Variability in intra-specific and monosporous isolates of *Volvariella volvacea* based on enzyme activity, ITS and RAPD

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Abstract Two parents and 15 monosporous isolates were morphologically characterized and were found to vary in their growth characteristics on malt extract agar medium. The isolates also varied in enzymes activity profile with respect to exoglucanase, endoglucanase, xylanase, laccase and polyphenol oxidase. Further in polymerase chain reaction (PCR) amplification of Internal Transcribed Spacer (ITS) region of 5.8S rDNA, an amplicon of same length (720 bp) was amplified from two parents and all the monosporous isolates, which revealed that all belong to the same species. The combined phylogenetic analysis of random amplified polymorphic DNA (RAPD) profiles obtained with five decamer primers (operon kit B) series primers also revealed intra-specific variation of 60% with in the two parent strains and their single spore isolates (SSIs). However, the variations between the parent strains and their SSIs were lesser and it was 50 and 32% in parent strains, OE-210 and OE-12, respectively. Based upon phylogenetic analysis, the isolates of parent strain, OE-210 formed 7 distinct phylogenetic clades, while of strain OE-12 formed 4 clades. The study elucidates that isolates showing variations in morphological growth characteristics and enzymes activity also showed variations in their RAPD profiles, revealed through phylogenetic analysis of RAPD profiles. It is also evident from the study

that morphological characterization along with enzymes activity assay of strains is essential before their use in yield evaluation trials with final authentication from molecular analysis.

Keywords Strain · Isolates · Variability · ITS · RAPD

Introduction

Volvariella volvacea (Bull ex Fr) Sing, belonging to the family Pluteacea (Kolt and Pouz) of Basidiomycetes [1], ranks sixth amongst the cultivated mushrooms [2] accounting about 5% of the total world mushroom production. This mushroom is being cultivated since 18th century mostly in China [2]; however, its biological efficiency is very low in comparison to other mushrooms. The low productivity of this mushroom is ascribed to low production of the hydrolytic enzymes [3, 4]. In addition to this, the regulation of cellulases and laccase enzymes plays a crucial role in substrate colonization and sporophore development in this mushroom [5–8]. Besides the need for enhanced level of hydrolytic enzymes in parent strains, isolation of high yielding monosporous isolates from parental lines has also been stressed upon for enhancing the productivity in this mushroom [9]. However, screening of isolates for selecting a higher yielder has always remained a cumbersome task mainly because of variations in the isolates of the same strain and needs screening of thousands of isolates to obtain an isolate with desired characters through grow out trials [10]. Present study is aimed to identify high yielding monosporous isolates of parent strains of *V. volvacea* by using tools like morphological characteristics, enzymes activity, Internal Transcribed Spacer (ITS) and RAPD profiles.

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Materials and methods

Parent strains and single spore isolates (SSI)

The parent strains of *V. volvacea* (OE-12 and OE-210) were obtained from Mushroom Culture Bank of our directorate and the crop was raised following the method of Verma [11]. The mushroom fruiting bodies of the parent strains were used for obtaining the spore print following the methods of Chang et al. [12] and the spore suspension was serially diluted up to 10^{-4} dilution. Monosporous isolates were obtained and maintained following the method described by Ahlawat et al. [8].

Morphological studies

Morphological growth characteristics of parent strains and monosporous isolates were recorded on malt extract agar petridishes. Initially, 42 SSIs of strain OE-12 and 38 SSIs of strain OE-210 were morphologically characterized and grouped into four different types. Representative isolates from each group were randomly selected for their detailed morphological characteristics, enzymes activity, ITS and RAPD profiles. Four replications were kept for each strain/SSI and radial growth data obtained were subjected to statistical analysis using AGRES software.

Enzyme assay

The activity of different enzymes of the parent strains and their SSIs 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 microfiber 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. [13] as modified by Sandhu and Kalra [14]. The reaction mixture for exoglucanase (FPase, EC 3.2.1.91) comprised of eight filter paper (Whatman No. 1) discs of 0.6 cm diameter 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 [15]. 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 carboxymethyl cellulose. Xylanase (EC 3.2.1.8) was

assayed at 40°C following a method described by Reese and Mandels [16].

Laccase (EC 1.10.3.2) was assayed by adding 0.3 ml enzyme source to 2.5 ml of 30 μ M Guaiacol in phosphate buffer (0.1 M) of pH 6.0 and ΔA 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 Chatechol as substrate in place of Guaiacol. One unit of laccase and polyphenol oxidase activity was calculated as change in absorbance of 0.001 per min/ml of enzyme source at 25°C, while that of FPase, CMCase and xylanase as the μ M glucose released per hour/ml of enzyme source.

