GENETIC VARIABILITY IN STRAINS OF *VOLVARIELLA VOLVACEA* COLLECTED FROM THE STATE OF ODISHA, INDIA

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ABSTRACT

Volvariella volvacea, the straw mushroom is an edible mushroom of tropics and sub-tropics, valued for its flavor, texture and nutraceutical properties. However, it is among the least studied mushrooms in India with respect to genetic variability and strain improvement programme. The present study aimed at studying the genetic variability in wild *Volvariella* germplasm collected from different regions of the state of Odisha, India. The mycelial cultures raised from *V. volvacea* fruit bodies, collected from nine different locations spread in seven districts of Odisha were used in the study. Out of total ten strains, seven were fast growing (>90 mm radial growth), while rest three were slow growing. The strains also exhibited variability in other mycelial growth characteristics and extracellular lignocellulolytic enzymes activity profiles. Fastest growing strain OSM-1 exhibited highest activity of exo-glucanase, low of endo-glucanase and superior levels of β-glucosidase and xylanase. Laccase activity was comparatively low in slow growing strains compared to fast growing strains. In grow out trials, four strains including three slow growing (OSM-5, OSM-8 and OSM-10) and one fast growing (OSM-2) did not colonize the spawn substrate. Highest fruit body yield was recorded in strain OSM-9 (23.60 kg/100 kg dry substrate), followed by strain OSM-3 (17.47 kg/100 kg dry substrate). Rest four strains gave negligible yield. The fruit bodies of strain OSM-9 were very light in weight (7.80 g) compared to strain OSM-3 (15.85 g). The strains formed two separate groups with respect to length of their 5.8S rRNA gene amplicons and the neighbor joining tree deduced from the 5.8S rRNA gene sequences. Group-I comprised of all fast growing strains (OSM-01 to OSM-04 and OSM-07), while group-II with three slow growing (OSM-5, OSM-8 and OSM-10) along with two fast growing strains (OSM-6 and OSM-9). The ClustalW2 analysis exhibited deletions at two base pairs, one each in ITS-1 and ITS-2 regions, and substitution at four different places in ITS-1 region in strains of group-II. The present study paves the way for further breeding programmes in this mushroom.

Keywords: *V. volvacea*, variability, morphological characteristics, enzymes activity, yield

INTRODUCTION

The genus *Volvariella* comprises about 50 species world over [1], including the cultivated *V. volvacea* (paddy straw mushroom). Using the 'strips' recognized by Singer [2], several morphological/ecological groups have been differentiated within this genus.The straw mushroom (*V. volvacea*) ranks sixth among the world's most important species in terms of production [3]. It has significant pharmacological properties, including anti-tumor polysaccharides, immunopromotive proteins and immune modulatory lectins [4-6]. It is a popular mushroom of tropics/subtropics, grows well between 30-35 °C and completes its cropping cycle very fast, within three weeks time. Compared to many cultivated species of mushrooms like *Agaricus bisporus, Lentinula edodes* and *Pleurotus* spp., the productivity of *V. volvacea* is low [7]. However, the productivity of a mushroom species is attributed to many factors, important among that are the hydrolytic enzyme production potential of the mushroom species [7], quality of substrate used, method of substrate preparation and the growing conditions. The hydrolytic enzyme production potential of a mushroom species has direct bearing on its mushroom production potential; the cellulases play role during substrate colonization stage, while laccase during sporophore development stage [8, 9]. This mushroom has also been found to produce an array of extracellular hydrolytic and oxidases enzymes - i.e., endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21) and laccase (EC 1.10.3.2) [10-15]. The enzyme copper binding regions and the N-terminal amino acid sequences have also been used to generate complete sequence of six number of laccase from this mushroom and out of these lac1 and lac4 have been suggested to play role in morphogenesis in this mushroom [16, 17].

Among morphological characteristics, the mycelial growth rate, mycelial growth intensity, and formation of aerial hyphae and chlamydospores are considered as important attributes for selecting a potential high yielding strain both for commercial mushroom growing as well as strain improvement programme [18]. However, selection of a strain based only upon morphological characteristics gives misleading results, as these characteristics vary under different cultural and growing conditions [18]. Recently, genetic characterization by using molecular markers has helped in phylogenetic characterization of the strains of different mushroom species and other related fungi without any effect of extraneous factors [19-23]. The present study was aimed at studying the variability in *V. volvacea* specimens collected from different locations of the state of Odisha, India and to use that variability for strainal improvement programme and for understanding the molecular mechanisms of substrate utilization and fructification in *V. volvacea*.

