope using 20X and 40X magnifications. Photographs were taken using Leica Application Suit (LAS Version 4.4.0) software.

Genomic DNA extraction and purification

Total genomic DNA from four *Pleurotus* species were isolated by N-cetyl-N,N,N-trimethyl-ammonium bromide (CTAB) method. Fungal mycelium (0.5 g) was taken and grinded with 25 mg PVPP using mini grinder, centrifuged at 10000 rpm for 2 min. The pellet was washed with sterile distilled water and again centrifuged at 10000 rpm for 20 min.. Then 675 µl of extraction buffer was added and incubated at 37^{0}C for 30 min. After that 75 µl of SDS (20%) was added and incubated at 65^{0}C for 2 hr and again centrifuged at 10000 rpm for 10 min at 4^{0}C.The supernatant was collected in a sterile micro centrifuge tube and equal volume of PCI (phenol: chloroform: isoamyl alcohol, 25:24:1) was added and shaken well. Then it was centrifuged at 10000 rpm for 10 min. at 4^{0}C and equal volume of chloroform: isoamyl alcohol (24:1) was added. It was again centrifuged at 10000 rpm for 10 min. at 4^{0}C. The aqueous phase was removed, taken in a sterile micro centrifuge tube, isopropyl alcohol was added, incubated at room temperature for 1hr and centrifuged at 10000 rpm for 10 min. The pellet was washed using 500 µl of 70% ethanol and centrifuged at 10000 rpm for 10 min at room temperature. Pellet was dried and dissolved in 20 µl sterile distilled water. For purification, genomic DNA was resuspended in 100 µl 1X TE buffer and incubated at 37^{0}C for 30 min with RNAse. After incubation the sample was re-extracted with PCI (phenol: chloroform: isoamyl alcohol, 25:24:1) and RNA free DNA was precipitated with chilled ethanol. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all samples produced clear sharp bands, indicating good quality of DNA.

PCR amplification of ITS region

Genomic DNA prepared from four *Pleurotus* species (*P. djamor*, *P. sajor-caju, P. ostreatus* and *P. floridanus*) were taken up for ITS-PCR amplification. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10µ 10 X Taq pol buffer, 1 µl of 1U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers (Table 1) and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94^{0}C for 5 min followed by 30 cycles of denaturation at 94^{0}C for 30 sec, annealing at 59^{0}C for 30 sec and extension at 70^{0}C for 2 min and the final extension at 72^{0}C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8µl) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis. PCR products were sent for sequencing to Credora Life Sciences, Bangalore.

18S rDNA sequencing

Sequencing of 18S rDNA was done bi-directionally using the ITS primer pairs by Credora Life Sciences, Bangalore. DNA sequence information was analysed using bioinformatics algorithms tool MEGA 4, as well as the few online software. Multiple sequence alignment of DNA sequences edited by using the software BioEdit and aligned with Clustral W algorithms.

BLAST analysis of the sequences

The DNA sequences were analysed using the alignment software of BLAST algorithm (http:// ingene2.upm.edu.my/Blast, Altschul *et al.*, 1997) for the different characteristic of DNA sequence for the identification of microorganism. Identification of microorganism was done on the basis of homology of sequence.

rDNA sequence submission and analysis

The DNA sequences were deposited to NCBI Genbank through Banklit procedure and approved

Table 1: PCR primers of ITS 4 and ITS6 used for sequencing

Oligonucleotide Sequences (5'- 3')		GC %	Tm Value ^{0}	Length	Product Size
ITS 4	TCCTCCGCTTATTGATATG	50	51.0 C	19	700 bp
ITS 6	GAAGGTGAAGTCGTAACAAGG	60	56.0^{0}C	21	

Table 2: 18S rDNA sequence of *P. djamor, P. sajor-caju, P. ostreatus and P. floridanus*

Species	Partial sequence of 18S rDNA	NCBI Accession No
P. djamor	CTGCGAAGGATCATTAATAATTACAAAAGCTTTTGAAGTTGTTTTGCTGGTCTCTAGGGACATTG TGCACGCTTCATTAGTTTCCACTTCATACCCCTGTGCACCTTTGATAGATTTCGGTTTGGGTTAT CCTTTGGTTTTTTTTTCTTAATTGAAAGGCCTTTGGTTTCCTTAAAACGACTTCTATACTATACCAC ACACCAAATGTATGTTTTATAATGAATGGTTTATAATGACAAGGCCATGACCTTATAAA CTTAATA CAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGT AATGTGAATTGCAGAATTCAGTGAATCATCGATCTTTGAACGCACCTTGCGCCCTTTGGTATTCC GAAGGCATGCCTGTTTGAGTGTCATTAAATTCTCAAACTCTATGACTTTATTGTTGTAGCTGTTTG GATTGTTGGGGGTTGCTGGCTTCTTTCTTTGAAGTCGGCTCCTCTTAAATGCAT TAGCGGGACTT TGTTGCCTCTGCGCATAGTGTGATAATTATCTACGCTAGACGCATGCAATTCTTATATTGTCCAG CTTTCTAATCGTCTCAAGGGACAATTACTTTGACAATTTGACCTCAAATCAGGTAGGACTACCCG CTCTGCGGAAGGATCATTAATGAATTCACTATGGAGTTGTTGCTGGCCTCTAGGGGCAGTGCACGC TTCACTAGTCTTTCAACCACCTGTG AACTTTTGATAGATCTGTGAAGTCGTCCTTCAAGTCGTCA GACTTGGTTTGCTGGGATTTAAACGTCTCGGTGTGACAACGCAGTCTATTTACTTAACACACCCC AAATGTATGTCTACGAATGTCATTTAATGGGCCTTGTGCCTATAAACCATAATACAACTTTCAACA ACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC	KT768094
P. sajorcaju	AGAATTCAGTGAATCATCGAA TCTTTGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCATG CCTGTTTGAGTGTCATTAAATTCTCAAACTCACATTTATTTGTGATGTTTGGATTGTTGGGGGTTG CTGGCTGTAACAAGTCGGCTCCTCTTAAATGCATTAGCAGGACTTCTCATTGCCTCTGCGCATG ATGTGATAATTATCACTCATCAATAGCACGCATGAATAGAGTCCAGCTCTCTAATCGTCCGCAAG GACAATTTGACAATTTGA CCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAG CGGAGGAGAGGACGGACATCTACCTGATTGAGGTCAATTGTCAATTGTCCTTGCGGACGATTG GAGAGCTGGACTCTATTCATGCGTGCTATTGATGAGTGATAATTATCACATCATGCGCAGAGGCAA TGAGAA GTCCTGCTAATGCATTTAAGAGGAGCCGACCTGTCAAGGCCAGCAGCCCCCAACAATCCAAAC	KT818506
P.ostreatus	ATCACAATTGGAAAATGGCAAAGGGCGTTTGTGTCTTCTACCCCCTCTGCTGCGCAAGTCCCTC ATATTACAACAAAGCTCATCTAGAATACTATGACCTGATCATCCAGCTCCTTATTGTGTATTCATC CTGACTTTTCATCCAGGAATCCACCATCACGGCAATTGAATCAAACGCCTTCCGCCCGCAGATT ATGCTCTGCAAGGTGACCATCTCCCCGCACATACCCCCCCATCACAAGAT TTCCTGATGTACTG CATATTTTTTTGCAGAACATTCTGCGGAAGGATCATTATGAATTCACTATGGAGTTGTTGCTGGCCTC TAGGGGCATGTGCACGCTTCACTAGTCTTTCAACCACCTGTGAACTTTTGATAGATCTGTGAAGTCGT CTCTCAAGTCGTCAGACTTGGTTGCTGGGATTTAAACGTCTCGGTGTGACTACGCAGTCTATTTACTTA CACACCCCAAATGTATGTCTACGAATGTCATTTAATGGGCCTTGTGCCTTTAAACCATAATACAACTTTCAA CAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG	KT768095
P.floridanus	AATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCATGCC TGTTTGAGTGTCATTAAATTCTCAAACTCACTTTGGTTTCTTTCCAATTGTGATGTTTGGATTGTT GGGGGCTGCTGGCCTTGACAGGTCGGCTCCTCTTAAATGCATTAGCAGGACTTCTCAT TGCCTC TGCGCATGATGTGATAATTATCACTCATCAATAGCACGCATGAATAGAGTCCAGCTCTCTAATCG TCCGCAAGGACAATTTGACAATTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCAT ATCAATAAGGCGGAGGAA	KT826605