DNA extraction

All strains as well as monosporous isolates were grown separately as liquid shake culture in 25 ml malt extract broth in flasks at $32 \pm 2^\circ\text{C}$ for 7 days. The genomic DNA was extracted from approximately 100 mg of freeze dried fungal mycelia by crushing in 1.5 ml microcentrifuge tubes using micropestles. Genespin Plant Genomic DNA Preparation kit was used for DNA extraction as per the protocol supplied by the manufacturer.

Amplification of 5.8S rRNA gene

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. [17] were used to amplify the ITS region of rDNA. PCR amplification was performed in a reaction mixture of 50 μ l, containing 0.4 μ l *Taq* DNA polymerase (6 U/ μ l), 5 μ l 10 $\times$ PCR buffer (100 mM Tris-HCl, pH 8.3, 15 mM MgCl_2 , 250 mM KCl), 0.4 μ l dNTP mix (25 mM each), 1 μ l each of ITS-1 and ITS-4 primers (0.01 mM), 2 μ l glycerol (5%) and 5 μ l of genomic DNA (40 ng). The reactions were performed in a PCR machine (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.

RAPD analysis

Initially 20 arbitrary decamer operon primers (kit B) series (Operon Biotechnologies, GmbH, Cologne, Germany) were used. Of these, five primers were selected for PCR amplification of all the strains/SSIs-based upon reproducibility of the banding patterns. Multilocus genotyping was performed by RAPD using primers, OPB-1 (5'-GTTTCGCTCC-3'), OPB-2 (5'-TGATCCCTGG-3')

OPB-3 (5'-CATCCCCCTG-3'), OPB-4 (5'-GGACTGGAGT-3') and OPB-5 (5'-TGCGCCCTTC-3'). Amplification was performed in 25 µl reaction mixture, containing 2 µl primer (50 pM), 0.2 µl dNTP mix, (25 mM each), 1 µl MgCl₂ (25 mM), 1 µl *Taq* DNA polymerase (6 U/µl), 2.5 µl 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 250 mM KCl), 2 µl glycerol (5%) and 12.3 µl of dH₂O. To this, 4 µl of genomic DNA (40 ng) was added and RAPD-PCR amplifications were performed in a PCR machine (Master Cycler Gradient) with initial denaturation at 94°C for 3 min followed by 38 cycles of 94°C for 40 sec, 40°C for 40 sec and 72°C for 1 min and final elongation at 72°C for 10 min with lid heating option at 104°C. The agarose gel was prepared in 1× Tris-acetate-EDTA (TAE) buffer (1.6 g in 100 ml buffer) and the PCR amplified products were electrophoretically separated by running the gel at 50 V for 3 h. Staining was done with ethidium bromide and the gel was visualized and photographed using Bio Imaging System. The analysis of strains and SSIs was carried out in triplicate with all primers to ensure minimum experimental errors of reading of wrong bands. The band profile of each gel was scored as '1' for presence and '0' for absence of bands and combined binary data matrix for all the markers was constructed. The binary data matrix was entered in the numerical taxonomy and multivariate analysis system package (NTSYS-pc) package [18, 19] and data was analyzed using SIMQUAL route to obtain Jaccard's similarity coefficients [20]. The similarity coefficients were used to construct dendrogram employing the Unweighted Paired Group Method using Arithmetic Averages (UPGMA) and the SAHN (Sequential Agglomerative Hierarchical and Nested Clustering) using the NTSYS software.

Results and discussion

Morphological characteristics

The morphological growth characteristics of five monosporous isolates and their parent strain, OE-12 revealed wide variations in their growth characteristics. Isolate, OE-1206 showed highest growth with plenty of aerial mycelia and chlamydospores in the middle and on the top of aerial mycelia, followed by OE-1216, while isolate; OE-1222 formed highest chlamydospores despite of being a slow grower. Isolate, OE-12-04 exhibited slowest mycelial growth along with lowest aerial mycelia and chlamydospores. Amongst parent strain, OE-210 and its 10 isolates, the isolates, OE-210-4, OE-210-12, OE-210-13, OE-210-29 and OE-210-30 showed faster radial growth, along with high intensity of aerial mycelia. However, the

