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MOLECULAR PHYLOGENETICS AND OPTIMIZATION OF GROWTH CONDITIONS OF INDIGENOUS EDIBLE AND THERAPEUTICALLY SIGNIFICANT *PLEUROTUS FLORIDANUS* FROM PAKISTAN

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Abstract

Poor agricultural practices and unavailability of nutritious food have developed a need to look for some non-conventional way of food production. Mushrooms and their cultivation are the best alternative way to achieve zero hunger and no poverty. In the present study, native *Pleurotus floridanus* a potentially edible and nutritious mushroom was characterized and evaluated for its cultivability and cultivation potential. *P. floridanus* is a new record for Pakistan. Maximum cultural growth was observed on the Compost Extract Agar (CEA) medium at 28°C followed by Potato Dextrose Agar medium (PDA) and Malt Extract medium (MEA). Cultured strains on CEA medium were used for the spawn production, Sorghum grains at 28℃ found as the best combination for spawn production. Cultivation potential was investigated on the mixed substrate of wheat straw and sawdust and pure wheat straw only. A mixed substrate was found better than pure substrate. Cultivation was only successful at 16°C that showed that *P. floridanus* is a lower temperature strain. These results showed that *P. floridanus* can grow on various types of substrates and medium and its cultivation on large scale can solve one of the major concerns of growing population that is the lack of nutritious and healthy food.

Key words: Molecular phylogenetics; Cultivability; Spawn; Cultivation potential; *Pleurotus floridanus*; Nutrient media; Temperature.

Introduction

Pleurotus (Fr.) P. Kumm genus exhibit the large range of plasticity in the phenotypic appearance that hindered the correct identification by old phenotypically dependent methods (Buchanan, 1993) but the molecular characterization have introduced some explication regarding the delimitation of species in genus (Vilgalys & Sun, 1994; Vilgalys, 1996; Alberto *et al*., 2002; Zervakis *et al*., 2004).

The *Pleurotus* spp. of the class basidiomycetes belong to a group known as "white rot fungi" (Bellettini *et al*., 2016) as they produce a white degraded biomass after decomposition are generally cultivated on non-composted lignocellulosic substrates (Xia *et al*., 2016). *P. ostreatus* (oyster mushroom), *P. eryngii* (king oyster or Cardoncello), *P. pulmonarius* (phenix mushroom), *P. djamor* (pink oyster mushroom), *P. sajor-caju* (indian oyster), *P. cystidiosus* (abalone oyster), *P. citrinopileatus* (golden oyster mushroom) and *P. cornucopiae* are commercially cultivated and have considerable economic, therapeutical and nutritional values (Zhang *et al*., 2016). These species are abundant source of minerals (P, Ca, K, Fe and Na), proteins and vitamin C, B complex (riboflavin, folic acid, thiamine and niacin) (Patil *et al*., 2010).

Pleurotus spp. stimulate immune system, prevent swelling or inflammation and tumor growth, possess antithrombotic, antimicrobial and hypoglycaemic properties, reduce blood lipid amounts, retard atherosclerosis and high blood pressure and possess various other activities (Gunde-Cimerman, 1999). *P. cystidiosus* and *P. floridanus* exhibit the strong antioxidant potential (Li *et al*., 2007; Blanche *et al*., 2019).

Worldwide, *Pleurotus* represented through twenty species (Menolli Junior *et al*., 2010). Eighty five percent of all oyster mushrooms is produced in China (Tesfaw *et al*., 2015).

In Pakistan and India, due to oyster like appearance, *Pleurotus* is usually called as Dhingri (Khan *et al*., 2013). Mushroom cultivation and marketing are around nonexistent in Pakistan. In Pakistan, mushrooms are cultivated in farmhouses, along with but not confined to the state-owned national logistic cell. Farm production shares around one percent of the total mushroom exports, while the remaining of it comes through the natural production in Khyber Pakhtunkhwa. (Razi, 2017). There is requirement to evolve enriched agronomic system in Pakistan. The agronomist should cultivate organoleptically accepted mushrooms like *Pleurotus* spp. with enthusiasm on large scale to meet the needs of a healthy diet. Financial status of cultivars can strengthen through applying the mushroom cultivation as a welcoming industry of agriculture in Pakistan (Shah *et al*., 2004). The aim of this research is the morpho-anatomical and molecular characterization and to evaluate the optimum temperature and nutritional requirements for the growth of native *P. floridanus* which is previously undescribed, Ishaq *et al*., (2018) conducted study on the exotic *P. floridanus* species, the strain they used was not the part of Pakistani mushroom flora. Cultivation of *P. floridanus* is rare unlike other species of *Pleurotus* such as *P. florida P. ostreatus* and *P. eryngii*. *P. floridanus* is a new record for Pakistan and also an addition to the edible agaric flora of the country.