MATERIALS AND METHODS

V. volvacea strains

The mycelial cultures of different strains were raised from the fruit bodies collected from nine different locations falling under seven districts of Odisha. The nine different locations belonged to five different agro-climatic regions. Maximum five strains were collected from East and South eastern coastal plain, two from mid-central Table land and one each from Northeastern ghat, North central plateau and West-central Table land (Table 1). The specimens were brought to pure culture by tissue culture method and coded as OSM-1 to OSM-10.

Morphological characteristics of strains

Strains were characterized for radial mycelial growth (dia. in mm) and colony morphology on malt extract agar (MEA) Petridishes and for downward mycelial growth as well as mycelial growth density and the extent of mycelial growth on pounded paddy straw filled in wide mouth test tubes. For each strain, the 6 mm (dia.) uniformly grown mycelial bit was placed in the center of the MEA Petridish and these were incubated at 34±2 °C for 7 days. Three replications were kept for each strain. For downward mycelial growth the pounded paddy straw was wetted overnight and the substrate with 70-72% moisture was filled in wide mouth test tubes, plugged and sterilized at 20 psi for 1.30 h. The sterilized paddy straw was inoculated with one mycelial bit of 6 mm dia./tube. The inoculated tubes were incubated at 34±2 °C for 9 days. The downward mycelial growth was measured in mm along the extent and the density of the mycelial growth. Three replications were kept for each treatment.

DNA extraction and 5.8S rRNA gene amplification

The mycelial cultures of all strains were grown separately on MEA Petridishes at 30±2 °C for 7 days. The mycelia from individual strain were scrapped and put in 1.5 ml micro-centrifuge tubes, kept at -85 °C for 2 h and freeze dried for 16-18 h. Genomic DNA was extracted from approximately 100 mg of freeze dried mycelia using DNeasy Plant Mini Kit (QIAGEN GmbH, D-40724 Hilden) following the protocol supplied by the manufacturer.

The polymerase chain reaction (PCR) primer ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') developed by White *et al.* [24] were used to amplify the ITS region along with 5.8S rDNA. PCR amplification was performed in a reaction mixture of 50 µl, containing 0.2 µl *Taq* DNA polymerase (5 U μl^{-1}), 5 µl 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 250 mM KCl), 5 µl dNTP mix (2.0 mM each), 1 µl each of ITS-1 and ITS-4 primers (0.01 mM), 1 µl glycerol (5%), 2 µl $MgCl_2$ (25 mM) and 2 µl of genomic DNA (50 ng). PCR reaction was performed in PCR Master Cycler Gradient in 36 cycles each of 95 °C for 1 min, 50 °C for 30 sec, 72 °C for 1 min 20 sec and final elongation at 72 °C for 10 min with lid heating option at 104 °C. The presence and yield of amplicons was ascertained on 2.0% agarose gel (w/v) prepared in 1% TBE. Gel electrophoresis was carried out at 90 V for 1.30 h in 0.5X TBE buffer. Staining was done with ethidium bromide and the gel was visualized and photographed using Bio Imaging System (Gene Genius, Syngene).

PCR products cleaning, sequencing, annotation and blasting

Amplicons of 5.8S rRNA of different strains were cleaned by using RCB kit (Banqiao City, Taipei County 220, Taiwan). Cleaned amplicons were got sequenced using 3730Xl (96 capillary) electrophoresis instruments from Bioserve Biotechnologies, Hyderabad, India. Received sequences in ab1/SCF format were cleaned to remove the misleading sequences and were improved upon using Chromas Lite 2.01 software (Copyright © 1998-2005 Technelysium Pty Ltd). The improved consensus sequences were blasted using BLASTn tool of NCBI [25] and the species against which highest similarity exhibited, was considered as the species identified.