growth type varied from dense thick to thin and restricted to equally distributed. The other isolates viz. OE-210-03, OE-210-09, OE-210-22, OE-210-23 and OE-210-28 showed growth at par with parent strain, OE-210 but slower than other isolates. Majority of the isolates did not bear chlamydospores on their mycelia, except isolate, OE-210-30. In earlier studies also the monosporous isolates of paddy straw mushroom have been reported to vary in their growth rates, abundance of mycelia, aerial hyphae and presence of chlamydospores [12], and based upon variations in their morphological growth characteristics, Chang et al. [12] divided monosporous isolates into five different groups ('NN', 'N', 'AN', 'A' and 'AA'). Although the presence of chlamydospores is considered as very important criterion while selecting a potential high yielding isolate. However, selecting isolates merely based upon morphological characteristics did not offer a fool proof solution to the problem as the isolates tend to change their characteristics from normal to abnormal type and vice versa [12]. Similarly Li and Chang [21] had also reported that the morphological characteristics couldn't be associated consistently with the size and shape of the mushrooms or with the vigor of the spawn in the beds.

Enzyme assay

The extracellular lignocellulolytic enzymes activity of the isolates and parent strains depicted in Table 1 reveal that monosporous isolates, OE-1204 and OE-1216 of strain, OE-12 exhibited many folds higher activity of exoglucanase, whereas all other SSIs exhibited lower activity than their parent strain. Similarly isolate, OE-210-3 exhibited much higher exoglucanase activity than its parent strain, OE-210.

In contrary to exoglucanase, the endoglucanase activity of the isolates of two strains was significantly lower than their parent strains (Table 1). The xylanase activity of the SSIs, OE-1216 and OE-1222 was higher than their parent strain, OE-12, while it was invariably higher in all the SSIs of parent strain, OE-210 than their parent. The laccase and polyphenol oxidase activity was higher in SSIs, OE-1203, OE-1206 and OE-1222 in comparison to their parent strain OE-12. The activity trend of laccase and polyphenol oxidase (PPO) in SSIs of parent strain, OE-210 was almost similar to that of SSIs of parent strain OE-12, with four SSIs showing 2- to 9-fold higher activity than their parent strain. The role of enzymes in mycelial colonization of substrate and sporophore formation in this mushroom have been highlighted in several earlier reports, where in the cellulases activity has been reported to be more during substrate colonization, while of laccase during sporophore formation [6, 8]. In the present study, the enzymes activity profiles of the parent strains and their SSIs showed great

Table 1 Cellulase, xylanase, laccase and polyphenol oxidase activity in parent strains and SSIs grown on paddy straw substrate under *in vitro* conditions

Parent strain/SSI	Enzyme activity*				
	Exoglucanase	Endoglucanase	Xylanase	Laccase	Polyphenol oxidase
OE-12	0.37	1.55	0.57	5.06	1.53
OE-1203	0.10	0.46	0.08	27.94	5.19
OE-1204	1.08	0.57	0.54	5.56	1.13
OE-1206	0.03	0.77	ND	36.20	5.92
OE-1216	0.74	0.83	1.68	8.02	2.64
OE-1222	0.04	0.67	0.95	30.58	5.69
OE-210	0.096	1.102	0.0032	10.67	7.42
OE-210-3	0.354	0.129	0.0129	ND	7.25
OE-210-4	0.042	0.256	ND	33.25	17.00
OE-210-9	0.035	0.046	0.0560	76.67	13.92
OE-210-12	0.012	0.249	0.0220	94.00	12.83
OE-210-13	ND	ND	0.0370	5.42	2.17
OE-210-22	0.009	0.001	ND	6.25	5.83
OE-210-23	0.006	ND	0.0180	ND	2.67
OE-210-28	0.080	0.054	0.0032	10.58	5.92
OE-210-29	0.050	0.050	0.4080	24.42	ND
OE-210-30	0.009	0.053	0.0039	15.17	9.42
	Mean = 0.1796	Mean = 0.3994	Mean = 0.2579	Mean = 22.929	Mean = 6.2671
	SEd = 0.034	SEd = 0.05	SEd = 0.048	SEd = 0.654	SEd = 0.1206
	CD(.01) = 0.094	CD(.01) = 0.135	CD(.01) = 0.131	CD(.01) = 1.78	CD(.01) = 0.329
	CV (%) = 23.47	CV (%) = 15.14	CV (%) = 22.76	CV(%) = 3.49	CV (%) = 2.36

SSI: single spore isolate; ND: Not detected; No. of replications – 3.

SEd: standard error of deviation.

*Exoglucanase, endoglucanase and xylanase - μ M glucose released per hour/ml; laccase and polyphenol oxidase - change in absorbance of 0.001 per ml/min.

variations, and can be grouped in various different groups based upon their enzymes activity profiles.

ITS profiling of 5.8S rDNA

PCR amplification of 5.8S rRNA gene region resulted in amplification of a single amplicon of approximately 720 base pair (bp) length in all the isolates and two parents. No intra-specific diversity was detected as far as the ITS length is concerned, confirming all the isolates belonging to the species *Volvariella volvacea*.

