Molecular characterization and optimization of parameters for cultivation of Pleurotus himalayaensis

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ABSTRACT: A new species of Pleurotus proposed as Pleurotus himalayaensis Dhancholia grows in association with a threatened plant of Ferula jaeschkeana Vatke at an altitude of 4000 mts and above in the Himalayas was collected from Lahaul valley. People in Lahoul valley found the mushroom to be highly delicious. Isolates were collected from ten different locations in the valley. In preliminary attempts it was found that mycelial run was fast on PDA and malt extract media among different growth media tested whereas temperature at 28°C with pH of 6 was found to be most congenial for vegetative growth. Cultivation technology for fruiting of this Pleurotus species was developed with locally available substrate i.e. Albizia chinensis sawdust + cotton seed cake supplemented with 10 per cent wheat bran at 500-800 lux and it was found to be best in terms of primordial initiation, number of primordia, fruit body expansion and development. Though the new species had different morphological characteristics but genetic sequence analysis based on internal transcribed spacer marker revealed it as closely related to P. eryngii.

Key words: Culture, media, molecular characterization, Pleurotus himalayaensis

Pleurotus (Fr.) P. Kumm (1871) is a genus of the edible “Oyster Mushroom” which is popularly referred to as “Dhingri” in India. Taxonomically, it belongs to the family Pleurotaceae of the order Agaricales, class Agaricomycetes, subphylum Agaricomycotina in the Phylum Basidiomycota and subkingdom Dikarya of Kingdom Fungi (Kirk et al., 2008). Species of Pleurotus are the third largest cultivated mushrooms in the world with an annual production of around 8,75,600 tonnes (USDA 2002) whereas in India production accounts for nearly 1500 tonnes. It has many health benefits such as anti-tumour, immuno-modulatory, antioxidant, anti-inflammatory, hypocholesterolaemic, anti-hypertensive, antiplatelet-aggregating, antimicrobial and antiviral (Khan and Tania 2012).

Recently, a novel species of Pleurotus was found growing in association with the threatened plant of Ferula jaeschkeana Vatke in the Lahaul Valley of Himachal Pradesh and was proposed as Pleurotus himalayaensis Dhancholia (Dhancholia 2013). The total production of this mushroom in the area around Sumnam village of Lahaul valley (major collection area) is only 8-10 quintals (Dhancholia 2013). The People in Lahoul valley found this mushroom highly delicious (Dhancholia 2013). As in barcoding of mushrooms, internal transcribed spacers (ITS) is the primary DNA barcode locus that is being utilized for the phylogenetic analysis for higher level classification of fungi. Though, the species was new, so far no cultural or cultivation studies have been conducted on it. Hence, the present study was undertaken to optimize the cultivation parameter with broad objective to develop cultivation technology for underutilized delicious mushroom.

MATERIALS AND METHODS

Isolation and identification Pleurotus species

Two isolates viz., DL27, DL501 of Pleurotus himalayaensis Dhancholia were isolated from fresh fruit bodies collected from village Kwarin (3400 amsl) Lahaul and Spiti on potato dextrose agar following standard isolation techniques. Morpho cultural characteristics were used to identify fungus based on description by Pegler (1976) and Dhancholia (2013). Different culture media (media malt extract, potato dextrose, yeast potato, dextrose yeast extract, Czapek’s dox and dimmick medium) were tested for better growth.

Total genomic DNA of fungal isolates was extracted using CTAB method (Murray and Thompson, 1980) with minor modifications. Amplified PCR products of four isolates (two collected from Lahaul valley and other two temperature sensitive strains of Lentinus and Pleurotus from Mushroom centre CSKHPKV) using ITS1 and ITS4 were amplified and products were sent for custom sequencing. The sequences obtained were analyzed using BLASTN program from the website http://www.ncbi.nih.gov/blast.