Materials and Methods

Sampling and characterization: Basidiomata of the specimen was collected from Himalayan moist temperate forests (khanaspur-Ayubia, KP) Pakistan. The Collected basidiomata were photographed using a Samsung camera and field notes were prepared. Systematic characterization was carried out by macro-microscopically and phylogenetically. The experiments were conducted in Fungal Biology and Systematics Research Lab, Department of

Botany, University of the Punjab, Lahore. Micro morphological characteristics were observed using a compound light microscope (MX4300H Techno Co., Ltd., Japan) with an oil-immersion lens at a magnification of 100X. Molecular characterization was done by following the Protocol (Gardes & Bruns, 1993) with little modifications. The Phylogenetic tree was built through molecular evolutionary genetic analysis using MEGA 6 software with default settings (Tamura *et al*., 2013). Specimen was deposited in the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH). DNA Sequence was submitted in GenBank under the accession number MT012089.

Estimation of culturability: Culturability of *Pleurotus floridanus* (LAH36079) was assessed according to the method described by Siddiq *et al*., (2018) on three different nutrient agar media i.e., Malt extract agar (2% MEA: agar 20g, malt extract 20g dissolved into 1000mL dH2O), Potato dextrose agar (2% PDA: thin potato slices 200g, glucose 20g, agar 20g per liter of dH2O) and Compost extract agar (CEA: 20g agar,10g glucose dissolved into 1000 mL wheat straw water based filtrate) at different temperatures i.e.,16^{o}C, 22^{o}C and 28^{o}C. Inoculated petri plates were sealed with parafilm and kept in incubator for 30 days. Mycelial characteristics of all species were observed on regular basis.

Prepared cultures were deposited in the Herbarium, Department of Botany, University of the Punjab, Lahore (LAH# 11320CA; On PDA medium, LAH# 11320CB; On MEA medium, LAH# 11320CC On CEA medium).

Spawn production: Pal & Thupa, (1979) described methodology was used to prepare spawn. Spawn was prepared on cereal grains (sorghum and wheat grains) in jars by inoculating the grains with prepared pure culture on CEA medium. Grains (sorghum and wheat) were taken and washed properly to remove dust, chaff or any other particles and soaked for 24 hours for maximum absorption of water. Soaked grains were again washed with water and boiled until they become soft. The washed grains were spread on the clean blotting paper to drain off the extra water from the grains. Two third of the sterilized jars were filled with the boiled grain (100g in each jar), then calcium carbonate (0.5g) and gypsum (1g) were added in each jar and mixed thoroughly and covered with the lid. Jars with grains were autoclaved. Autoclaved jars were placed under the sterilized laminar airflow cabinet and allowed to cool at room temperature. Grains were inoculated with activated pure mycelium colony. Three discs of the active mycelium of 1cm size of the cultured strains were inoculated in one jar under the aseptic condition. Jars were incubated at different temperatures and mycelium extension rate on the grains (wheat and sorghum) was observed until grains were completely covered with the mycelium and mushroom seeds prepared.

Substrate production: Wheat straw and sawdust were used as the raw materials. Fresh wheat straw collected from the field area of University of the Punjab, Lahore and sawdust collected from the furniture shop at barket market, Lahore. Two types of substrates were prepared, one of pure wheat straw and one is the mixture of wheat straw and sawdust of equal ratio. Raw materials were soaked in water for 24 hours then made pile of them to make the substrate suitable for mycelial running and 65% moisture maintained during the decomposition process of eight days. Chicken manure and gypsum (one fourth of the substrates) were added as supplements for nitrogen and carbon source. Substrate and supplements were piled up and covered properly with polythene sheet to prevent air borne contaminants while turning of the pile was done after every two days. When substrates were prepared, they were filled in polypropylene bags and autoclaved for 3 to 4 hours to remove any type of contamination if present and allowed to cool. Polypropylene bags of 20 x 15cm were used for the cultivation. 1kg of the substrate was filled in each bag.

Spawning: Sterilized compost bags were inoculated with the spawn prepared on sorghum grains at the rate of 25g per 1kg bag at the sterilized surface and bag mouths were tied with the rubber bands and placed on sterilized shelves in the lab under the dim light.

Spawn running, pin head emergence, fruiting and flushes: Cultivation potential in the form of spawn running period, pinhead emergence, fruiting time, and no. of flushes appeared on both types of substrates were observed at different temperatures.