RAPD analysis

Distinct banding pattern was recorded for each primer and the RAPD profiles obtained with primers, OPB-2 and OPB-3 for SSIs of strain OE-12 and with OPB-4 for SSIs of strain OE-210 have been depicted as Figs. 1 and 2 as the

representative profiles. A combined phylogenetic analysis of RAPD profiles of SSIs and two parent strains revealed wide intra-specific variations (50% in OE-210 and 32% in OE-12) within the SSIs of two parent strains. The two parent strains and their SSIs showed highest dissimilarity of 60% (Fig. 3). The phylogenetic analysis showed at least 14–20% dissimilarity among the progenies and their own parent strains, whereas parent, OE-12 and its monosporous isolates could be divided into 4 distinct phylogenetic clades. The parent strain, OE-12 representing clade 8 showed similarity of 78% with isolates in clade 9 and 10, while it showed similarity of 68% with isolates in clade 11. Similarly, the phylogenetic analysis of the parent, OE-210 and its isolates formed 7 distinct phylogenetic clades (Fig. 3). Out of 10 isolates, the isolate OE-210-4 showed highest similarity of 85% with the parent strain and formed same clade as of the parent. The parent showed similarity of 83% with isolates in clades 2 and 3, 71% with clade 4, 64% with clades 5 and 6, and 50% with clade 7. The intra-specific diversity in ITS region of 5.8S rRNA gene and RAPD

Fig. 1 RAPD profile of *Volvariella volvacea* parent strain OE-210 and its SSIs with primer OPB-4.

Fig. 2 RAPD profiles of *Volvariella volvacea* parent strain OE-12 and its SSIs with primer OPB-2 and OPB-3.

Fig. 3 Combined clustering tree of *Volvariella volvacea* parent strains, OE-12, OE-210 and their SSIs.

profiling has been abundantly used by several workers for genetic characterization of the mushroom species as well as strains [22, 23] and *V. volvacea* in particular [22, 24]. However, none has correlated the three very important parameters together to differentiate the SSIs for their selection in mushroom yield trial. Only in recent Ahlawat et al. [25] have used these parameters in selection of potential high yielding parent strains of *V. volvacea*. The detailed biochemistry and molecular biology of this mushroom has been recently studied by Buswell and Chen [26] but they also did not use morphological characteristics, enzymes activity and DNA markers together for the selection of potential high yielding monosporous isolates.

In the present study, the isolates differing in morphological growth characteristics were also recorded to differ in phylogenetic analysis using RAPD markers according to which the parents and the SSIs of two strains belonged to 4 and 7 distinct clades, respectively. SSIs, OE-1203 and OE-1206 belonging to one phylogenetic group (clade 9) also showed very much similar activity profile of five enzymes. The other two isolates, OE-1204 and OE-1222 forming one group (clade 11) with 80% similarity also matched in activity of endoglucanase. The isolate, OE-1216 forming a distinct group, showed 3-fold higher activity of xylanase than the parent. The two isolates, OE-210-13 and OE-210-22 of parent strain, OE-210 forming one group (clade 3) showed almost similar activity profile of different enzymes except of polyphenol oxidase. Almost similar was the case of isolates, OE-210-9 and OE-210-29 forming clade 5. The isolate, OE-210-12 with maximum diversity (50%) with parent, exhibited highest activity of laccase and polyphenol oxidase. The other higher laccase and PPO producers, OE-210-09 and OE-210-29 from clade 5, and OE-210-30 from clade 4 also showed quite high dissimilarity of 29 and 26%, respectively with the parent strain, OE-210. However, some deviation from the trend has also been recorded and the isolate, OE-210-04 forming same group with parent, OE-210 (clade 1) exhibited 3-fold higher activity of laccase and PPO than its parent.

The highest dissimilarity in phylogenetic analysis shown by the hyper laccase producer SSIs with their two parents and formation of distinct clades reveal some relationship of the enzymes activity with genetic distinctness of the monosporous isolates. In earlier studies, the intra-specific diversity in ITS region of 5.8S rRNA gene, RAPD profiling and enzymes markers have been commonly used for genetic characterization of mushroom strains. However, except Ahlawat et al. [25], none has correlated the three very important parameters together to differentiate the parent strains for their selection in mushroom yield trial. A comparatively fast growing isolate with high density of aerial hyphae and chlamydospores along with higher

activity of cellulases and ligninases forming distinct clade in phylogenetic analysis can prove to be potential high yielding isolate.

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