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BIOCHEMICAL DIVERSITY IN MONOKARYONS PRODUCED FROM *PLEUROTUS OSTREATUS*

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ABSTRACT
In the present study, we have isolated fifty-eight single spore isolates from *P. ostreatus*, identified as monokaryon. We measured mycelial growth rate on PDA plates, *in-vitro* extracellular laccase enzyme activity, dye decolorization capability on solid media plate, and nutrients (proteins, carbohydrates) and antioxidant (phenolics) content in the mycelial phase of this fungus. We selected monokaryons of *Pleurotus ostreatus* due to variability in them as already proved by many researchers; and vast importance of this mushroom in not only industries but also at domestic level. This mushroom produces millions of spores having immense variability in their morphological, biochemical and molecular characters due to its bifactorial tetrapolar genetic system. From the experimental results, the mycelial growth rate was found in range of 5.01-0.48 with mean of 2.62 mm/day, whereas carbohydrates, proteins and phenolics content were in range of 4.66-3.69, 25.15-18.80 g/100g & 0.16-0.12 mg/100g, respectively. Decolorization of Malachite green and Bromophenol blue was also assessed; however, all the isolates were able to decolorize the dyes with variable extents. Four mating types were also identified by inter-crossing amongst single spore isolates on the basis of their mating compatibility. The mating type dependence of growth rate in monokaryons can be helpful for marker-assisted selection of fast-growing monokaryons to be used in the construction of dikaryons able to colonize the substrate faster. Statistical analysis could be try to suggest that it would be better to select the isolates on the basis of their phenotypic variance than genotypic for further in breeding and production at industrial scale.

KEY WORDS: Oyster mushroom; Malachite green; Bromophenol blue; radial growth; mushroom breeding; mating types.

INTRODUCTION

Species of genus *Pleurotus*, *Pleurotus ostreatus*, belongs to gilled basidiomycete. It is also known as Oyster mushroom due to oyster shape of fruiting body. This mushroom has an ability to grow directly on unfermented natural lignocellulosic agricultural and can converted them into useful products (Rajarathnam and Bano 1989; Hestbjerg *et al*., 2003). It also has the vast ability to degrade many xenobiotics including many reactive textile dyestuff like-Malachite green, Bromophenol blue & other toxic industrial waste materials and converted in to less toxic materials (Kwang and Chang, 1998) due to its power of non-specific enzyme systems (*e.g.* laccase) present in this mushroom. This conversion property is one of the most economically viable processes for the bioconversion of lignocellulosic and other toxic wastes materials (Bogan *et al*., 1999; Cohen *et al*., 2002; Singh *et al*., 2011). Besides the bioconversion property of this *P. ostreatus*, it also produces mushrooms that have many medicinal, nutritional, biotechnological and environmental applications (Cohen *et al*., 2002; Patel *et al*., 2012). Fruiting bodies of *P. ostreatus* can produce millions of spores after meiosis (Okuda *et al*., 2009) and these monokaryotic spores are very important in sexual reproduction of this mushroom for generating diversity due to spore`s self-sterility nature. Two compatible spores produced heterothallic homo- basidiomycete whose mating is controlled by a bi-factorial (tetra-polar) genetic system (Casselton and Olesnicky, 1998). In this system two unlinked loci -named as *A* and *B*, subsequently named as *MatT* – (transcription factor) and *MatP*- (pheromone & pheromone receptor) by Brown and Casselton (2001), were engaged in the controlling of formation of sporocarp. These two mating loci control different steps of hyphal fusion, nuclear migration, and nuclear sorting during the onset and progress of the dikaryotic growth (Larraya *et al*., 2001). The availability of morphological and biochemical markers in monokaryons can be helpful for marker-assisted selection of fast-growing monokaryons to be used in the construction of dikaryons able to colonize the substrate faster as reviewed by Kothe (2001). So, objectives of the present study were isolation of single spore isolates (SSIs) and identification of them as monokaryons; and assessment the diversity in morphological (radial growth), biochemical (total crude protein, carbohydrate and phenolics), enzymatic (laccase activity) and dyes (Malachite green and Bromophenol blue) decolorization capacity of selected monokaryons. Finally, in

the present study, we have identified four mating types from isolated monokaryons by intra-crossing of monokaryons.

MATERIALS & METHODS

Fungal culture and growth condition

Pleurotus ostreatus (ITCC: 1088), used in the present study was procured from Forest Research Institute, Dehradun, Uttarakhand, India. Culture of *P. ostreatus* (after here known as parent culture) and its single spore isolates (SSI) were maintained on PDA (Dextrose- cat. No.: GRM077-500G; Agar powder- cat. No.: GRM026-500G) slants and preserved at 4ºC for 2-3 months. After this period, sub-culturing was needed. The mycelial mat of parent culture and its SSIs was harvested, freeze-dried, and ground in cold mortar & pestle containing desired buffer after growing in potato dextrose broth medium at 28±1°C. All the relevant chemicals were procured from Himedia Laboratory, Mumbai, India.

Fructification and determination of Mating Types

The fruiting body of parent culture was cultivated in the mushroom production house, established in Department of Biotechnology, Veer Bahadur Singh Purvanchal University, Jaunpur (India) at optimum growing condition (temperature- 25 to 30°C and humidity- 55 to 85%), a fruit body was selected and then collected SSI as described below.