Biological efficiency (yield): Biological efficiency of both types of substrates was observed as per kg of the substrate bag. It used to determine the growth potential of the oyster mushroom based on the following formula:

Biological efficiency dry weight of substrate = Weight of fresh mushroom x 100 %

Statistical analysis of the data: All the experiments were conducted in triplicates and data are expressed as mean value ± S.E. Analysis of Variance (ANOVA) with Duncan's multiple range tests (p<0.05) by using co-stat software (Version 3.03) was employed to analyze the effect of different treatments on mycelium extension rate and cultivation potential.

Results and Discussion

Morpho-anatomical analysis: ***Pleurotus floridanus*** Singer, *Pap. Mich. Acad. Sci.* 32: 134 (1948) [1946] (Fig. 1: A-H).

Basidiomata: Found in pairs, creamy white to light gray (8/1 7.5 YR) to yellow (8/6 5Y). Pileus: Smooth margins, color ranged from creamy white to light gray (8/1 7.5 YR) to yellow (8/6 5Y), bivalve shape, undulate. Lamellae: Deeply decurrent, yellow (8/6 5Y), crowded.

Basidiospores: (7.70-) 8.5–9.56(-13.97) x (3.99) (4.40) – (4.8) (5.024) µm, avl x avw =10.842 x 4.70µm, epiculated, subcylinderical, thin walled, inmayloid, hyaline. BASIDIA: (19.11-) 19.57–24.57(-26) x (5.19-) 5.55–6.0(-6.1) µm, avl x avw =21.121 x 5.615µm, clavate, two to four spored, thin walled, basal clamp connection, Basidiols present.

CYSTIDIA: (19.42-) 20.44–25.868(-26.42) x (4.38-) 4.88–5.50(-5.60) µm, avl x avw = 23.70 x 5.14 µm, clavate, thin walled. PILEUS HYPHAE: (2.45-) 3.671–7.239(-7.39) µm, avw = 5.298µm, irregular, thin walled, clamp connection present. STIPE HYPHAE: (3.00-) 3.75–3.96(-4.32) µm, avw = 3.9272, irregular, thin walled, clamp connection present.

Material Examined: Pakistan, Khyber Pakhtunkhwa, Ayubia, Khanaspur, KP 2575 m a.s.1, on decayed log, 28 December 2018. A.R. Niazi (LAH36079), GenBank accession number; MT012089.

It was the first attempt to describe the *P. floridanus* on both morpho-anatomical and molecular basis from Pakistan. Its micro morphological features clearly distinguished it from its genetically closely related *P. ostreatus* species (Menolli Junior *et al*., 2014).

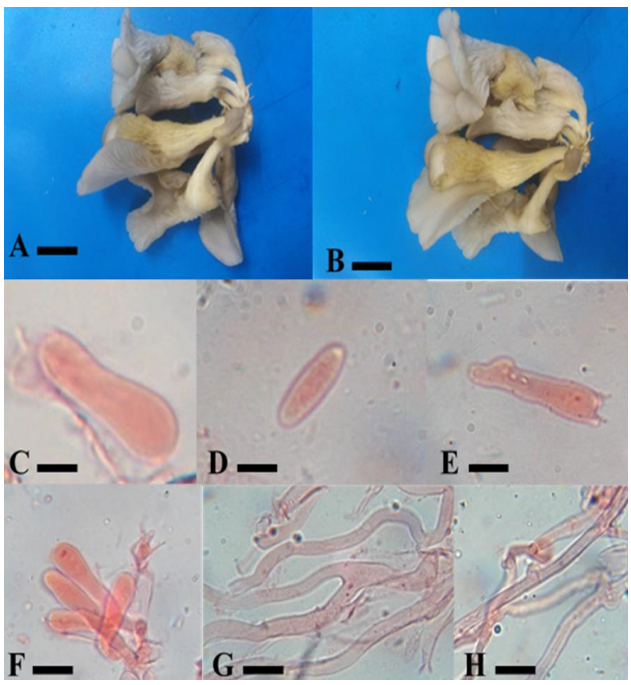

Fig. 1. **A – H: Macroscopic and Microscopic Morphology of *Pleurotus floridanus*** (LAH36079)**.** A-B. Basidiomata of *Pleurotus floridanus;* C. Basidioles; D. Basidiospores; E. Basidia F. Cystidia; G. Pileus hyphae; H. Stipe hyphae. **Scale bars:** A - B = 2.5cm, C = 9.5µm, D = 5.2µm, E = 13.4µm, F = 11.6µm, G = 20.12µm, H = 22.7µm.