as the ITS sequence after complete annotation and given accession numbers. All the conserved regions of 18S r DNA sequences with other ex-type isolate sequences obtained from NCBI GenBank data base were analyzed using the bioinformatics tool BioEdit. A multiple sequence alignment was carried out that included the ITS region, including gaps and the complete sequences align. From the sequence alignment observed between conserved region of isolates the evolutionary history was inferred using UPGMA method (Sneath and Sokal, 1973). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*. 2004). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.* 2007) and MEGA11.

Preparation of ethanolic extract of oyster mushroom

For the estimation of different antioxidant activity, ethanolic extract was prepared from dried powder

Table 3: Genbank accession numbers of the Ex-Type strains of *P. djamor* that showed the homology with the isolate.

GenBank Accession No	Species	Strain or Isolate	rDNA sequence (bp)	Origin
EF437221	*P. cystidiosus*	P-19"	636	India
DQ978222	*P. cystidiosus*	X 652	682	India
KP164598	*P. cystidiosus*	ZYB 2013	651	China
KF280325	*P. djamor*	MBsn	604	Brazil
JX262249	*P. djamor*	CBE 11	560	India
KF932719	*P. djamor*	1526	666	Russia
JN637828	*P. djamor*	B-36	657	Cuba
EU424287	*P. djamor*	CBS 100134	687	China
KJ831854	*P. djamor*	IB36	603	Peru
HM770895	*P. djamor*	IUM1794	625	South Korea
JQ837487	*P. djamor*	Z1	675	Russia
KJ754112	*P. djamor*	7	687	Kenya
GU722277	*P. djamor*	ECS-01130	571	Mexico
KT818506	*P. sajor-caju*	IPL/MC/PS-1	656	India
HM998809	*P. ferulaginis*	LGMACC 850404	630	Hungary
KT768094	*P. djamor*	IPL/MC/PD1	655	India

Table 4: Genbank accession numbers of the Ex-Type strains of *P. sajor-caju* that showed the homology with the isolate

GenBank Accession No	Species	Strain or Isolate	rDNA sequence (bp)	Origin
KM985672	*L. sajor-caju*	BPSM35	585	India
JX965409	*L. sajor-caju*	pau3	620	India
JQ814757	*L. sajor-caju*	CS-32	577	Russia
KJ654407	*L. sajor-caju*	E882B	606	Australia
KT956122	*L. sajor-caju*	EB1001	698	Thailand
KR908737	*L. sajor-caju*	NCIM 1133"	531	India
KM267726	*L. sajor-caju*	JMH36	488	Tanzania
KR673684	*L. sajor-caju*	KA13-1213	588	South Korea
DQ077888	*P. eryngii*	PHZAU2	639	China
HM998809	*P. ferulaginis*	LGMACC 850404	630	Hungary
AY265831	*P. ostreatus*	ASI 2016	638	Korea
KP455495	*Pleurotus* sp.	DL501	636	India
FJ608594	*Pleurotus* sp.	AG X	848	Czech Republic
KT818506	*L. sajor-caju*	IPL/MC/PS-1	656	India

of four *Pleurotus* species. One g dried powder was suspended in 100 ml 95% ethanol and stirred for 24 h at room temperature. The suspension was filtered with whatman no 1 and the filtrate was evaporated by rotary evaporator at 40^0C. The extraction was further resuspended in 95% ethanol and used for further use.