Isolation of single spore isolates (SSIs)

For the isolation of single spore isolates, a fresh and mature pileus (upper part of fruiting body) was put in sterilized Petri dish covered with a sterilized black paper sheet at 28±1°C for 2 -4 hours. After shedding off the spores, paper was dried at 37ºC (now called as spore print) and stored at room temperature in sealed plate for further use. The spores collected in the form of spore print (plate a, Fig. 1) were dissolved and diluted in sterilized normal saline water (1.8% NaCl – cat. No.: MB023-500G) to obtain 10-12 spores on a plate and 200 µl of this suitable diluted spore solution was placed and spreaded on water agar plates (agar-agar powder in distilled water @ 2%) and incubated at 28±1°C for sprouting of spores. After visualizing the filaments surrounded to spores (plate b, Fig. 1), they were picked by fine needle (size: 0.61mm in diameter) in separate PDA plates as single spore isolates (SSI). All the developed SSIs were examined under 40X magnification (Make: Nikon Labo Phot 2) after staining with trypan blue (cat. No.: GRM263-5G) stain for the presence/ or absence of clamp connections (Fig. 2). SSIs without clamps indicated their monokaryotic nature and were labelled accordingly for the isolation of four mating types representing the segregated progeny; on the other hand, SSIs with clamp connection were discarded. The monokaryotic lines were designated as bracketed numerals from 1 to 58 (*e.g.* [1],[2]…and so). All the selected monokaryons were stored in PDA slant at 4ºC for further work. Monokaryotic cultures were sub-cultured after every 2 months periodically.

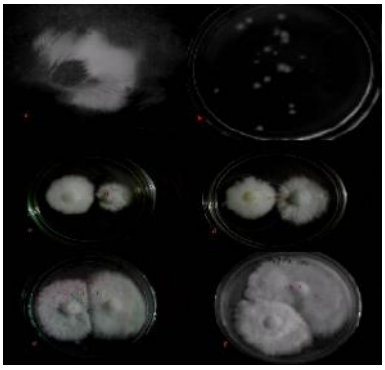

FIGURE 1: Different stages of isolation of SSI

FIGURE 1a- spore print, **b-** single spore isolates on the water agar plate, **c-** inoculums from two different SSIs, **d-** Growing mycelia towards each-other, **e-** meeting of the growing mycelia, and **f-** contact zone formation at the midpoint of two inoculums.

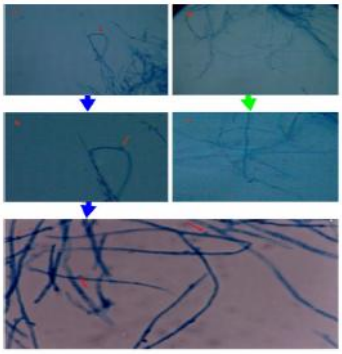

FIGURE 2: Crossing and appearance of clamp connection between two monokaryotic mycelia

Photograph showing characteristics features of mono- and di- karyotic hyphae. Red arrow indicates clamps of di-karyotic hyphae

Mating compatibility studies

For the isolation of mating types or for testing the compatibility of monokaryons, mycelia of different monokaryons (all 58) were inter-crossed in all possible combination with each other. All crosses were conducted on 90mm PDA plates by inoculation of the mycelia from two different SSIs in the center of the plate ~1.0 cm apart from each other and incubated at 28±1°C until the mycelia grew to meet (plate c, Fig. 1). After this growth, a piece of medium was cut off from plate and stained with trypan blue solution and examining for a clamp connection (dikaryon) or their compatibility (*i.e.* present or absence of clamp connection). Formation of clamp connections was the primary criterion for mating compatibility study (Eger, 1978). If clamp-connections were observed in the contact zone, mycelia in the outer edges of paired colonies were also examined. When clamp-connections were also seen in the edge of paired colonies, sexual compatibility of the mated pair was scored positive as described by Kazuhiro *et al*. (2005). Four mating types were isolated by four round of monokaryotic- monokaryotic (mon-mon) crossing. The plan of these crossing is given in table (Table 1).

TABLE 1: Intra-crosses among the SSIs (monokaryons) for mating compatibility test

1st Round Crossing				2nd Round Crossing		3rd Round Crossing		4th Round Crossing	
Cross	Rxn	Cross	Rxn	Cross	Rxn	Cross	Rxn	Cross	Rxn
1 × 1	-	1 × 30	+	7 × 7	-	2 × 2	-	19 × 19	-
1 × 2	-	1 × 31	+	7 × 1	+	2 × 19	+	19 × 22	+
1 × 3	+	1 × 32	-	7 × 2	-	2 × 22	-	19 × 28	+
1 × 4	+	1 × 33	+	7 × 19	-	2 × 26	+	19 × 29	+
1 × 5	+	1 × 34	-	7 × 21	+	2 × 28	-	19 × 41	+
1 × 6	+	1 × 35	-	7 × 22	-	2 × 29	-	19 × 42	+
1 × 7	+	1 × 36	+	7 × 23	+	2 × 34	+	19 × 45	+
1 × 8	+	1 × 37	+	7 × 25	+	2 × 41	-	19 × 46	+
1 × 9	+	1 × 38	+	7 × 26	-	2 × 42	-		
1 × 10	+	1 × 39	+	7 × 28	-	2 × 43	+		
1 × 11	+	1 × 40	+	7 × 29	-	2 × 45	-		
1 × 12	+	1 × 41	-	7 × 32	+	2 × 46	-		
1 × 13	+	1 × 42	-	7 × 34	-				
1 × 14	+	1 × 43	-	7 × 35	+				
1 × 15	+	1 × 44	-	7 × 41	-				
1 × 16	+	1 × 45	-	7 × 42	-				
1 × 17	+	1 × 46	-	7 × 43	-				
1 × 18	+	1 × 47	+	7 × 44	+				
1 × 19	-	1 × 48	+	7 × 45	-				
1 × 20	+	1 × 49	+	7 × 46	-				
1 × 21	-	1 × 50	+	[+ve sign showing Clamp connections (cross compatible), –ve sign showing absence of Clamp connections (cross incompatible)].					
1 × 22	-	1 × 51	+						
1 × 23	-	1 × 52	+						
1 × 24	+	1 × 53	+						
1 × 25	-	1 × 54	+						
1 × 26	-	1 × 55	+						
1 × 27	+	1 × 56	+						
1 × 28	+	1 × 57	+						
1 × 29	-	1 × 58	+						