Molecular and phylogenetic analysis: Barh *et al*., (2019) and Adedokun *et al*., (2016) reported previously it from India and Nigeria respectively. The specimen was sequenced using ITS1F/ITS4 region of nrDNA. Targeted ITS regions of *P. floridanus* yielded 662 base pairs. Initial blast of our amplified Pakistani strain revealed 98% similarity with *P. floridanus* (MK281340). In the phylogenetic analysis three clades were formed. All *P. floridanus* species including Pakistani strain clustered in clade I. Clade III contains all fives species that placed in subgenus *Coremiopleurotus* due to their distinct anatomical features and anamorphic stage during their cultural growth that are absent in all the species of Clade I. *Hohenbuenelia petalodes* (AF139956), was used for rooting purpose (Fig. 2). *P. floridanus* is genetically closely related to the *P. ostreatus* as shown in fig 2, but Li *et al.*, (2017) clearly demonstrate that it is a distinct species from *P. ostreatus*.

Effect of temperature and nutritious media on cultivability: Like every living organism mushrooms also require food to show their growth potential in the form of mycelium extension rate. Three different nutrient media were used to check their growth potential. Mycelium texture and growth pattern on all the media used are shown in (Table 1). Mycelium extension rate (mm/day) on different media at different temperatures was significantly different at (p<0.05) showing in (Table 2). Mycelium took almost the same no. of days to completely colonize the culture plate on CEA and PDA media while on MEA media mycelium took more days to completely colonize the petri plate (Fig. 3). All the media proved the supportive media for the growth of mycelium of *P. floridanus* but the growth is relatively slow on MEA which might be due to nutritious requirement of the *P. floridanus* mycelium fulfilled quickly by the PDA and CEA media as compared to the MEA medium. The current results were in agreement with the Zervakis *et al*., (2004) and Sardar *et al*., (2015) studied the mycelium extension rate of *Pleurotus* species more at high temperatures and found the Potato dextrose agar medium (PDA) as the best medium for the growth of mycelium of various *Pleurotus* species. Our results were also similar to the Atri *et al*., (2013). The current study was also in concurrent with the Ishaq *et al*., (2017) from Pakistan, they studied the cultural characteristics of the exotic *P. floridanus* but we studied the mycelial characteristics of the native *P. floridanus.*

Effect of temperature and grains on the spawn production potential: Grains are the rich source of carbohydrates and proteins. Small discs of mycelium fully colonized the grains by using the stored food in the grains but the colonization rate vary depending on the grains size and stored food. Colonization rate was checked at 16°C, 22°Cand 28°C temperature for both wheat and sorghum grains. Data (Table 3) showed that the colonization rate (mm/day) on sorghum and wheat grains at different temperatures was significantly differed at (p<0.05). Mycelium colonized more quickly on sorghum grains as compared to wheat grains at all three temperatures because of their smaller size and have greater surface area. Mycelium found more points for colonization so the colonization rate is higher on sorghum grains as compared to wheat grains. Our findings were similar with the Tinoco *et al*., (2011) and Tsegaye & Tefera, (2017).

Table 1. Mycelial cultural characteristics of *Pleurotus floridanus* on different media.

Types of media	Mycelium characteristics		
	Texture	Growth	Color
CEA	Cottony	Irregular	Creamy white
MEA	Cottony	Irregular	Creamy white
PDA	Cottony	Irregular	Creamy white

CEA, Compost extract agar; **MEA**, Malt extract agar; **PDA**, Potato dextrose agar