DPPH Scavenging activity

Free radical scavenging activity of mushroom extracts were estimated by DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity. Ethanolic extract (100 µl) of four species of oyster mushroom (*P. djamor*, *P. sajor-caju, P. ostreatus* and *P. floridanus*) was added with DPPH solution (5µl) and incubated for 30 min in dark. After incubation absorbance was taken at 517nm against a control. DPPH scavenging activity was measured using the following formula

$$\text{Inhibition \%} = (A_{control} - A_{sample})/A_{control} \times 100$$

Ferric reducing antioxidant power assay

Twenty five ml 0.2 M Phosphate buffer (pH6.6), 2.5 ml of 1% potassium ferricyanide, 1 ml of distilled water and 1 ml of ethanolic extract of the test sample was taken and incubated at 50^0C for 20 min in water bath. Then 2.5ml of 10% trichloro acetic

Table 5: GenBank accession numbers of the ex-type strains of *Pleurotus ostreatus* that showed the homology with the isolate

GenBank Accession No	Species	Strain or Isolate	rDNA sequence (bp)	Origin
DQ077888	*P. eryngii*	PHZAU2	639	China
HM998809	*P. ferulaginis*	LGMACC 850404	630	Hungary
AY450345	*P. ostreatus*	6689	1551	Austria
EU622256	*P. ostreatus*	NW446	652	China
EU622249	*P. ostreatus*	NW423	648	China
GQ249947	*P. ostreatus*	PU001	563	India
HM067973	*P. ostreatus*	COIR PTK	635	India
HM138675	*P. ostreatus*	PAK1	635	India
KC782771	*P. ostreatus*	PLO6	575	Brazil
KT968336	*P. ostreatus*	PoVF8	677	Korea
KT968340	*P. ostreatus*	PoVF18	677	Korea
KJ020935	*P. ostreatus*	ST	632	Italy
KT818506	*L. sajor-caju*	IPL/MC/PS-1	656	India
KT956122	*L. sajor-caju*	EB1001	698	Thailand
KT768095	*P. ostreatus*	IPL/MC/PO-1	204	India

Table 6 : Genbank accession numbers of the ex-type strains of *P. floridanus* that showed the homology with the isolate

GenBank Accession No	Species	Strain or Isolate	rDNA sequence (bp)	Origin
JQ731605	*P. ostreatus*	VKESR1	625	India
JX429937	*P. floridanus*	FPFMK	668	Malaysia
JN234837	*P. floridanus*	FTCW1 (PFW1)	654	Malaysia
KF373566	*P. floridanus*	LCJ 155	683	India
GU721058	*P. ostreatus*	PF101	671	India
HM998809	*P. ferulaginis*	LGMACC 850404	630	Hungary
DQ978222	*P. eryngii*	X 652	682	India
KM375930	*P. floridanus*	AAU-SAP	1835	India
KT968336	*P. floridanus*	FLO-01	652	Korea
KT968340	*P. floridanus*	Flo-01	667	Korea
KJ020935	*P. floridanus*	Pfu-652	432	China
KT826605	*P. floridanus*	IPL/MC/PF-1	665	India

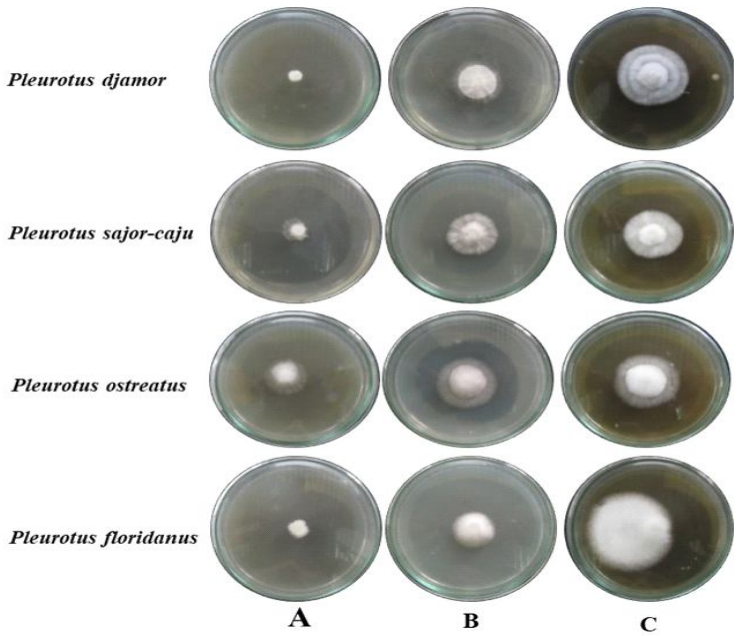

Fig 1: Mycelial growth rate of *Pleurotus* spp. on (A) Water Agar (B) Potato dextrose Agar (C) Malt extract Agar

acid was added, mixed well, centrifuged at 10000 rpm for 15 min and the upper layer of the mixture was collected. 2.5ml of reaction mixture (2.5 ml) was taken and to it 2.5 ml of distilled water and 300µl of 1% ferric chloride was added and mixed well. Then the absorbance was taken at 700nm in spectrophotometer.

Estimation of flavonoid content

Flavonoid content of the mushroom extracts was done following the method as described by Barros *et al* (2008). Mushroom extract (100µl) was mixed with 500µl distilled water, 30µl of 5% sodium nitrate was added to it, incubated for 5min and then 60µl of 10% aluminium chloride added and incubated at room temperature for 6 min. After the incubation, 200µl of 1M NaOH and 110 µl distilled water was mixed well and absorbance was taken at 510nm.

RESULTS

Three different media has been used to evaluate the mycelia growth rate of four *Pleurotus* species (*P. djamor*, *P. sajor-caju, P. ostreatus* and *P. floridanus*) and it was observed that all the four *Pleurotus* species grow fast in Malt extract agar when incubated at 27^0 C (Fig. 1). Increased growth rate was also observed in case of Potato dextrose agar but very slow growth was observed in case of water agar. At first, large amount of hyaline areal mycelium was observed which become whitish after some time. In case of *P. djamor*, the colour of mycelia was white in early stage but it becomes light pink at maturity. The mycelia cover the media with regular wavy mat with distinct margins. On the other hand, the mycelia of *P. floridanus* growing irregular with thin margin. Growth of *P. sajor-caju* and *P. ostreatus* was white cottony mat growing in concentric manner. Optimum temperature for mycelial growth was recorded at 25^0 C.