Assessment of radial growth rate

Radial growth rate (mm/day) was studied on PDA plates for each SSI in triplicate. A mycelia plug (4.0 mm diameter) was prepared by cork-borer from all culture plate and placed in the centre of the PDA plates. Plates were incubated at 28±1°C in BOD incubator (Make: NSW, New Delhi, India) and followed by the measurement of radial growth from the second day of inoculation (Fig. 3). Growth measurement was performed after every 24 hours till the fully covered the plates fully and the measurement was done by meter scale with magnification lens (hand handled) in millimetre.

FIGURE 3: Measurement of mycelia radial growth

Measurement of mycelia radial growth on PDA plate by meter scale in lab condition

Determination of protein, carbohydrate and phenolics in mycelia

Total proteins, carbohydrates and phenolics of monokaryotic mycelia were determined by standard methods as described by Naraian *et al*. (2014) and Hsu *et al.* (2003). The total protein content present in mycelia was analyzed by the standard method of Bradford (Bradford, 1976). The protein concentration was finally determined using standard curve plotted for bovine serum albumin (BSA) and calculated for mg/g of the mycelium sample. Total carbohydrate content available in mycelium was determined using the phenol-sulfuric acid method of Dubois *et al.* (1956), using standard curve plotted for pure glucose and calculated for mg/g of the mycelium sample. However, total amount of phenolics content of methanolic extract of dried mycelia was measured as per standard method developed by Singleton *et al.* (1965). Extract from each SSIs mycelium (1.0 ml) was added into 1.0 ml of Na_2CO_3 (Cat. No.: GRM3951-500G) and also 400 µl Folin-Ciocalteau Reagent (Cat. No: ML059-500ML) was mixed in solution. Finally volume was maintained up to 10.0 ml with distilled water and then incubated at 27°C in incubator for 1.0 hour. After incubation period, absorbance was recorded at 765 nm by UV-visible spectrophotometer (Make: Systronics India Limited). The total phenolics concentration was finally determined using standard curve plotted for Gallic acid and calculated by mg/g of the mycelium sample.

Enzyme Assay

Preparation of Enzyme Extract

Crude enzyme extract was obtained by crushing 0.5 g of mycelia from 3 - 5days young culture in1.0 ml phosphate buffer (0.1 M; pH 6.8). Mycelia and solid substrate were removed by filtration followed by centrifugation at 10,000 rpm in a cooling centrifuge (Make: Remi, India) for 5 min. The supernatant obtained was used for enzyme assay.

Determination of laccase enzyme activity

Laccase activity was determined via the oxidation of o-methoxyphenol catechol monomethylether (guaiacol) as substrate. The reaction mixture contained 100 mM guaiacol (Make: Himedia, Cat. No.: RM1118-250G) in 0.1 M sodium phosphate buffer (pH 6.8) and 1.0 ml of crude enzyme solution was incubated at 37°C for 10 min. The oxidation was followed by the increase in absorbance and this change in absorbance was measured at 470 nm in UV-visible spectrophotometer (Make: Systronics India Limited). One active unit was defined as 1.0 μmol of guaiacol oxidized per minute. The activity was calculated as per the formula:

IU/ml= ΔA@470nm/0.001

Dye degradation on solid medium

Dye degradation on solid medium was carried out according to the method of Machado *et al.* (2005) by the using of potato dextrose agar media for Malachite green dye (MG-cat. No.: S020) with five different concentrations as 20, 40, 60, 80 and 100 ppm); whereas, the concentration of Bromophenol blue (cat. No.: MB123-50G) was 50, 100, 150, 200, 250 and 300 ppm. Point inoculation was performed on dye rich medium from 5 days old fungal culture. Point inoculation was performed by placing mycelial discs at the centre of PDA media, known as inoculums (size: 4mm in diameter), obtained from actively growing culture in it and incubated at 28°C in the incubator for 10 days. A parallel experiment was also performed with the media plates without any dye. Results were observed after 5 days and 10 days, as clear zone was appeared against green/blue background. Decolorization was calculated considering the color of the non-inoculated control to be 100%. The dye plates were analyzed for decolorization. The decolorization of dyes was observed by the formation of decolorization zone under and around developing mycelia. The results were observed after 5 days and 10 days and compared with control plates.

Statistical Analysis

Analysis of variance was performed using the GLM procedure of SAS 9.2 (SAS Institute, 2007). The phenotypic and genotypic coefficient of variation were calculated according to Burton and De Vane (1953), heritability was calculated following Johnson *et al*. (1955). All the statistical analyses were conducted using the SPAR v. 2.0.