Sequence alignment, phylogeny and evolutionary relationships

The improved consensus sequences of 5.8S rDNA of different strains were studied for variability in their nucleotide sequences by using ClustalW2 tool of European Bioinformatics Institute (EBI). Phylogenetic and molecular evolutionary analyses for strains were conducted using MEGA version 5.0 [26]. The evolutionary history was inferred using the neighbor-joining method [27].

Enzyme assay

The activity of different enzymes of the different strains was studied first by growing them on sterilized paddy straw substrate with 70% moisture in flasks. The enzymes were extracted from the mycelium-colonized substrate in 50 ml phosphate buffer (0.1 M), pH 7.0 by keeping the buffer mixed substrate at 40 °C for 30 min in an incubator shaker maintained at 100 rpm. The extract was filtered through glass microfibre filter (GF-C) and stored at 4 °C for further use. The enzyme assay was carried out in triplicate for all the enzymes and data were subjected to statistical analysis using AGRES software.

The cellulases were measured according to the method of Mandels *et al.* [28] as modified by Sandhu and Kalra [29]. The reaction mixture for exoglucanase (FPase, EC 3.2.1.91) comprised of eight filter paper (Whatman No. 1) discs of 0.6 cm dia in 0.5 ml acetate buffer of pH 5.0 and 0.25 ml of the enzyme source. The reaction mixture was incubated at 40 °C for 4 h and the reducing sugars released were measured by Nelson Somogyi method [30]. The endoglucanase (CMCase, EC 3.2.1.4) activity was measured following the above method, replacing filter paper discs with 0.5 ml of 5 mg ml^{-1} carboxy methyl cellulose. Xylanase (EC 3.2.1.8) was assayed at 40 °C following a method described by Reese and Mandels[31].

Laccase (EC 1.10.3.2) was assayed by adding 0.3 ml enzyme source to 2.5 ml of 30 μM guaiacol in phosphate buffer (0.1 M) of pH 6.0 and Δ A was read at 470 nm after incubating the reaction mixture for 30 min at room temperature against zero time control. Polyphenol oxidase (EC 1.10.3.1) was assayed using catechol as substrate in place of guaiacol. One unit of laccase and polyphenol oxidase activity was calculated as change in absorbance of 0.001 min^{-1} ml^{-1} of enzyme source at 25 ^{o}C, while that of FPase, CMCase and xylanase as the μ mol glucose released h^{-1} ml^{-1} of enzyme source.

Mushroom spawn preparation and crop raising

The ready to mix spawn of different strains was prepared by using chopped paddy straw following the method of Ahlawat *et al*. [32].The strains were evaluated for fruit body yield using compost prepared with paddy straw + cotton ginning mill waste in 1:1 ratio (w/w). Composting was carried out by following the method of Ahlawat *et al*. [32]. Chicken manure and $CaCO_3$ were added @ 5.0% and 1.5% (dry wt. basis), respectively on day 0 and day 3 of stacking, while turnings were given on day 1, 2, 3 and 4 of outdoor composting. Beds of 180 cm x 70 cm x 12 cm (l x w x h) size were prepared with 35 kg wet substrate on shelves of iron racks in cropping room. Six replications were kept for each strain and the experiment was conducted in a randomized block design. The data recorded was subjected to statistical analysis using AGRES software. The fresh mushroom yield was recorded in g/20 kg of ready to use composted substrate. Data was also recorded for the time taken for first harvest in days post-spawning and mean fruiting body wt (g).

RESULTS AND DISCUSSION

Morphological characteristics

A total of 10 strains cultured from mushroom specimens collected from nine different locations falling in seven different districts and five agro-ecological regions were used for the study (Table 1). The strains were recorded for radial and downward mycelial growth, type of growth and formation of aerial hyphae as well as intensity of chlamydospores on MEA medium in Petridishes and pounded paddy straw in wide mouth glass test tubes (Table 2).