Morphological and anatomical study of Pleurotus species

Cultivation of oyster mushroom is very common practice in North Bengal and the environmental condition is very much suitable for the cultivation. All four *Pleurotus* species cultivated in North Bengal of which Pink oyster mushroom (*P. djamor*) is very commonly cultivated in the north-western part of India. *P. djamor* looks very gracious on bed. Fruiting body wide, 3-4 cm diameter, wavy outer edge with no particular shape, thickness is about 3-4mm outer edge. *Pileus* is pink in colour with very small stipe, sometimes stipe is absent, diameter is about 2-3.5

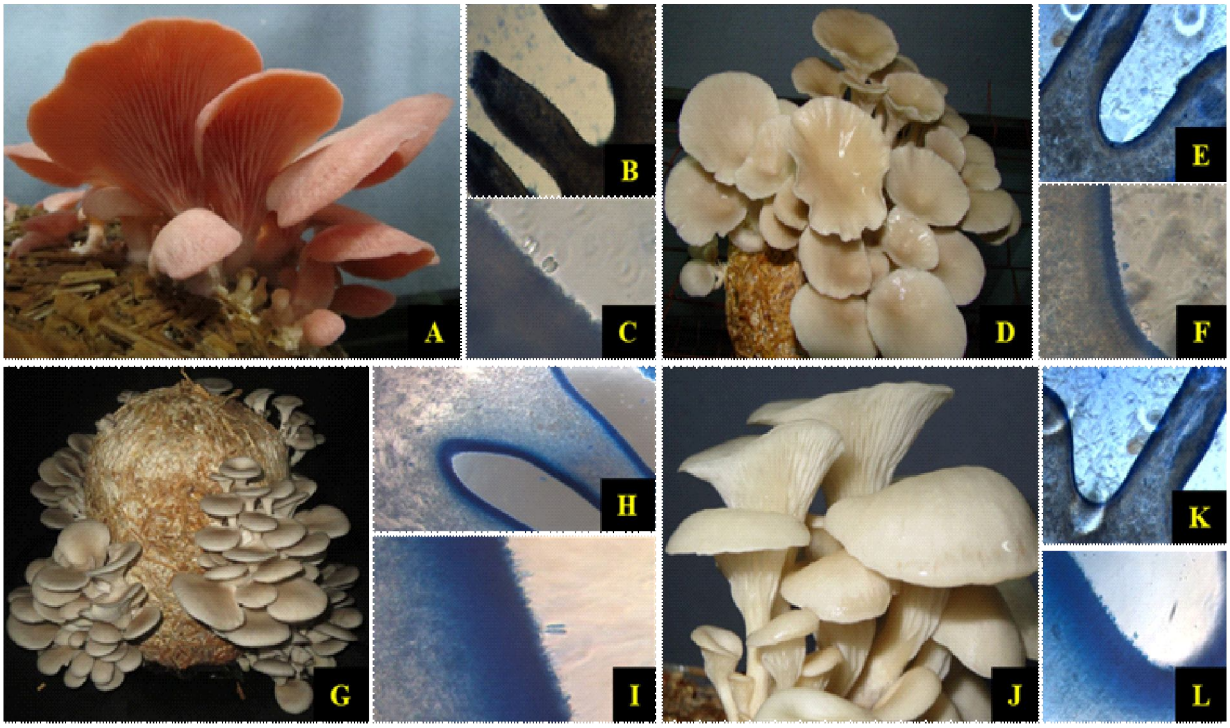

Fig 2: Morphological and anatomical features of Oyster mushroom showing basidia containing basidiospore. (A-C) *Pleurotus djamor* (D-F) *P. sajor-caju* (G-I) *P. ostreatus* (J-L) *P. floridanus*

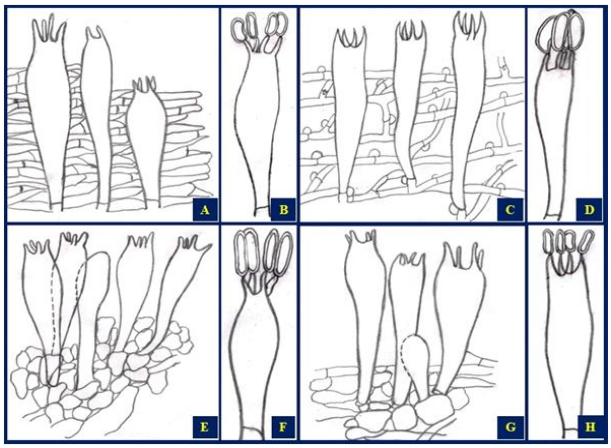

Fig. 3: Hand drawing of basidium attached with the hymenophore and basidiospore attached to basidium of (A&B) *P. djamor* (C&D) *P. sajor-caju* (E&F) *P. ostreatus* (G&H). *P. floridanus*

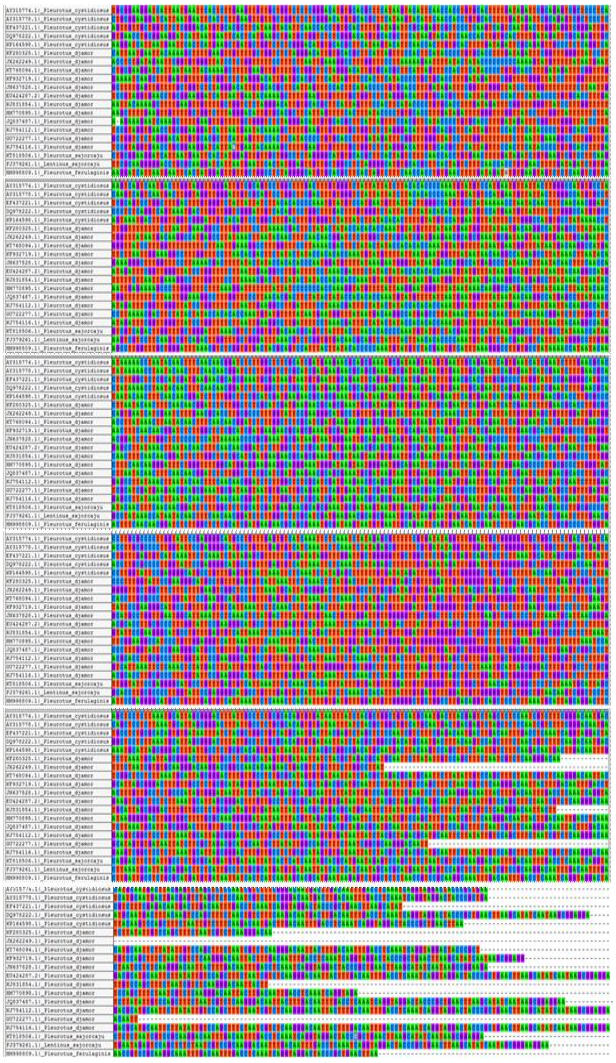

Fig. 4: 18S r DNA sequence alignments of *P. djamor* (IPL/MC/PD-1) with other ex-type isolates. The conserved regions of the gene are demonstrated in different colours