RESULTS

Screening of mating types

After sprouting of spores on water agar plate (just after 2 - 3days of incubation), individually sprouted spore (called as: single spore isolates) were collected on individual fresh PDA plates and allowed to further growth at 28±1°C. Initially a total of sixty single spore isolates (SSIs) were collected and checked for presence/ or absence of clamp connection. Fifty eight out of sixty collected single spore isolates were identified as monokaryons; and then they were preserved on PDA slants with a defined bracketed numerals from 1 to 58 (e.g. [#1], [#2]…and so). All the selected monokaryons were stored in PDA slant at 4ºC for further work and they were sub cultured after every 2 months periodically. For the identification of mating types, three sets of intra-crosses were made within all SSIs. The outcome of all three sets of pairings were consistent with a bi-factorial tetra-polar mating system and as a result of mating compatibility test by intra-crossing of all (fifty eight) selected SSIs as given in table (Table 1), all were categorized in to four types: I, II, III & IV as given in table (Table 2). From the table 2, it was cleared that Mating type I & II could say for SSI [#1] & [#7], however, mating type III & IV could be considered for SSI [#2] & [#34]. In other word, monokaryons [#1] & [#7] could be categorized as A_1B_1 and monokaryons [#2] & [#34] as A_2B_2.

TABLE 2: Confirmation of mating-types

	1	7	2	34
1	-	+	-	-
7	+	-	-	-
2	-	-	-	+
34	-	-	+	-

Foot note:*- denotes not compatible while **+** denoted compatible mating-type

TABLE 3: Grouping of monokaryons based upon their growth rate

Radial growth rate (mm/day) of mycelia after 7th day				
0 – 1.0 (mm/day)	1.1-2.0 (mm/day)	2.1-3.0 (mm/day)	3.1-4.0 (mm/day)	4.1-5.0 (mm/day)
54	4, 9, 12, 15, 22, 26, 32, 36, 39, 47, 48, 56, 58,	3, 6, 7, 8, 10, 14, 16, 18, 19, 20, 21, 25, 28, 29, 31, 40, 41, 43, 46, 49, 50, 51, 52, 53, 55, 57	5, 11, 13, 23, 30, 34, 35, 37, 38, 42, 44	1, 2, 17, 24, 27, 33

Radial growth

Radial growth rate (mm/day) of monokaryotic mycelia at 7th day of incubation is represented in table (Table 3). From the table (Table 3), it is revealed that on the basis of their growth rate, SSIs could be comprised into four groups. In the first group, the lowest growth rate was SSI [#54] and it is equal to 1.0 mm/day. Second group comprises total thirteen SSIs with 1.1 to 2.0 mm/day growth rate, and third group comprises a total of twenty six SSIs with 2.1 to 3.0 mm/day growth rate, however, fourth group comprises a total of five SSIs with 4.1 -5.0 mm/day growth rate.

Nutritional content

Many nutritional parameters were measured for both types of cultures- parent as well as SSIs in their mycelial stage of life cycle. The measurements of total crude proteins, carbohydrates as well as phenolics have great importance in nutritional and functional characterization. Mean of all the measured nutritional content is given in table (Table 4). From the table 4, it is revealed that the total crude proteins was found in a range of 25.15-18.80 g/100g of mycelial weight with a mean of 22.33±1.23 and carbohydrates was found in a range of 4.66-3.69 g/100g of mycelial weight with a mean of 4.12 ±0.21. However, the total phenolics content was in range of 0.16-0.12 mg/100g of mycelial weight with a mean value 0.14 ±0.01 as given in table (Table 4).

Laccase Activity

Laccase activity was determined by the oxidation of o-methoxyphenol catechol monomethylether (guaiacol) as substrate for the assessment of lignolytic activity, detected in the supernatants when *P. ostreatus* and its monokaryotic mycelial cultures grown in aerated culture. Laccase activity was detected only when the culture grown in liquid media and the mean value of activity is given in table (Table 4). At the fifth day, the laccase activity was noted peak 19.13 U/100g mycelia, while 3.58 U/100g was observed as lowest activity with a mean of 9.69 U/100g of mycelia in broth culture media as appeared in the table (Table 4).

TABLE 4: Mean performance of *P. ostreatus* and its monokaryons for different mushroom traits