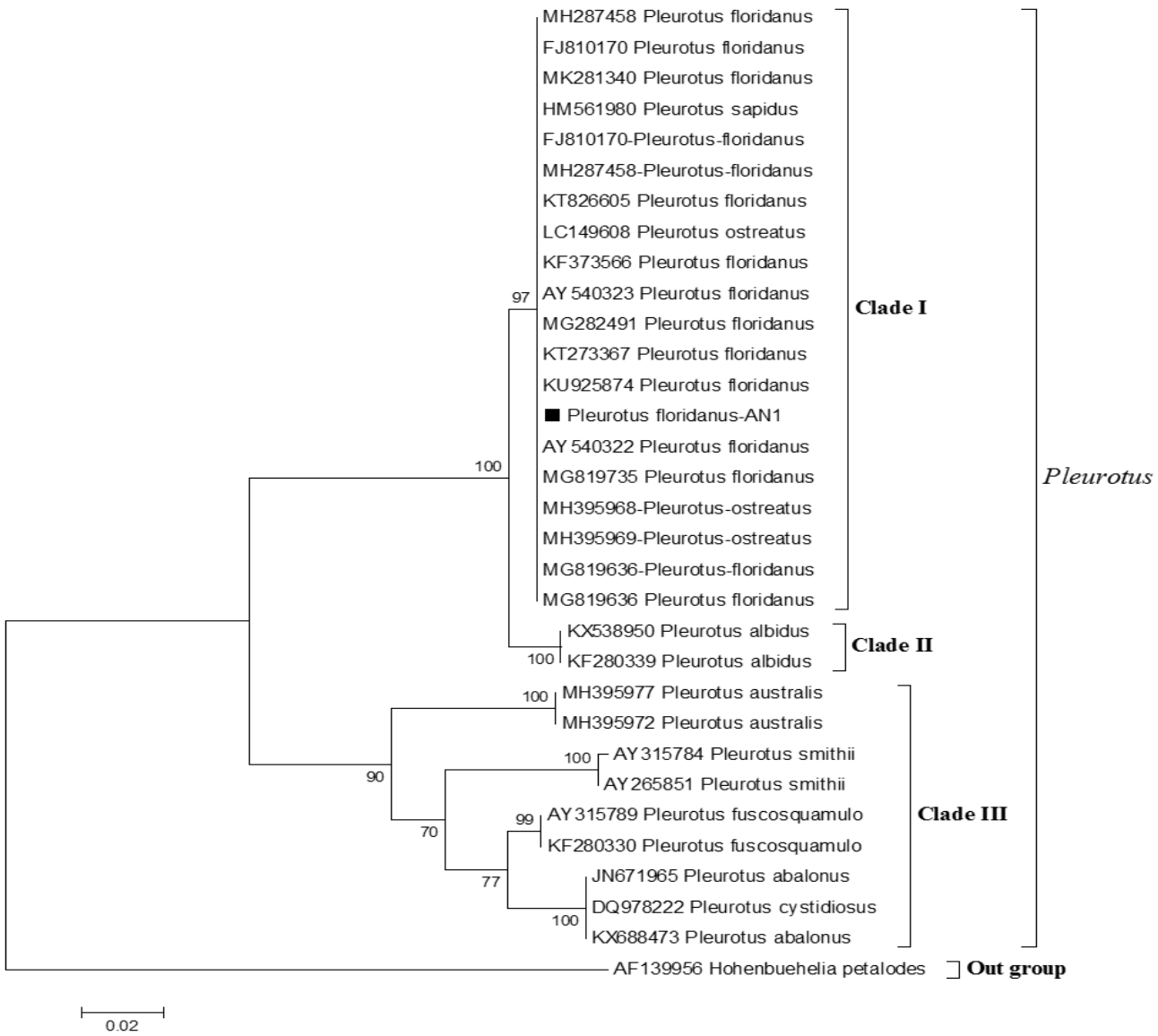

Fig. 2. Maximum likelihood (ML) analysis was performed using Jukes-Cantor model in MEGA6 software to test the phylogeny at 1000 bootstraps. The analysis involved 32 nucleotide sequences along with *Hohenbuehelia petalodes* used as an outgroup. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6. Sequences generated during this study are represented with ■

Table 2. Mycelium extension rate of *Pleurotus floridanus* at different temperatures on different media.

Temperature (°C)	Mycelial extension rate (mm/day)		
	CEA	MEA	PDA
16	4 ± 0.288e	2.5 ± 0.251f	3.7 ± 0.145e
22	9 ± 0.288c	7 ± 0.210d	8.6 ± 0.145c
28	14.5 ± 0.287a	9.9 ± 0.208b	15.06 ± 0.233a
LSD		0.701	

*The results reported were run in triplicates and stated as mean ± Standard error. *LSD stands for the least significant difference *Different alphabets indicate significant (p<0.05) difference between the mean according to Duncan's new multiple range test while ± indicates standard error. **CEA**, Compost extract agar; **MEA**, Malt extract agar; **PDA**, Potato dextrose agar

Table 3. Effect of temperature on the mycelium extension rate on the wheat and sorghum grains.

Types of grains	Mycelial Extension Rate (mm/day)		
	16°C	22°C	28°C
Wheat grains	7.76 ± 0.145e	9.66 ± 0.176d	14.8 ± 0.166b
Sorghum grains	9.83 ± 0.166d	12.5 ± 0.288c	17.8 ± 0.166a
LSD		0.584	

*The results reported were run in triplicates and stated as mean ± Standard error. *LSD stands for the least significant difference *Different alphabets indicate significant (p<0.05) difference between the mean according to Duncan's new multiple range test while ± indicates standard error