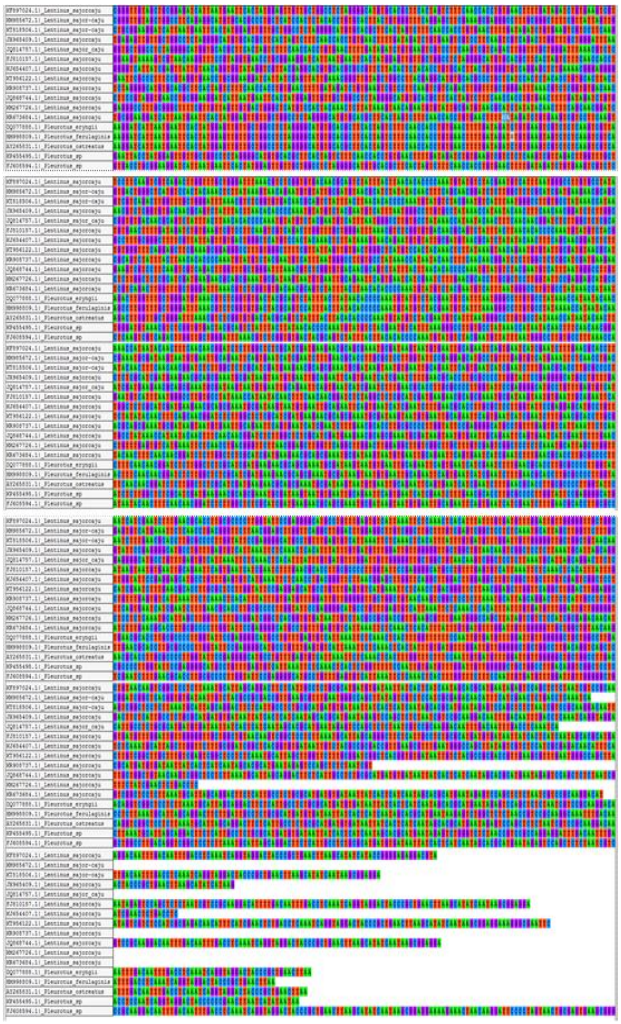

Fig. 5:18S r DNA sequence alignments of *P. sajor-caju* (IPL/MC/PS-1) with other extype isolates. The conserved regions of the gene are demonstrated in different colours

cm, fleshy with light aroma, gills are decurrent. *Basidiospores* are oval to kidney shaped and four spores attached with the basidium. Spore length 1.8-3.5 µm. *P. djamor* had been cultivated during the autumn to spring as it requires lower temperature (18-20^0 C) with 75-85% relative humidity (Fig 2A-C; Fig 3 A&B).

On the other hand, *P. sajor-caju* is one of the major oyster mushrooms cultivated in North Bengal on a very large scale. This mushroom is very popular for its large size which increases the production rate. This species can be cultivated throughout the year for its wide range of environmental requirement. It grows at higher temperature about 25-30^0 C. The *Pileus* fan shaped, diameter is very large (4-5cm) with a long stipe. The *pileus* is fleshy, prominent edges with distinguish grey colour. Anatomical study reveals that the *basidiospores*

Fig. 6: 18S r DNA sequence alignments of *P ostreatus* (IPL/MC/ PO-1) with other ex-type isolates. The conserved regions of the gene are demonstrated in different colours

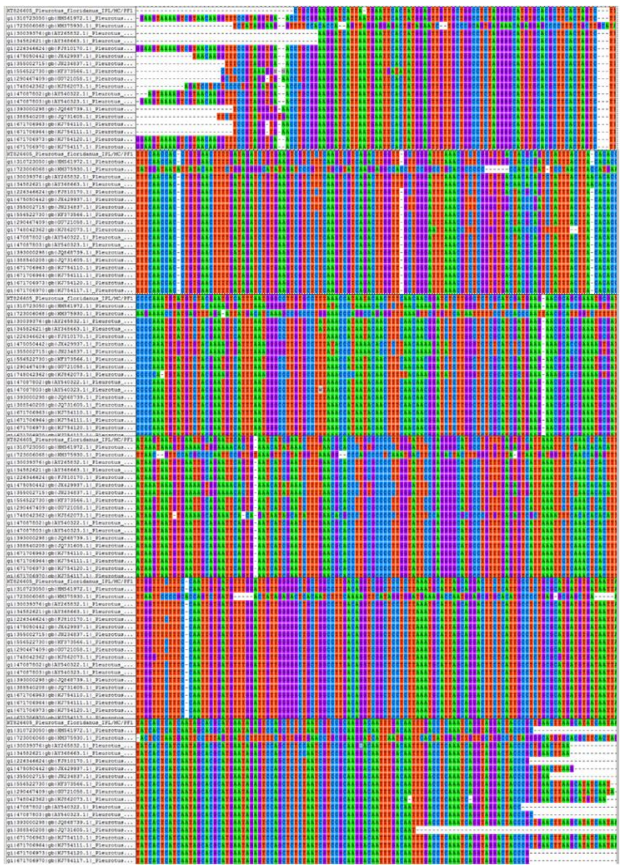

Fig.7: 18S r DNA sequence alignments of *P floridanus* (IPL/MC/ PF-1) with other ex-type isolates. The conserved regions of the gene are demonstrated in different colours

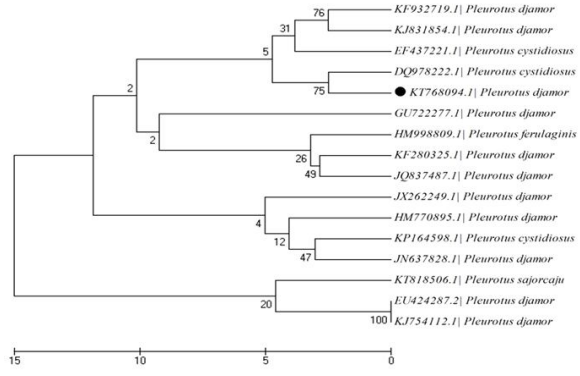

Fig. 8: Phylogenetic placement of *P. djamor* (IPL/MC/PD-1) with other ex-type strain sequences obtained from NCBI Genbank Database