Table 1. Particulars of *Volvariella volvacea* isolates collected from different agro-climatic situations of Odisha (2010-11)

Isolates	Place of collection	Date of collection	Agro-climatic zone
OSM-1	Athgarh, Cuttack	06.09.2010	Mid-Central Table Land
OSM-2	Bhubaneswar, Khurda	09.08.2010	East and South Eastern Coastal Plain
OSM-3	Bhubaneswar, Khurda	12.08.2010	East and South Eastern Coastal Plain
OSM-4	Salepur, Cuttack	14.08.2010	East and South Eastern Coastal Plain
OSM-5	Lambodarpur, Dhenkanal	13.09.2010	Mid-Central Table Land
OSM-6	Aska, Ganjam	26.07.2010	North Eastern Ghat
OSM-7	Nalapari, Kendrapara	23.08.2010	East and South Eastern Coastal Plain
OSM-8	Balakati, Khurda	1.08.2010	East and South Eastern Coastal Plain
OSM-9	Betnoti, Mayurbhanj	5.09.2010	North Central Plateau
OSM-10	Padiabahal, Sambalpur	2.08.2010	West-Central Table Land

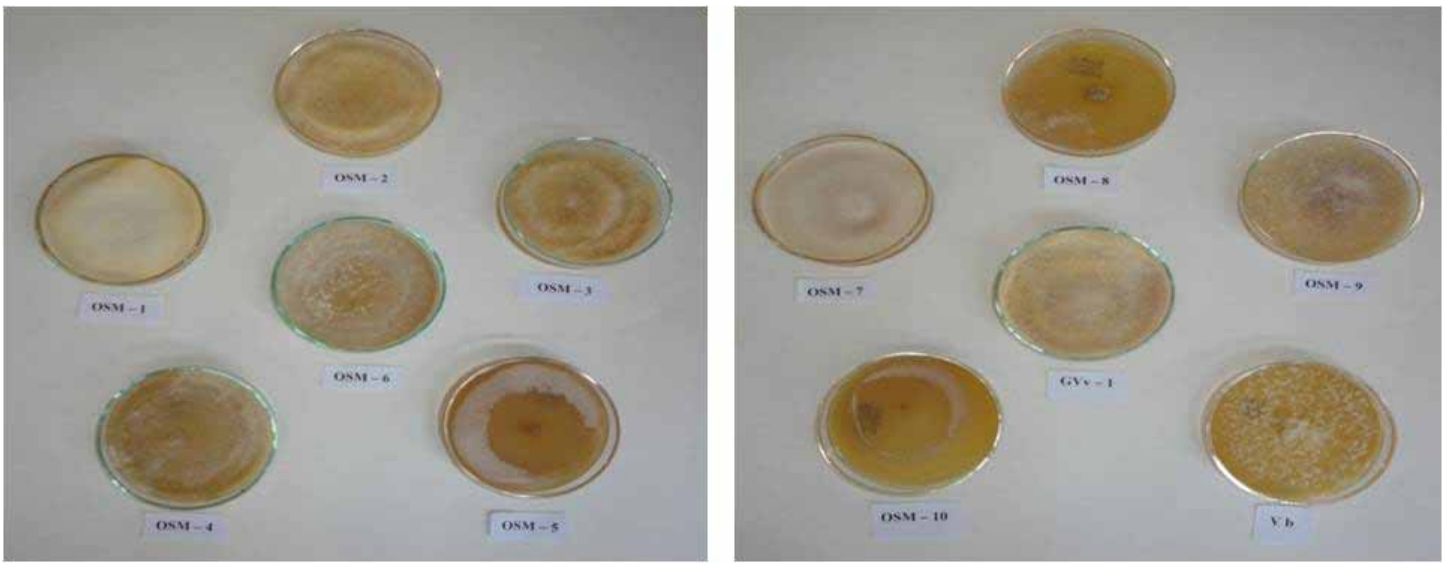

Figure 1. Mycelial growth of strains grown on Malt Extract Agar in Petridishes

The data presented in Table 2 reveal that strains OSM-1, OSM-2, OSM-3, OSM-4, OSM-6, OSM-7 and OSM-9 were fast growing as compared to strains like OSM-5, OSM-8 and OSM-10. The strains also varied in density and extent of aerial mycelial growth. The aerial mycelia were almost absent in slow growing strains. Strains, OSM-1 and OSM-7 exhibited highest mycelial growth density, followed by strains OSM-2, OSM-3 and OSM-9. The data for the downward mycelial growth on pounded paddy straw reveal that it was highest in strain OSM-1 (106 mm), followed by strains OSM-7 (85 mm), OSM-3 (78 mm), OSM-2 and OSM-9 (77 mm) and OSM-4 (68 mm). It was lowest in strains OSM-8 and OSM-10 (22 mm), followed by OSM-5 (44 mm). The majority of the fast growing strains formed creamy

white colonies, except of strains OSM-1 and OSM-6, which formed white coloured colonies. These low growing strains OSM-5, OSM-8 and OSM-10 formed white, creamy and yellowish colonies (Table 2).