Phylogenetic analysis

In order to ascertain the unique identity of identified species, phylogenetic relationship of four sequenced isolates along with 12 reference sequences were analyzed in MEGA 4.1 (Tamura et al., 2007). Pair wise evolutionary divergence was calculated by maximum composite likelihood method in MEGA 4.1 (Tamura et al., 2007). Out of four sequenced isolates, two isolates representing Pleurotus himalayaensis Dhancholia (DL 27, DL 501) and two temperature sensitive strains viz.,
Substrate evaluation

Locally available straw (paddy, soybean, wheat, corn cobs), weeds (Lantana, Eupatorium, Ageratum) and sawdust (Albizia chinensis, Dalbergia sissoo, Pongamia pinnata, Mangifera indica) in various combinations supplemented with cotton seed cakes, and wheat bran were evaluated.

Pre-wetted substrates at 65 percent moisture level were supplemented with calcium sulphate (gypsum) and calcium carbonate (chalk powder) to obtain pH of 7.0. The mixed substrates (500 gm wet) were filled in polypropylene bags, plugged and autoclaved in three replications. Casing was done after a span of four month by exposing the top surface of polypropylene bags. Cropping conditions (28°C temperature, 1500 ppm CO₂, 500-800 lux light, 85-90% RH) were maintained for pinning and fruiting. Reduced relative humidity of 80-85%, light (500 lux) and fresh air was introduced after the pin head changed to fan shape (Pileus).

Temperature shock

After 120 days of incubation, bags fully colonized with mycelium were taken out of incubator and kept in the refrigerator at 4°C in order to give temperature shock for primordia initiation. Bags were kept at 2, 4, 16, 24, 48 and 72 hrs separate with three replications each.

RESULTS AND DISCUSSION

Identification of Pleurotus species

Sequence analysis upon BLASTN at NCBI gene bank revealed that the isolates DL27 (KP455494), DL501 (KP455495) showed 99 per cent sequence homology to P. eryngii and P. nebrodensis respectively whereas the remaining two references isolates viz. BM1 and BM2 were identified as L. sajor-caju, and P. ostreatus. Two of the sequences generated in present investigation belonging to Pleurotus himalayaensis were submitted at NCBI Gene-Bank. DL27 (KP455494) (representing Pleurotus himalayaensis Dhancholia) having close resemblance to P. eryngii was selected for optimization of cultural and cultivation parameters.

Phylogenetic analysis

Phylogenetic analysis of the isolates showed three major clusters in which DL27 and DL501 were clustered in a single group (Fig. 2) along with P. eryngii. Thus, revealing close relatedness to P. eryngii. Variation with respect to deletion in nucleotide sequence of four sequenced isolates was observed and compared with reference Pleurotus species based on sequence alignment. A quantitative representation of four isolates showed that DL27 was more similar to DL501 (0.007) compared to BM2 (0.008) and BM1 (0.017).

In estimating the genetic distance of DL27 isolate, maximum evolutionary divergence of 0.017 was found with L. sajor-caju whereas it was least in P. eryngii (0.00).
Table 1. Mycelial characteristics of *Pleurotus himalayaensis* Dhancholia on various substrates combinations after 120 days of spawn run

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Substrate combinations</th>
<th>Mycelial characteristics</th>
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<tbody>
<tr>
<td>C1.</td>
<td>67% cotton seed cake + 30% <em>Albizia chinensis</em> saw dust + 1% lime + 2% gypsum</td>
<td>Complete dense, quite thick and compact growth</td>
</tr>
<tr>
<td>C2.</td>
<td>67% cotton seed cake+30% <em>Mangifera indica</em> saw dust + 1% lime + 2% gypsum</td>
<td>Nil</td>
</tr>
<tr>
<td>C3.</td>
<td>67% cotton seed cake + 30% <em>Dalbergia sissoo</em> saw dust + 1% lime + 2% gypsum</td>
<td>Complete dense, quite thick and compact growth</td>
</tr>
<tr>
<td>C4.</td>
<td>67% cotton seed cake + 30% <em>Pongamia pinnata</em> saw dust+1% lime + 2% gypsum</td>
<td>Complete dense, quite thick and compact growth</td>
</tr>
<tr>
<td>C5.</td>
<td>80% cotton seed cake + 13% wheat bran + 5% corn powder + 1% lime + 1% gypsum</td>
<td>Nil</td>
</tr>
</tbody>
</table>