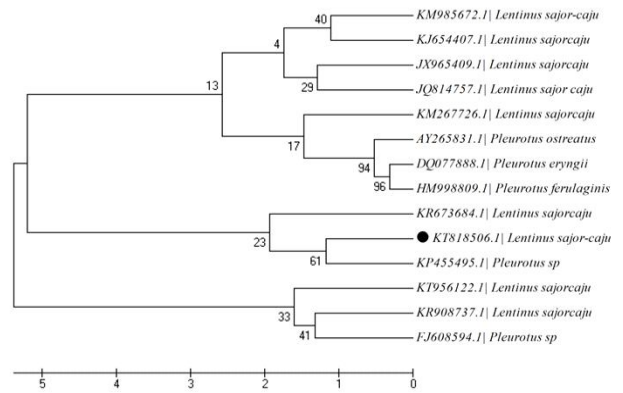

Fig. 9: Phylogenetic placement of *P sajor-caju* (IPL/MC/PS-1) with other ex-type strain sequences obtained from NCBI GenBank Database

are long, oval shaped with quite prominent edges. Spores are attached with the basidium by the corner of the basal part of the spores, size about 2.5-5 µm (Fig.2 D-F; Fig 3 C&D). *P. ostreatus* widely cultivated in North Bengal for its shape, texture, taste and the environmental condition is very much favourable for its cultivation. Fruiting body initiated at 18-22^0 C with 80-85% relative humidity. *Pileus* is thick, fleshy with a very short stipe. Pinhead appears light blackish thus it is popularly known as Black oyster mushroom. Diameter of the pileus is about 2-3cm and the whole part of the fruiting body edible. *Basidiospores* are long, oval to kidney shaped about 2-4µm long and four spores attached with the basidium (Fig. 2 G-I; Fig. 3 E&F). *P. floridanus* also cultivated widely in this area

during the winter season as it requires low temperature for its fruiting body initiation with very low amount of relative humidity and about 16-20^0 C requires for fruiting initiation. *Pileus* is bright white, fleshy, thick and about 2-3cm diameter. *Stipe* is long with decurrent gills. Fruiting initiation occurs in a cluster. *Basidiospores* are long oval shaped attached with basidium. Basidiospores are about 2-3.5µm attached with basidium (Fig 2 J-L; Fig 3 G&H)

Molecular characterization of Pleurotus spp. 18S rDNA sequence

18S rDNA sequencing of four *Pleurotus* spp. were done and sequences of the identified species (Table 2) were submitted in NCBI GenBank under the accession number KT 768094 for *P. djamor*, KT818506 for *P. sajor-caju*, KT768095 for *P. ostreatus* and KT826605 for*P. floridanus*.

Multiple sequence alignment

A multiple sequence alignment of ITS gene sequences of *P. djamor* , *P. sajor-caju, P. ostreatus* and *P. floridanus* were conducted. Sequences of other strains obtained from NCBI GenBank database showing maximum homology with our strains was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA4 software. Genbank accession numbers of the Ex-type strains of *P. djamor* (Table 3),*P. sajor-caju* (Table 4), *P.ostreatus* (Table 5) and *P. floridanus* (Table 6) that showed the homology with the respective isolate have been presented. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours for *P. djamor* (Fig 4), *P. sajor-caju* (Fig. 5), *P. ostreatus* (Fig 6) and *P. floridanus* (Fig. 7).

Phylogenetic analysis

The evolutionary history was inferred using the UPGMA method The optimal tree with the sum of branch length is 97.46225950 for *P. djamor* (Fig. 8). There was a total of 559 positions in the final dataset. The optimal tree with the sum of branch length is 31.00795241 for *P. sajor-caju* as shown in Fig 9.. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Figs.8 & 9).

Codon positions included were 1st+2nd+3-rd+Noncoding. There were a total of 487 positions in the final dataset for *P. sajor-caju*. The BLAST query of the 18S rDNA sequence of T/ITS4 and T/ITS6 (for *P. sajor-caju*) against Genbank database confirmed their identity The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). The optimal tree with the sum of branch length is 55.72288571 and there was a total of 203 positions in the final dataset for *P. ostreatus* (Fig 10) , while branch length is 1886.85763040 for *P. floridanus* and there was a total of 622 positions in the final dataset (Fig.11). Phylogenetic placement of all four *Pleurotus* species with other ex-type strains obtained from NCBI GenBank was compared using UPGMA method in MEGA11 software and result has been presented in Fig 12.

Antioxidant activity of Pleurotus spp.

The extracts of different species of oyster mushroom showed positive antioxidant activity by fading the violet colour of DPPH solution to yellow to pale violet colour. Results revealed that the scavenging activity of DPPH were directly proportional with the concentration of the samples used. As the concentration of the sample was increased, the scavenging activity of towards DPPH radicles also elevated (Fig. 13A). Different concentrations were used for the evaluation of DPPH scavenging activity and it was observed that *P. djamor* showed maximum activity among the other species in respect to all the concentrations. It was found that *P. djamor* showed about 88% DPPH scavenging activity in 20 mg/ml concentration while *P. floridanus* showed lowest scavenging activity (77%) among the other species. All the mushroom species showed appreciable reducing power activity in different concentrations (5-20 mg/ml). Highest amount of reducing power ability was observed in case of *P. djamor* at 20 mg/ml concentration while

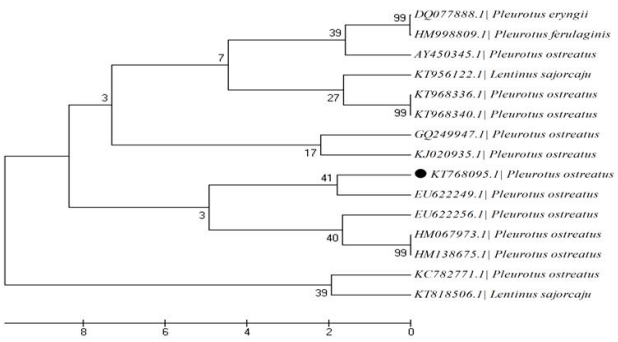

Fig.10: Phylogenetic placement of *P. ostreatus* (IPL/MC/PO-1) with other ex-type strain sequences obtained from NCBI GenBank Database