Table 2. Morphological growth characteristic of different strains of *Volvariella volvacea* on malt extract agar medium.

Strains	Mycelial growth characteristics				
	Radial mycelial growth (Dia. mm)	Downward mycelial growth (mm)	Aerial mycelia		Colony colour
			Mycelial density	Extent	
OSM - 1	90	106	++++	+++++	White
OSM - 2	90	77	+++	++++	Creamy White
OSM - 3	90	78	++	++++	Creamy White
OSM - 4	90	68	-	+++	Creamy White
OSM - 5	44	44	-	+	White
OSM - 6	90	61	+	+++	White
OSM - 7	90	85	++	+++++	Creamy White
OSM - 8	57	22	-	++	Creamy
OSM - 9	90	77	+	++++	Creamy White
OSM - 10	35	22	-	+	Yellowish

Least - +; highest - +++++; absent –

In present study majority of the strains exhibited the characteristics of a 'typical' *V. volvacea* strain (vigorously growing mycelia with abundant aerial and horizontal hyphae, mycelia usually thick at the margins of agar plate) [18], excepting 3 strains (OSM-5, OSM-8 and OSM-10), which showed quite 'atypical' characteristics matching characteristics of single spore isolates of *V. volvacea* [9, 18]. The variations in morphological characteristics in strains of *V. volvacea* and changes in their morphological growth characteristics at different stages of growth have also been reported earlier [15, 18]. So, broadly two different types of strains came in picture; one comparatively fast growing both on MEA and paddy straw and second quite slow growing on both types of media. Within the group, the strains also varied in their type of growth and colony characteristics.

Phylogenetic analysis of different strains

Nucleotide BLAST of Sequences: As the BLAST of all 10 sequences done, the first hit strain or species of each query sequence from which the query sequence was maximum aligned is given here. All strains were found to belong to the species *V. volvacea*.

Diversity analysis using CLUSTALW: The diversity analysis exhibited 5.8S rRNA gene sequences of two different sizes. The first group of five strains (all fast growing) comprised of strains OSM-1, OSM-2, OSM-3, OSM-4 and OSM-7 exhibited amplicons of 638 bp long, while the second group of five strains (three slow and two fast growing) comprised of strains OSM-5, OSM-6, OSM-8, OSM-9 and OSM-10 exhibited ampliconsof 636 bp long (Fig. 2). There were deletions at two different places in second set of

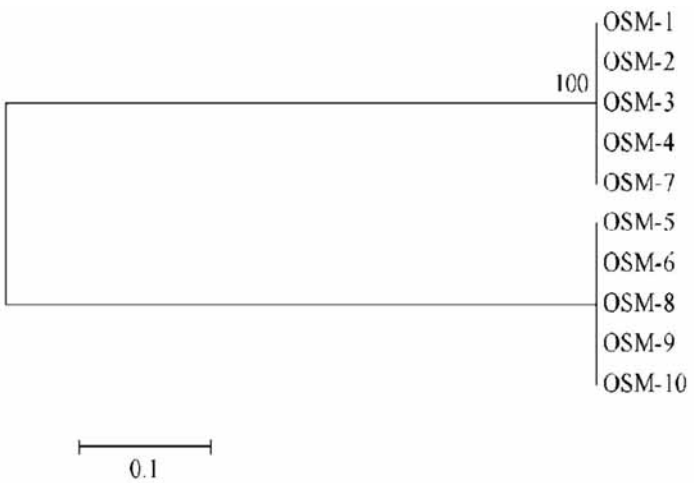

Figure 2. Neighbor-Joining T reededuced from 5.8S rRNA gene sequences of different strains

strains, one each in ITS-1 and ITS-2 regions. The sequences in two sets of strains also showed substitution at four different nucleotides in ITS-1 region (Fig. 3). In several earlier studies variations have been reported both in parent strains as well as in SSIs of the strains based upon their RAPD profiles [21, 22] and the laccase gene sequences [33]. However, only in a recent study, the variations in 5.8S rRNA gene sequences have been used for studying the variability in SSIs of *V. volvacea* [34].