Sample	Growth rate (mm/day)	Carbohydrate (g/100g)	Protein (g/100g)	Phenolics (mg/100g)	Laccase activity (U/g)	Malachite Green (0.008%)		Bromophenol Blue (0.1%)	
						5th Day	10th Day	5th Day	10th Day
Parent	0.48	4.23	24.25	0.14	18.40	1.87	33.71	17.59	75.14
SSI 1	5.01	4.27	23.93	0.14	19.13	21.57	43.27	18.98	74.65
SSI 2	4.61	4.30	20.80	0.14	18.63	16.57	33.28	18.42	71.83
SSI 3	2.21	4.39	20.97	0.15	8.83	8.97	17.97	20.72	73.27
SSI 4	1.56	4.35	23.59	0.15	7.57	8.03	16.11	21.10	78.20
SSI 5	3.06	4.33	21.28	0.12	4.80	11.60	23.27	19.66	76.49
SSI 6	2.18	4.26	20.77	0.14	6.83	8.93	17.86	18.34	77.32
SSI 7	2.91	4.11	20.93	0.14	10.03	10.71	21.50	19.07	75.38
SSI 8	2.40	4.07	19.89	0.14	9.28	7.80	15.69	17.90	79.57
SSI 9	1.83	4.04	21.26	0.14	6.10	8.70	17.50	18.65	78.88
SSI 10	2.89	4.09	21.73	0.15	10.12	10.60	21.25	19.97	73.64
SSI 11	3.19	4.01	21.10	0.14	12.40	10.70	21.43	19.47	71.67
SSI 12	1.75	3.94	23.46	0.13	6.13	6.91	13.85	19.78	68.12
SSI 13	3.24	3.92	24.71	0.14	12.67	6.18	12.38	19.15	68.93
SSI 14	2.28	3.82	23.95	0.14	7.17	5.44	10.91	17.49	72.41
SSI 15	1.73	3.84	21.89	0.14	6.57	4.71	9.44	19.59	71.72
SSI 16	2.66	3.83	24.12	0.14	9.15	3.98	7.98	20.25	75.41
SSI 17	5.00	3.89	22.38	0.15	18.67	3.25	6.51	20.19	70.23
SSI 18	2.80	3.94	19.29	0.14	9.83	2.52	5.04	20.34	75.75
SSI 19	2.44	3.99	20.47	0.13	9.20	1.79	3.58	20.40	75.92
SSI 20	2.84	4.10	24.52	0.14	10.22	1.06	2.11	19.41	74.65
SSI 21	2.43	4.21	22.80	0.16	9.43	0.33	0.64	18.74	68.55
SSI 22	1.12	4.01	22.87	0.15	9.83	4.53	9.11	17.63	65.52
SSI 23	3.31	4.19	22.46	0.14	12.93	11.47	23.02	17.46	63.63
SSI 24	4.13	4.23	20.59	0.14	14.90	14.97	30.04	19.72	64.64
SSI 25	2.46	4.22	21.29	0.14	10.10	13.33	26.74	17.81	66.61
SSI 26	1.14	4.27	24.33	0.14	4.03	5.40	10.83	18.69	68.50
SSI 27	4.43	4.52	18.80	0.14	18.20	20.97	42.00	19.28	75.61
SSI 28	2.57	4.51	25.15	0.16	10.40	9.60	19.25	18.59	85.46
SSI 29	2.71	4.16	22.54	0.15	10.42	10.43	20.94	19.87	84.07
SSI 30	2.86	4.12	22.50	0.13	10.29	11.53	23.15	20.15	77.20
SSI 31	2.58	4.06	23.29	0.14	9.47	9.48	19.04	19.69	74.73
SSI 32	1.46	4.03	24.83	0.14	6.24	6.47	12.95	19.37	73.00
SSI 33	4.20	3.99	24.87	0.14	13.51	16.30	32.70	17.89	72.76
SSI 34	3.34	4.20	21.40	0.14	18.28	10.60	21.26	16.39	69.82
SSI 35	3.21	4.11	21.41	0.15	11.96	10.90	21.87	18.20	70.38
SSI 36	1.76	4.03	23.90	0.15	6.14	5.97	11.99	17.20	76.53
SSI 37	3.19	3.97	22.66	0.12	12.84	12.03	24.14	18.98	75.12
SSI 38	3.75	3.94	20.65	0.15	11.97	11.70	23.48	20.52	75.61
SSI 39	2.40	3.91	20.95	0.14	6.80	13.30	26.68	21.41	70.30
SSI 40	2.20	3.89	20.09	0.13	6.70	7.83	15.71	20.53	72.10
SSI 41	2.51	3.95	22.23	0.15	9.71	8.50	17.09	18.89	67.08
SSI 42	3.22	3.96	20.92	0.15	10.57	12.47	25.03	18.22	63.78

SSI 43	2.74	3.99	20.90	0.13	9.94	12.67	25.40	20.01	65.96
SSI 44	3.77	4.20	22.82	0.14	12.29	13.50	27.09	20.17	67.03
SSI 45	1.51	3.95	23.24	0.15	5.62	6.20	12.47	19.36	73.88
SSI 46	2.92	3.69	23.20	0.15	9.98	11.27	22.62	18.66	75.00
SSI 47	1.33	3.74	20.95	0.14	4.79	4.47	8.98	19.13	80.33
SSI 48	1.46	3.81	22.53	0.15	4.37	5.10	10.23	18.78	80.15
SSI 49	2.18	3.93	22.70	0.15	7.77	7.57	15.16	19.85	74.31
SSI 50	2.47	4.02	24.30	0.14	9.35	8.67	17.41	19.24	71.73
SSI 51	3.06	4.10	19.97	0.15	10.95	11.77	23.62	19.59	67.44
SSI 52	2.90	4.23	24.89	0.15	10.16	11.27	22.62	18.58	67.60
SSI 53	2.48	4.33	23.38	0.14	9.03	8.83	18.78	17.43	65.60
SSI 54	0.50	4.53	23.14	0.14	3.58	2.30	4.64	18.69	66.53
SSI 55	2.59	4.66	23.06	0.15	9.65	9.89	19.87	18.96	73.72
SSI 56	1.77	4.63	24.50	0.15	6.53	6.40	12.83	19.92	73.68
SSI 57	2.89	4.65	21.61	0.14	10.73	11.27	22.62	18.86	75.46
SSI 58	1.77	4.50	20.78	0.14	6.52	5.30	10.62	19.86	71.54