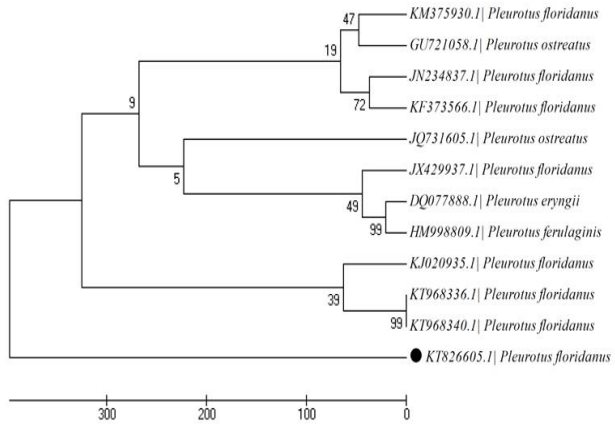

Fig.11: Phylogenetic analysis of *P floridanus* (IPL/MC/PF-1) with other ex-type strain sequences obtained from NCBI Genbank Database

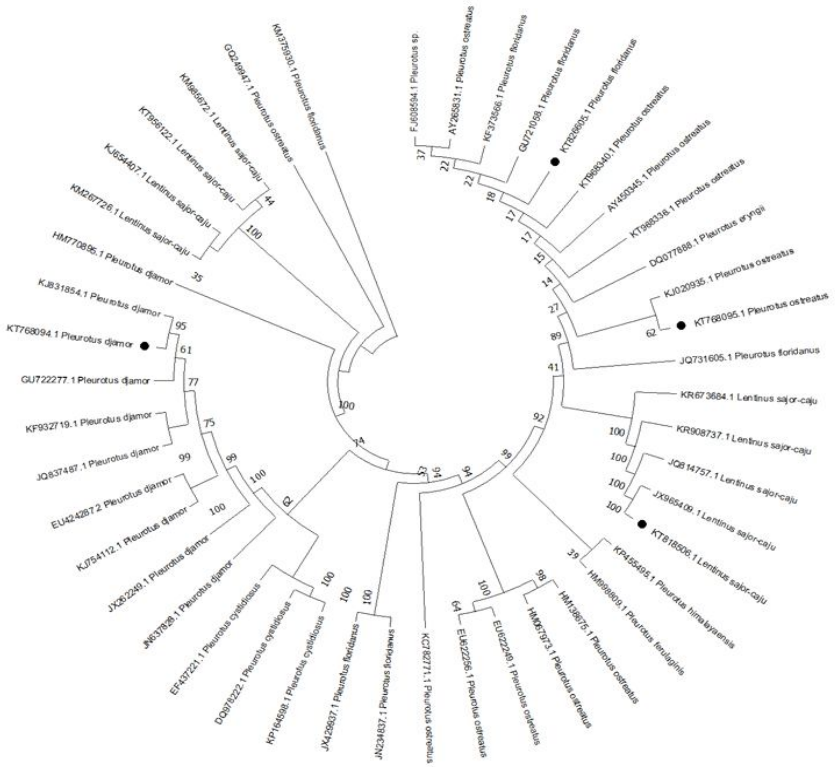

Fig.12: Comparison of phylogenetic placement of four *Pleurotus* species with other 41 ex-type strains obtained from NCBI GenBank using UPGMA method in MEGA11 software

P. ostreatus and *P. sajor-caju* showed lowest amount of reducing power activity at 20 mg/gm tissue concentration. Free reducing power activity was estimated using the different concentrations of four species of oyster mushroom and the highest activity was observed in *P. djamor* whereas in case of *P. ostreatus* and *P. sajor-caju* the activity is quite lower than that of the others (Fig. 13B). Antioxidant is an important parameter and mushroom is one of the major sources of antioxidant compounds. Total flavonoid content and carotenoid content is also an important compound showing antioxidant activity. Total flavonoid content of four different species was assessed and the results revealed that all the species were showing significant amount of flavonoid content. Different concentrations were

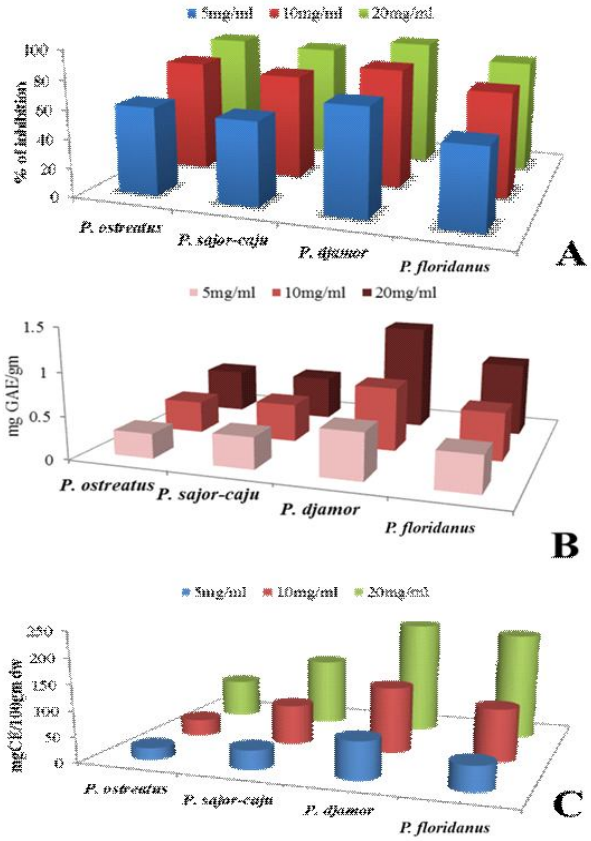

Fig.13: Antioxidant activity of *Pleurotus* species. (A) DPPH scavenging activity (B) Free radicle antioxidant power assay (C) Flavonoid activity

taken into consideration and it was observed that *P. djamor* and *P. floridanus* showed highest amount of flavonoid in compare to other two species (Fig 13 C). The results were also revealed that the higher concentration of the sample helps in increasing the flavonoid content and thus highest flavonoid content activity was found in case of *P. djamor* and *P. floridanus* in 20 mg/ml concentration in compare to 5 gm/ml and 10 mg/ml concentration.