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OSM-1       CAGAATCGAACGCGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC          60
OSM-2       CAGAATCGAACGCGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC          60
OSM-3       CAGAATCGAACGCGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC          60
OSM-4       CAGAATCGAACGCGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC          60
OSM-7       CAGAATCGAACGCGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC          60
OSM-8       CAGAATCGAACGTGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC          60
OSM-6       CAGAATCGAACGTGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC          60
OSM-5       NNNNNNNNNNNNTGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC          60
OSM-10      NNNNNNNNNNNNTGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC          60
OSM-9       NNNNNNNNNNCGTGGGTCGGGCTGATTGCTGGCTCCTCGGAGCAGGTGCACGCCCTCCCC          60
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OSM-1       GACGCCTTCCATTCTCCACGTCCCCACCTGTGCACCTTCTGTAGGCCGTGAAGCCGCCTC          120
OSM-2       GACGCCTTCCATTCTCCACGTCCCCACCTGTGCACCTTCTGTAGGCCGTGAAGCCGCCTC          120
OSM-3       GACGCCTTCCATTCTCCACGTCCCCACCTGTGCACCTTCTGTAGGCCGTGAAGCCGCCTC          120
OSM-4       GACGCCTTCCATTCTCCACGTCCCCACCTGTGCACCTTCTGTAGGCCGTGAAGCCGCCTC          120
OSM-7       GACGCCTTCCATTCTCCACGTCCCCACCTGTGCACCTTCTGTAGGCCGTGAAGCCGCCTC          120
OSM-8       GACGCCTTCCATTCTCCACATCCCCACCTGTGCACCTTCTGTAGGCCATGAAGCCGCCTC          120
OSM-6       GACGCCTTCCATTCTCCACATCCCCACCTGTGCACCTTCTGTAGGCCATGAAGCCGCCTC          120
OSM-5       GACGCCTTCCATTCTCCACATCCCCACCTGTGCACCTTCTGTAGGCCATGAAGCCGCCTC          120
OSM-10      GACGCCTTCCATTCTCCACATCCCCACCTGTGCACCTTCTGTAGGCCATGAAGCCGCCTC          120
OSM-9       GACGCCTTCCATTCTCCACATCCCCACCTGTGCACCTTCTGTAGGCCATGAAGCCGCCTC          120
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OSM-1       GTGTTCTTGTACGACCGGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA          240
OSM-2       GTGTTCTTGTACGACCGGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA          240
OSM-3       GTGTTCTTGTACGACCGGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA          240
OSM-4       GTGTTCTTGTACGACCGGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA          240
OSM-7       GTGTTCTTGTACGACCGGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA          240
OSM-8       GTGTTCTCGTACGAC-GGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA          239
OSM-6       GTGTTCTCGTACGAC-GGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA          239
OSM-5       GTGTTCTCGTACGAC-GGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA          239
OSM-10      GTGTTCTCGTACGAC-GGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA          239
OSM-9       GTGTTCTCGTACGAC-GGGGACCCCTCGTCGGCCCCATAGACATACCAATACAACTTTCA          239
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OSM-1       GCCCCCCCGCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG          600
OSM-2       GCCCCCCCGCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG          600
OSM-3       GCCCCCCCGCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG          600
OSM-4       GCCCCCCCGCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG          600
OSM-7       GCCCCCCCGCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG          600
OSM-8       GCCCCCC-GCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG          598
OSM-6       GCCCCCC-GCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG          598
OSM-5       GCCCCCC-GCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG          598
OSM-10      GCCCCCC-GCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG          598
OSM-9       GCCCCCC-GCGGCCGCACTCAGCGTGGCTCGGCTTCGAACCGTCCGGCCCCTCGAGCCGG          598
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Figure 3. ClustalW analysis of the 5.8S rRNA gene sequences of different strains