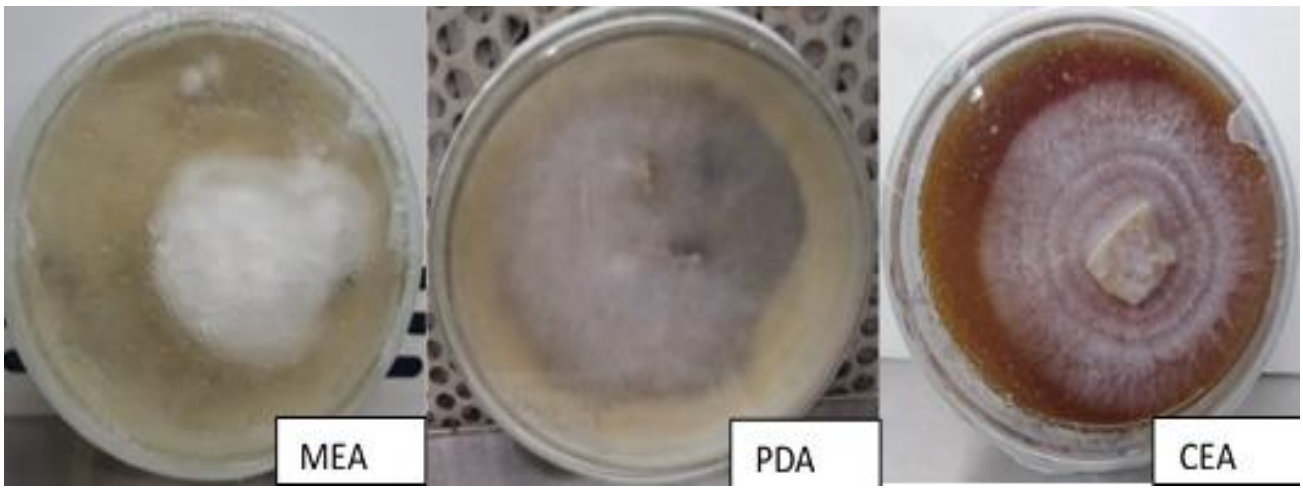

Fig. 3. Mycelium extension rate of *Pleurotus floridanus* (LAH36079) on different media at 28°C after 25 days of inoculation.

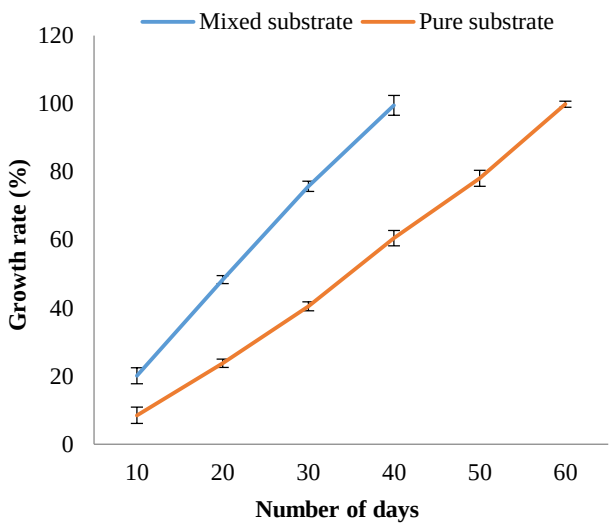

Fig. 4. Graph showing the frequency of spawn running completion on both type of substrates.

Effect of temperature and lignocellulosic substrates on the cultivation potential: Temperature is a very crucial factor for the successful fruiting of any mushroom. Only Optimized temperature and humidity can convert the dikaryotic mycelium into the fruiting body. Spawn prepared on the sorghum grains was used to determine the cultivation potential on the two types of substrates at various temperatures. There was no fruiting on both types of substrates bags kept at 22°C and 28°C while the substrates bags kept at 16°C successfully completed their spawn running period and initiated primordia formation that proved that *P. floridanus* is a lower temperature strain Data (Fig. 4) showed that Spawn run rate was faster on the substrate of wheat straw and sawdust combination as compared to the pure wheat straw only. Lignocellulosic content high in sawdust as compared to the wheat straw that was used by the mycelium as the protein and carbohydrate source for its growth that's why spawn running period completed earlier on the mixed substrate than the pure wheat straw substrate. Our results were similar to the Das *et al*., (2015) and Obodai *et al*., (2003) found the positive correlation between lignin-cellulosic contents and yield of *P. ostreatus*. After spawn running completion, specific yellowish- white outgrowths appeared on all the replicates of both types of substrates and then degenerate, after it primordia appeared. That yellowish outgrowths may act as the initiator of the primordia or pinhead emergence (Fig. 5). Time taken from the pinhead appearance to the harvesting was more on the pure substrate than the mixed substrate.