DISCUSSION

Oyster mushroom is one of the most popular mushrooms in North Bengal and a large number of growers are now cultivating oyster mushroom throughout the year. Three growing media, such as potato dextrose agar (PDA) and malt extract agar (MEA) and water agar (WA) were used to evaluate the mycelial growth characters of four species of oyster mushrooms (*P. djamor, P. sajor-caju, P. ostreatus* and *P. floridanus*). Their mycelial growth completed on the 8th day at 27^{0}C. In the present study, results revealed an increase in the growth rate as the incubation temperature was increased from 24 to 28^{0}C which was also reported by Thulasi *et al* (2010). Malt extract agar showed highest growth rate followed by the potato dextrose agar, while water agar was lowest. Initiation of mycelial growth was also analysed and it was found that the growth of *P. floridanus* initiated earliest (20 h of incubation) in malt extract agar while in case of potato dextrose agar it took 23 h but in water agar it took longest period to initiate the colonization. Average maximum growth of *P. sajor-caju* was also recorded on malt extract agar (MEA) than on potato dextrose agar (PDA) medium at 25 ºC under humid (65 – 80% RH) conditions (Asghar *et al.*, 2007)

In the present study, four different species of oyster mushroom were taken into consideration and their morphological, antomical as well as molecular characterizations have been discussed. *P. ostreatus* commonly known as black oyster mushroom was characterized depending upon their morphological structure such as light blackish fruiting body, smaller pileus structure as well as the basidium consisting four of basidiospore. Spores were very small and oval shaped. On the other hand, *P. sajor-caju*, commonly called as grey oyster mushroom characterized based on its greyish fan shaped large fruiting body with small oval or kidney shaped basidiospore attached with tetrasporic basidium. Besides, the white oyster mushroom *P. floridanus* was characterized by the bright white fruiting body along with its decurrent gills and kidney shaped small spore attached to the basal part with the basidium. Pink oyster mushroom, *P. djamor* was also characterized with its distinct pink fruiting body with very small stipe. It was also observed that sometimes, the stipe absent or very small in size. The results revealed that spore of all the species were very small (1.8-5µm) and spores of *P. djamor* was found to be smallest among these *Pleurotus* species. All four *Pleurotus* species were identified using 18S rDNA sequencing and they all were identified as *P. djamor, P. sajor-caju, P. ostreatus* and *P floridanus* and subsequently all the sequence have been deposited in the NCBI Genbank. 18S rDNA sequence alignments of *P. djamor, P. sajor-caju, P. ostreatus* and *P floridanus* with other ex-type isolates have been presented and the conserved regions of the gene are demonstrated in different colours. Phylogenetic placement of all four *Pleurotus* species with other 41 ex-type strains

obtained from NCBI GenBank was compared using UPGMA method in MEGA11 software and result has been presented.

Antioxidant compound is very important for human health. In this study anti-oxidant activities includes DPPH radical scavenging activities, free radical antioxidant power activity and total flavonoid activity. *P. djamor* and *P. ostreatus* showed higher DPPH scavenging activity in comparison to other two species. Results also indicated that DPPH scavenging activity increases directly proportional to the concentration. The present investigation also includes the ferric reducing antioxidant power and the results revealed that *P. djamor* and *P. floridanus* showed higher FRAP activity and *P. djamor* showed highest activity in 20 mg/ml. concentration. Chaturvedi *et al* (2011) stated that oyster mushrooms are a potential source of antioxidant compounds and the antioxidant activity were concentration dependent. Dubost *et al*., (2007) explained that the methanol extract of fruiting bodies of *P. ostreatus* showed reducing power and high antioxidant properties. Menaga *et al* (2013) reported that the methanolic extract of *P. floridanus* showed the most potent radical-scavenging activity at a maximum concentration of 100 μg/ml and the scavenging effects on DPPH radicals. The radical scavenging activity of *P. ostreatus* mushroom has been reported (Ramkumar *et al*., 2010) to be higher (6 mg/ml) than those of other mushrooms like *Agaricus bisporus, Volvariella volvaceae* and *Calocybe indica.* Ethanolic extract of *P. florida* showed higher chelating activity against ferrous ion when compared to *P. ostreatus* (Md. Imran *et al*., 2011). IC_{50} value of DPPH scavenging ability of aqueous extract of *P. sajor-caju* showed 9.01 % (Finimundy *et al.* 2013) and the EC_{50} values of *P. abalones* in DPPH radicals scavenging ability and reducing power were 8.68 and 4.68 mg/ml respectively (Wang *et al.,* 2012). Deshmukh and Shinde (2014) compared the cold water and hot water extracts of *P. florida* and *P. sajor-caju* and found that the cold water extract of both the species showed higher antioxidant activity. Antioxidant and antidiabetic activity of *P. djamor* has been reported (Roy et al. 2018). Antiradical activity and ferric reducing antioxidant power of *P. pulmonarius, P. floridanus* and *P. sajor-caju* formulations extracts *in vitro* have been discussed by Blanche *et.al.,* (2019), whereas characterization and potent application of *P. floridanus* trypsin inhibitor (PfTI) has been elucidated by Pannippara *et.al*.(2020).

Niazi and Ghafoor (2022) have optimized the growth conditions of *P.floridanus* , an indigenous edible and therapeutically significant mushroom from Pakistan and its molecular phylogenetics have been reported. Guo *et al* (2022) have focused on nutritional qualities and antioxidant activity of *P. floridanus* grown on composted peach sawdust substrate with different composting time. Crude polysaccharide of treatment D4 showed highest scavenging ability toward both radical 3 ethylbenzthiazoline-6-sulfonic acid and hydroxyl radical suggests that composting processes is suitable for its cultivation based on nutritional and antioxidant qualities of fruiting bodies. Recently Andrew (2023) has explained that addition of nutrient rich foliage of *Leucaena leucocephala* on rice straw shortened cultivation time of *P. floridanus* and increased protein and mineral contents.

CONCLUSION

In the present study, four different species were taken into consideration, and they were separated depending upon their morphological as well as molecular characterizations. Molecular characterization of all four species were done and submitted NCBI GenBank under the accession number KT768095, KT818506, KT 768094 and KT826605 for *P. ostreatus, P. sajor-caju, P. djamor* and *P. floridanus* respectively. It was also observed that in compared to all four species *P. djamor* shows higher amount of antioxidant activity in terms of DPPH scavenging activity, FRAP activity and flavonoid content. The present investigation revealed that the four different species cultivated in North Bengal were distinct in morphology and molecular characters and all the species were therapeutically important for human health.

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