Extracellular lignocellulolytic enzymes activity profile: The crude enzyme extract extracted from fully mycelial colonized paddy straw was used as an enzyme source. Exoglucanase activity was highest in fast growing strain OSM-1, followed by slow growing strain OSM-5 and another fast growing strain OSM-2. It was least in fast growing strain OSM-9, followed by two slow growing strains OSM-9 and OSM-10. Endoglucanase activity was highest in fast growing strain OSM-6, followed by strain OSM-9 and two slow growing strains OSM-10 and OSM-8. It was least in strain OSM-2. β-glucosidase activity was highest in strain OSM-2, followed by strains OSM-3, OSM-4 and OSM-1. Its activity was lowest in strain OSM-9. Activity of xylanase was highest in strain OSM-6, followed by strains OSM-4 and OSM-1. Its activity was lowest in slow growing strain OSM-5. Laccase activity was highest in strain OSM-7, followed by OSM-2 and slow growing strain OSM-8. Its activity was lowest in strain OSM-10. Similarly, activity of PPO was highest in strain OSM-7, followed by OSM-2 and least in OSM-3 (Table 3).

Table 3. Extracellular lignocellulolytic enzymes activity of strains of *Volvariella volvacea*

Strains	Enzyme activity					
	Exo-glucanase	**Endo-glucanase**	**β-glucosidase**	**Xylanase**	**Laccase**	**Polyphenol oxidase**
OSM- 1	0.0066	0.0045	0.0093	0.0066	164.911	54.588
OSM- 2	0.0054	0.0038	0.0114	0.0049	371.005	100.773
OSM- 3	0.0048	0.0052	0.0107	0.0050	90.916	47.910
OSM- 4	0.0049	0.0064	0.0107	0.0070	84.815	51.071
OSM- 5	0.0056	0.0053	0.0074	0.0046	43.938	57.610
OSM- 6	0.0050	0.0121	0.0072	0.0075	74.572	58.110
OSM- 7	0.0053	0.0064	0.0095	0.0060	388.694	122.316
OSM- 8	0.0041	0.0087	0.0094	0.0064	263.70	64.638
OSM- 9	0.0036	0.0110	0.0068	0.0048	47.527	57.932
OSM-10	0.0037	0.0105	0.0070	0.0050	1.549	56.094

Units of measurement: Exo and Endo-glucanase/Xylanase - µ mole glucose released/min/ml of filtrate; PPO/Laccase - change in absorbance by 0.001/min/ml of filtrate; β–glucosidase - µ mole p-nitrophenol released/min/ml of filtrate.

In extracellular lignocellulolytic enzymes activity profiles, the strains did not exactly behaved as per extant of their mycelial growth on MEA and paddy straw. In case of β–glucosidase and xylanase, the highest activities were exhibited by fast growing strains, however, mixed responses were recorded in laccase, polyphenol oxidase and endo-glucanase activities. Here again the superior activity of an individual enzyme was not found sufficient to support the fast mycelial growth on MEA and paddy straw as in case of an earlier study [21]. Like a few earlier studies with monosporous isolates [9] and with parent strains of *V. volvacea*. [8], several fast growing strains were recorded to exhibit quite low level of activities of laccase and polyphenol oxidase. Here, again it is proved that mycelial growth in *V. volvacea* strains is not regulated by any one specific enzymes and laccase in particular. The variability in activities of extracellular enzymes in *V. volvacea* strains along with role of cellulases in mycelial colonization and laccase in sporophore formation have also been reported earlier by other workers [5, 7].

Mushroom yield parameters and yield potential: Out of ten strains selected in the beginning and used in other *in vitro* studies, only six strains, which showed good mycelial growth on master spawn and commercial spawn substrates, were selected for yield evaluation trials. Out of six strains selected, superior spawn run was recorded in strain OSM-1, followed by strains OSM-3 and OSM-9 (Table 4). In rest three strains it was almost of same level. Pinning intensity was highest in strain OSM-9, followed by OSM-3, while pinning was not recorded in strain OSM-6. Highest mushroom yield was recorded from the beds of strain OSM-9 (1416 g/bed). It was followed by yield in beds of strain OSM-3 (1048.12 g). Strains OSM-1, OSM-6 and OSM-7 gave only few scattered mushrooms.