Biological efficiency: Biological efficiency (yield) obtained high in amount from the substrate of wheat straw and sawdust combination as compared to the pure wheat straw only (Table 4, Fig. 6). 57.80±0.137 g yield per 1kgbag was obtained from the mixed substrate while only 35.7±0.115g per 1kgbag was obtained from pure wheat straw substrate showed that *P. floridanus* require sawdust substrate for its optimum growth. Our findings were in agreement with the Adedokun & George-David, (2016). Our results were also in concurrent to the Ishaq *et al*., (2018) who optimize the biological efficiency of the exotic *P. floridanus* but we optimize the growth requirements of the indigenous *P. floridanus* species from Pakistan to get maximum economic viability by its cultivation at large scale.

Table 4. Yield obtained from pure and mixed substrate at 16°C.

Types of substrates	Biological efficiency (yield)			
	1st Flush yield (g)	2nd flush yield (g)	3rd flush yield (g)	Total yield (g)
Mixed substrate	18.6 ± 0.152c	20.85 ± 0.175c	18.577 ± 0.295c	57.80 ± 0.137a
Pure wheat straw	17.93 ± 0.145c	17.7 ± 0.115c	Not appeared	35.7 ± 0.115b
LSD	0.891			

*The results reported were run in triplicates and stated as Mean± Standard error. *LSD stands for the least significant difference *Different alphabets indicate significant (p<0.05) difference between the mean according to Duncan's new multiple range test while ± indicates standard error