Dye degradation

Parent culture (*P. ostreatus*) and its 58 SSIs were analyzed regarding their ability to decolorized two triphenylmethane category of dyes (Malachite green and Bromophenol blue) on solid medium. All the cultures showed variable dye decolorization capability at 5th and 10th days of incubation as appeared in table (Table 4). From the table 4, Malachite green (MG) dye decolorization capacity of *P. ostreatus* (parent culture) and its SSIs was checked by adding different concentration of dye, *e.g*. 20, 40, 60, 80 and 100 ppm (parts per million) into culture medium. *P. ostreatus* could decolorize 1.87% at 5th day of incubation and 33.71% at 10th day of incubation for 80 ppm concentration, however, its SSIs could be able to decolorized 21.57 to 0.33% at 5th days of incubation and 43.27 to 0.64% at 10th days of incubation, on average. The maximum decolorization of this dye was achieved by SSI [#1] and minimum decolorization was by SSI [#21]. However, in case of Bromophenol blue (BB) dye, the concentration was 50, 100, 150, 200, 250 and 300 ppm into culture medium. *P. ostreatus* could decolorize 17.59% at 5th day of incubation for 100 ppm and 75.14% at 10th day of incubation for the same concentration; however, its SSIs could be able to decolorized 21.41 to 16.39% at 5th day of incubation for 100 ppm concentration and 85.46 to 63.63% at 10th day of incubation for the same concentration of dye. Maximum decolorization was achieved by SSI [#40], while minimum decolorization was showed by SSI [#35] at 5th day of incubation, whereas, maximum degradation was demonstrated by [#29] and minimum by SSI [#24] at 10th day of incubation.

DISCUSSION

Oyster mushroom (*P. ostreatus*) is commonly cultivated in many Asiatic countries like China, Japan, Taiwan and India, due to its simple cultivation technique and wide range of temperatures (25~35ºC). This mushroom possesses many important properties not only for humankind but for other animals also (Albores *et al.*, 2006). *P. ostreatus* can produce millions of spores (Okuda *et al*., 2009) and they shedded under the pileus of fruiting bodies, forming a layer of almost white-colour area. A spore suspension was obtained by removing a sporocarp, kept at sterilized paper and agitating them in sterilized water as similar steps were described by Choi *et al.* (1999). Serially diluted spore suspensions were placed on water agar and after incubation at 28±1°C many of them were sprouted. Sixty eight separated mycelia generated from a single spore were transferred on fresh PDA media plate and each of them was checked for the presence of clamp connection. And finally 58 SSIs as monokaryons were preserved for further studies.

The key role of saprophytic macro-fungi, such as *P. ostreatus* is in water sequestration, nutrient cycling, human nutrition and bioremediation of agro-industrial waste materials has been well studied over a number of decades (Villas-Boas *et al.* 2002; Zhang *et al*., 2002); and the production of over a millions of spores due to bifactorial tetrapolar genetic system is also well established (Larraya *et al.*, 2001). However, morphological and biochemical profiling of monokaryons generated from *P. ostreatus* have not well documented but these are available for different species of *Pleurotus* including *P. ostreatus*. An understanding of growth response and other biochemical aspects about many SSIs could improve the acquaintance about SSIs generated from a single sporophore as a result it will be very useful in the production of compatible dikaryotic strains with improved mushroom`s characters.

Average values of total radial growth up-to 7th day incubation of individual SSI are presented in table (Table 3). It was observed that different isolates exhibited different mycelia growth and on the basis of their growth rate isolates could be categorized into five groups having different growth rate in laboratory studies as in table (Table 3). Many other workers also have performed similar experiments on different fungal species to study their behaviours on

different culture media and environmental factors. Kim and Xiao (2005) assess the similar effect on *Sphaeropsispyri putrescens*, the causal agent of Sphaeropsis rot of pears and apples. In the present study, the highest growth rate was 4.1 to 5.0 mm/day of total six monokaryons (*i.e.* #1, #2, #17, #24, #27, #33) and the lowest growth rate was showed by only a single monokaryon, *i.e*. #54 as presented in table (Table 3), however, the mycelial growth rate for other five species of genus *Pleurotus* was 8.0 to 13mm/day at 25°C as reported by Atri *et al*. (2012). Radial-growth rate has been shown to be a good measurement approach for the discrimination of faster and slower monokaryons for breeding purposes.

From the table 2 and table 3, it is recognized that distribution of mating types does not depend upon the growth factor`s distribution. Two of the four identified mating type were belong to highest growth rate group however others two shown different growth rate among them. The present observation is in accordance to Larraya *et al.* (2001), stated that the edible fungus *P. ostreatus* (oyster mushroom) is an industrially produced heterothallic homobasidiomycete whose mating is controlled by a bi-factorial tetra-polar genetic system.

There are millions of spores formed from a fruiting body. All the spores having almost different qualitative attributes like radial growth behaviours. All the spores are belonging to four categories: as called mating types (e.g. four mating types are occurs in *Pleurotus* species). Mating type analysis would be used to differentiate one closely related basidiomycetes fungi, *P. ostreatus.* Isolated mating types would be beneficial for the study of segregation of quantitative properties. Mating types would be used in the identification of species by di-mon crossing. The mating type dependence of growth rate in monokaryons can be helpful for marker-assisted selection of fast-growing monokaryons to be used in the construction of dikaryons able to colonize the substrate faster.