Table 4. Yield potential of different strains on composted substrate of paddy straw + cotton ginning mill waste (1:1, w/w).

Strains	Mycelial colonization of substrate	Pinning	Time taken for 1st harvest (days post spawning)	Yield (1st week) (g/number/bed of 20 kg substrate)	Average wt. of fruit bodies (g)
OSM- 1	5.0 +	0.166 +	15.66	13.16/0.5	26.32
OSM- 2	—	—	—	—	—
OSM- 3	3.87 +	0.333 +	15.48	1048.12/66.12	15.85
OSM- 4	2.0 +	1.0 +	15.00	19.6/1.66	11.84
OSM- 5	—	—	—	—	—
OSM- 6	2.33 +	—	17.33	55.5/4.16	13.34
OSM- 7	2.50 +	0.5 +	14.00	26.66/1.66	16.06
OSM- 8	—	—	—	—	—
OSM- 9	3.83 +	1.0 +	14.41	1416/181.5	7.80
OSM- 10	—	—	—	—	—

+: visible, —: not visible

The strains were also evaluated for time taken for first harvest (day post spawning) and the mean fruiting body wt. Lowest time for first harvest was recorded in strain OSM-7 (14.0 days), closely followed by strain OSM-9. In rest strains, it was almost same. The fruit body weight was highest in strain OSM-1 (26.32 g), followed by strain OSM-7 (Table 4). Amongst good yielding strains, the fruit body wt. was highest in strain OSM-3 (15.85 g) and just almost half in strain OSM-9 (7.80 g).Yield evaluation trials for selecting a better performing strain or single spore isolate have also been performed earlier by several workers [9, 15, 32, 35, 36] but only few of them have correlated the morphological and biochemical characteristics of a strain with its yield potential and have helped in selecting several high yielding strains [15, 32].

The present study reveals a relationship between the origin of the strains and its phylogenetic belongingness and it can be attributed to the origin of strains from a common parent in that area. There are only very few reports on using the lignocellulolytic enzymes activity or the protein profiles of the strains in studying polymorphism in different mushrooms and that too are restricted to use of laccase polymorphism in *Agaricus bitorquis* [37] and protein profiles in *A. bisporus* and *A. bitorquis* [38]. The relationship between origin of strains and the variability existed in them have also been studied earlier [21] and reported variability in strains originated from Odisha and Kerala states of India. However, in present case variability in the strains obtained from different regions of same state Odisha has been highlighted, which will strengthen the hypothesis of existence of variability in the strains of *V. volvacea*, which otherwise is considered as primary homothallic leaving very less scope of variability in this species. The fast growing strains like OSM-1 and OSM-7 did not gave fruiting even after exhibiting quite fast and dense mycelial growth on two media, contrary to medium level growing strains like OSM-3 and OSM-9 forming not very dense mycelial mat on media but gave fruiting in fruiting trials. These two strains even did not exhibit very high activities of any specific enzymes except of endo-glucanase in OSM-9 and β–glucosidase in strain OSM-3. The two strains were also isolated from far distant places and falling in two phylogenetically distinct groups. The present study nullifies the notion that fast growing attribute is the deciding factor for fruiting body formation or higher fruit body yield. Very few studies of this type have been conducted earlier, Chen *et al*. [8] and Ahlawat *et al*. [9], who had reported the role of cellulases in substrate colonization and laccase in sporophore formation in this mushroom. The present study has provided an insight into the subject, which will help in developing a solid base for existence of variability in *V. volvacea* growing under natural conditions.

CONCLUSION

The specimens collected were found to vary both at mycelial growth characteristics and their 5.8S rRNA gene sequences levels, and broadly can be placed under two different groups. The strains exhibited varied levels of extra cellular lignocellulolytic

enzymes activities and not much correlation between the mycelial growth characteristics and the enzymatic activity profiles was recorded. Wide variations were also recorded at the levels of mycelial colonization of spawn and cultivation substrates and the fruiting pattern, as the slow growing strains along with one fast growing strain OSM-2 did not colonize the wheat grain based spawn substrate, while many fast growing strains did not gave fruiting. The strains have provided a good germplasm for understanding the biology of substrate colonization and fruiting, and their use in strain development programme.

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