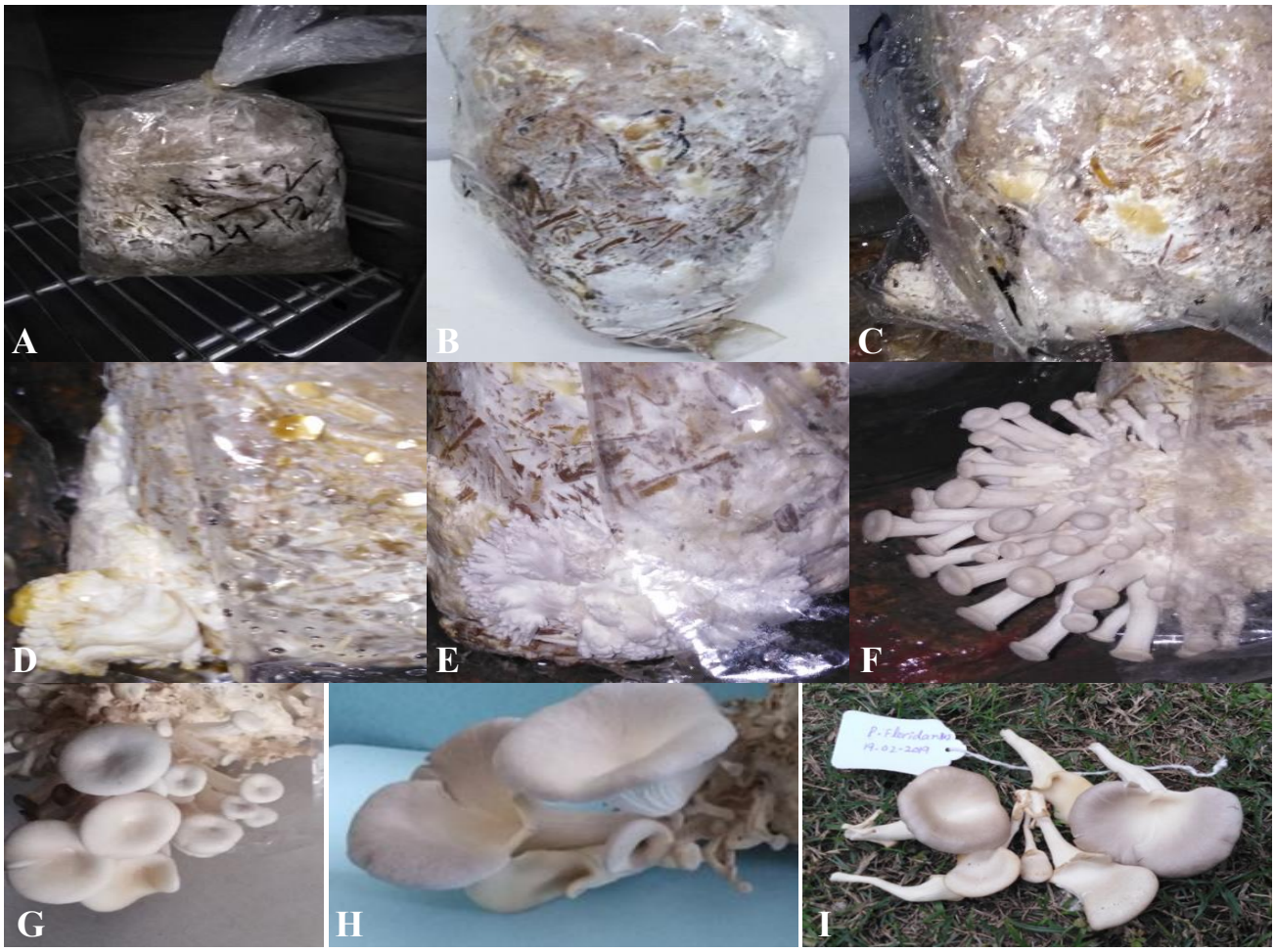

Fig. 5. All stages of cultivation of *Pleurotus floridanus* from spawn running to harvesting; (A) Spawn Running (B) Fully spawned compost (C) Yellowish white outgrowth appeared (D) Outgrowth developed fully (E) Pin head emerged (F) Growing Pin heads (G) Growing Fruiting bodies (H) Matured fruiting bodies (I) Harvesting.

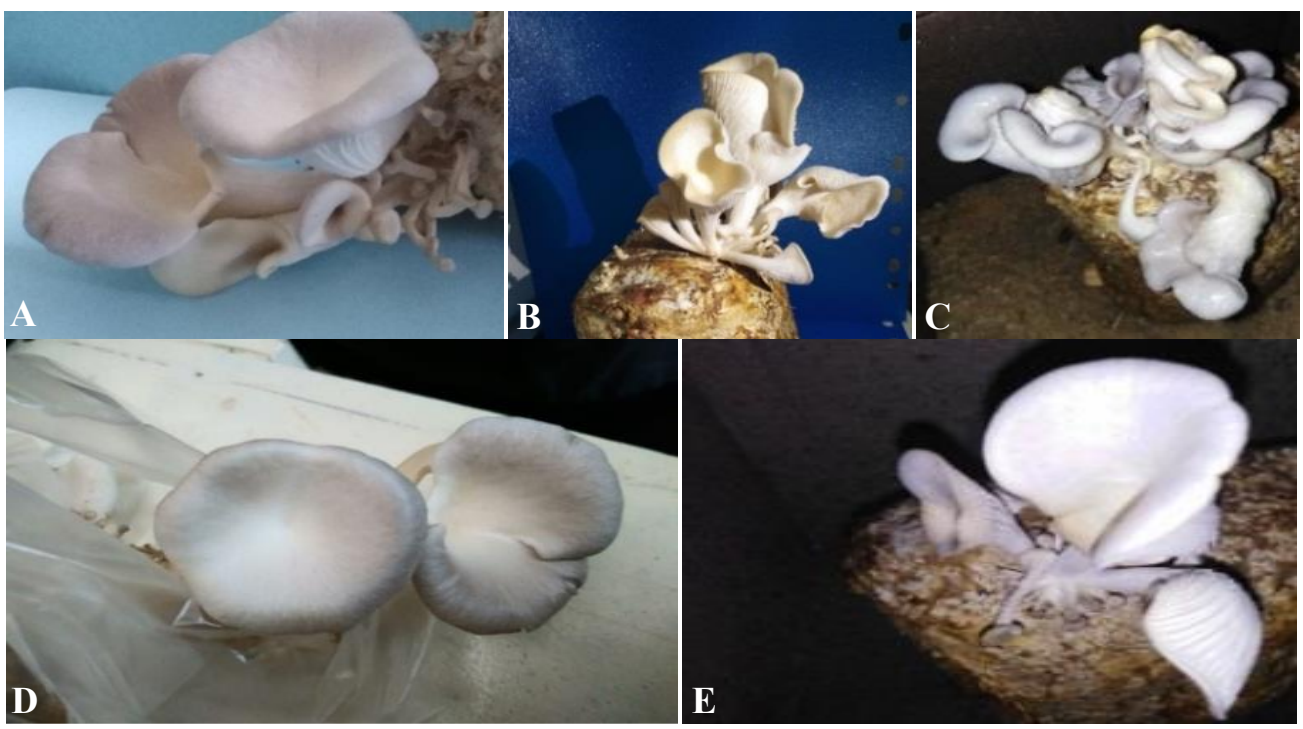

Fig. 6. Flushes observed on mixed and pure substrates; A-C, 1st, 2nd and 3rd flush on the mixed substrate; D-E, 1st and 2nd flush on the pure substrate.

Conclusion

In conclusion, *P. floridanus* have great potential to cultivate on a variety of substrates. It is a superfood and its cultivation on a large scale can help in strengthening the agro-economic status of low-income countries. Furthermore, it is previously an unreported species from Pakistan and an addition to the edible flora of the country.

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