The measurements of total crude protein, carbohydrate as well as phenolics have great importance in nutritional and functional characterization. Among the 58 monokaryons considerably variation were observed for all important nutritional attributes under study. In the present study, all the monokaryons were in around to their parent culture for their protein, carbohydrate and phenolics contents in mycelia stage as shown in table (Table 4). The total protein content was found in line with earlier report by Yang *et al.* (2001) but in the present study, total protein content is higher than reported by Reis *et al.* (2012), when they studied nutritional contents in many mushroom`s fruiting body. The nutrient contents were found to be distributed un-even regarding to its SSIs and mating types. A scatter plot was plotted by the total crude protein content and the radial growth rate, the resulted plot showed no correlation between the total protein content and radial growth rate. Value of correlation coefficient between total crude protein content and the radial growth rate was found -0.228. When the data of total protein and radial growth rate were analyzed by simple linear regression then it was with a slope of -0.1399 and the intercept (a) was 5.74.

Although, activity of laccase enzyme and radial growth rate was showed a strong positive correlation on scatter plot and correlation coefficient (r) was 0.736, however, the data of laccase activity and radial growth rate were analyzed by simple linear regression then it was with a slope of 2.8899 and the intercept (a) was 2.39. Again, scatter plot was plotted between laccase activity and total protein but it was found that there is no correlation between the total protein content and laccase activity in scatter plot. The value of correlation coefficient was calculated and it was -0.102. When the data of total protein content and laccase activity were analyzed by simple linear regression then it was with a slop of -0.2458 and the intercept (a) was 15.45. Since, laccase is a unique enzyme, having considerable value not only research purposes but also for industrial level due to its broad range of substrate specificity (Singh *et al*., 2013). Laccase enzyme production and radial growth rate were also assessed by Naraian *et al.* (2014) and they were also reported that a positive correlation between them, however, they reported very low level of enzyme activity as compared to the present study.

From the Table 4, the carbohydrates content in mycelia of all isolates was found 4.12 ±0.21 g/100 g. This value is lower as reported by Reis et al. (2012), when they studied nutritional contents in many mushrooms. Total phenolics content of parent culture and its SSIs were in range of 0.16-0.12 (mg/100g) with a mean value of 0.14 ±0.01 (mg/100g). All the SSIs showed almost similar content of phenolics to their parent as revealed in table (Table 4). The value of total phenolics found in present study was lower than Jeena *et al.* (2014); they reported 0.68 ±0.01 (mg/100g) in the fruiting body of *P. ostreatus*. Many earlier reports reported that fruit bodies of *P. ostreatus* contains anti oxidant property, and it was due to Phenolics, are the major naturally occurring antioxidant components found in many mushroom (Tsai *et al.* 2006).

In the present study, microbial degradation of two hazardous dyes, Malachite green and Bromophenol blue, in semi-solid medium was also carried out to examine the degrading ability of *P. ostreatus* and its 58 monokaryotic isolates. Decolorization of even higher concentration of Bromophenol blue was generally greater at 5th Day of incubation than the lower concentration of Malachite green G. It could be proved that the Malachite green is more potent dye than Bromophenol Blue.

Analysis of variance (Table 5) revealed significant difference for growth rate, total protein content, decolorization of Malachite green at 5th and 10th day of incubation, however, carbohydrate & phenolics content, laccase activity and decolorization of Bromophenol blue at 5th and 10th days of incubation appeared not significant. Variance in different SSIs was highly significant for all the traits indicating the presence of sufficient variability in the SSIs selected for this study.

Mean performance of parent and monokaryotic cultures

Genetic variability is the basic need for not only the breeders of mushroom but also for industrial applications. Among the characteristics studied under this study, most important mushroom trait, i.e. growth rate, was observed a wide range of variation from 5.01 to 0.48 mm/day. SSI [1] was found to grown fastest in its mycelial stage whilst parent culture`s growth rate was slowest. Wide variation was observed with reference to laccase activity, total protein content, and decolorization of Malachite green and Bromophenol Blue at 5th & 10th days of incubation.

TABLE 5: Analysis of variance (ANOVA) for mushroom and other traits of *P. ostreatus* and its monokaryons

Source		GR	CT	TP	PH	LA	MG @5	MG @10	BB @5	BB@10
Replication	DF	2	2	2	2	2	2	2	2	2
	MS	0.12	0.04	74.13	0.000052	0.13	0.45	169.50	1.87	57.82
	f-statistics	8.69	0.32	16.25	0.28	1.69	7.92	161.12	0.49	0.89
	p > f	0.0003	0.72	0.00001	0.75	0.18	0.0005	0.00001	0.61	0.41
Treatment	DF	58	58	58	58	58	58	58	58	58
	MS	2.86	0.15	7.63	0.000018	44.12	59.74	242.32	3.23	70.07
	f-statistics	201.36	1.19	1.67	1.003	548.44	1047.15	230.34	0.84	1.08
	p > f	0.00001	0.21	0.009	0.48	0.00001	0.00001	0.00001	0.75	0.34
Error	DF	116	116	116	116	116	116	116	116	116
	MS	0.01	0.13	4.55	0.00018	0.08	0.05	1.05	3.82	64.38

GR-Growth rate (mm/day)**, CT-** Carbohydrate**, TP**-Total Protein**, PH**-Phenolics**, LA**-Laccase activity**, MG@5**-Malachite Green @ 5 days**, MG@10**- Malachite Green @ 10 days**, BB@5**-Bromophenol Blue @ 5days**, BB@10-** Bromophenol Blue @ 10days.

Coefficient of variability and heritability

Phenotypic and genotypic coefficients of variation were assessed and after analysis, it gave a clear picture on the magnitude of variations presents in the studied isolates (Table 6). High PCV (>30%) and GCV (>30%) were observed for mycelial growth rate, laccase enzyme activity, decolorization of 80 ppm Malachite green at 5th days of incubation and also decolorization of same dye with same concentration at 10th days of incubation (Fig. 4). Phenotypic coefficient of variation was higher in extent than genotypic coefficient of variation for all the characters under study. The high magnitude of variability in the present study may be due the fact that the formation of monokaryotic spores by bi-factorial tetra-polar genetic system, so it can say that SSIs were segregates during meiosis by different allelic combinations. Low GCV & PVC (≤10%) were recorded for carbohydrate, total protein and phenolics contents; and for decolorization of Bromophenol blue (100 ppm) at 5th days of incubation and also decolorization of same dye with same concentration at 10th days of incubation. Since the differences between the PVC and GVC were not higher in all characters, thus selection on the basis of phenotype will be effective.

TABLE 6: Estimates of genotypic and phenotypic coefficient of variation, heritability, genetic advance and genetic gain for different mushroom traits

Character	Mean±SE	Range	GCV (%)	PCV (%)	Heritability (%)
Growth rate (mm/day)	2.62±0.06	5.01-0.48	37.21	37.49	99
Carbohydrate (g/100g)	4.12 ±0.21	4.66-3.69	2.23	9.12	6
Protein (g/100g)	22.33±1.23	25.15-18.80	4.53	10.57	18
Phenolics (mg/100g)	0.14 ±0.01	0.16-0.12	0.31	9.49	00
Laccase activity (U/g)	9.96 ±0.16	19.13-3.58	38.46	38.56	99
Malachite Green (0.008%) at 5th day	8.92 ±0.13	21.57-0.33	49.99	50.05	100
Malachite Green (0.008%) @ 10th day	18.43 ±0.59	43.27-0.64	48.67	48.98	99
Bromophenol blue (0.01%) @ 5th day	19.13±1.12	21.41-16.39	2.31	9.95	5
Bromophenol blue (0.01%) at 10th day	72.71±4.63	85.46-63.63	1.89	11.19	3

GCV- Genotypic coefficient of variation, **PCV-** Phenotypic coefficient of variation

Heritability measures the strength of the relationship between phenotype and genotype of any individual. Here, in the present study, heritability was assessed and it was > 95% in growth rate, laccase activity, decolorization of 80 ppm Malachite green dye, lower in case of carbohydrate, protein content and decolorization of Bromophenol blue, however, zero for the phenolics content. So, by this point of view, isolates may be selected on the basis of characters showed higher heritability.

Strain improvement for the purpose of breeding and process of identifying fungi does not rely solely on morphological features for identification. The use of fungal cultures,

obtained from single spore isolations, is fundamental to the identification of many fungi including *Pleurotus* species. Since millions of spores are generated from a single fruit body and all the spores may have variable characteristic features. It would be easy to select desirable characters from a list of isolates, so from the breeding and improvement program, this study could be very helpful.

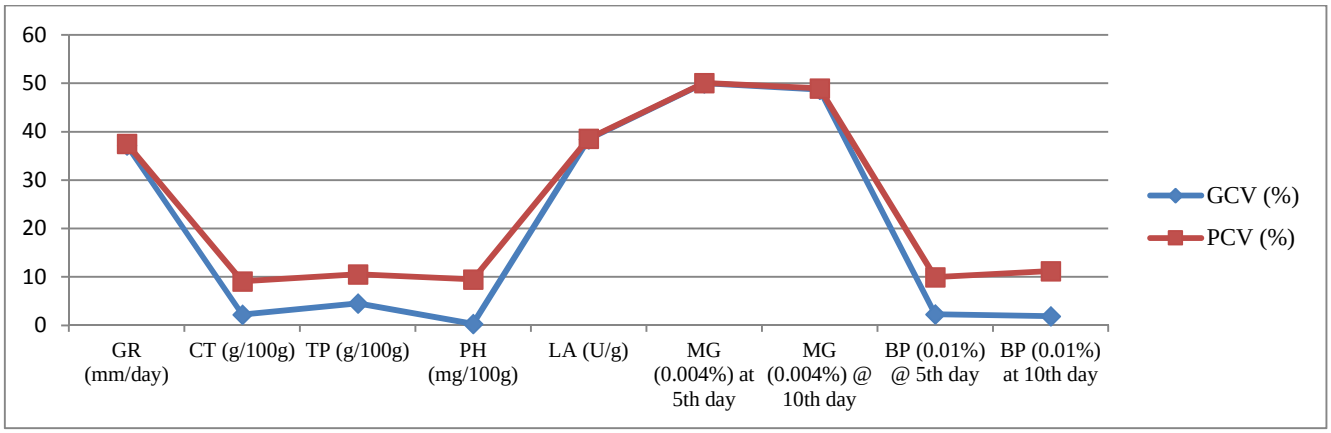

FIGURE 4: GCV (genotypic coefficient of variation) and PCV (phenotypic coefficient of variation) of different mushroom`s characters

GR-Growth rate (mm/day)**, CT**- Carbohydrate**, TP**-Total Protein**, PH**-Phenolics**, LA**-Laccase activity**, MG@5-**Malachite Green @ 5 days**, MG@10**- Malachite Green @ 10 days**, BB@5-**Bromophenol Blue @ 5days**, BB@10**- Bromophenol Blue @ 10days.

CONCLUSION

Spores producing from a single sporocarp have shown diversity in their many properties, so, it would be better to select the isolates on the basis of their phenotypic variance than genotypic for further in breeding and production at industrial scale.

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