DL27 strain (psychrophilic, naturally growing in cold deserts, Zone 4) was having evolutionary closeness to BM2 which is low temperature loving strain of *Pleurotus* i.e. (0.008) in comparison to BM1 (0.017), a moderate to high temperature loving strain grown in summer. Since, it is a new *Pleurotus* species and systematic work on its cultivation refinement is under progress thereby no literature pertaining to study was available. In one of studies by Avin et al. (2012), partial rDNA sequences, including the internal transcribed spacer I-5.8S rDNA internal transcribed spacer II, was used for molecular identification and assessment of phylogenetic relationships between selected edible species of the Basidiomycetes.

**Mycelial growth optimization**

Growth parameters such as media, temperature and pH were evaluated for test isolate (DL27). Among six media, malt extract (89.5 mm) was found the best followed by potato dextrose (86.5 mm) and yeast potato dextrose (77.7 mm). A temperature range of 28-30°C and pH 6.0 were optimum for the growth of DL 27.

Our results are in agreement with the earlier workers where malt extract medium supported good growth of *P. ostreatus* (Balazs et al., 1987; Cedano, 1993, Gardezi, 1994; Zagrean et al., 2009). In other studies involving different *Pleurotus* species, workers had optimized temperature for mycelial growth in range of 25-30°C specifically 25°C for *Pleurotus* spp. (Zadrazil, 1968, 1976) and 25°C and 20°C for Spanish and Kabulian isolate of *Pleurotus eryngii* (Sharma and Jandaik, 1984). Liu et al. (2009) studied different temperature range for different *Pleurotus* spp. 25-30°C for *P. florida*, 28°C for vegetative growth of *P. eryngii* and 27°C for *P. ferulae* has been reported.
Pleurotus spp. could grow on a wide range of pH between 3-8 as reported by various workers. They reported retarded growth of mycelium above and below this pH range. Different pH has been suggested by various workers for different species of Pleurotus i.e. 6-8 for P. ostreatus by Li-Ming (1995), pH 5.5 for initial mycelial growth and pH 7.0 for final mycelial growth of P. ostreatus by Bugarski et al. (1997) and pH 6 has been reported to be the best for maximum mycelial growth of P. eryngii (Miyauchi et al., 1998).

Substrate evaluation

Mycelial colonization behaviour: Three substrates i.e. C1 (67% cotton seed cake + 30% Albizia chinensis + 1% lime + 2% gypsum), C3 (67% cotton seed cake + 30% Dalbergia sissoo saw dust + 1% lime + 2% gypsum) and C4 (67% cotton seed cake + 30% Pongamia pinnata saw dust + 1% lime + 2% gypsum) showed the complete spawn run while C2 and C5 were not at all colonized and were, therefore, discarded (Table 1).

Higher yields of oyster mushroom were reported on wheat stalk, cotton stalk, millet stalk, soybean stalk and lentil straw. Out of which soybean stalk proved to be the best substrate (Dundar and Yildiz, 2009). Ashraf et al. (2013) evaluated different substrates viz. cotton waste, wheat straw and paddy straw for cultivation of P. sajorcaju, P. ostreatus and P. dijmor and observed cotton waste as the best substrate for fastest spawn running, primordial initiation, harvesting stage, maximum number of fruiting bodies and maximum yield.

Primordial initiation: The primordial initiation took place at 45 days of casing after temperature shock of 24, 48 hrs followed by gradual decline at higher duration for 72 hrs. However, duration of 2, 4 and 16 hrs was found ineffective for primordial initiation (Table 2).

Fruiting behaviour: Morphological features of developing basidiocarps were variable for traits such as stipe length, pileus diameter, pileus colour and pileus surface (Table 3). Few desirable and unique traits such as fruit body with dimidiate shape, caespitose growth habit of stipe was observed on C1 whereas in C3 and C4, the pileus was convex. In all the substrates, fruit bodies had white, thin, decurrent lamellae with entire edged lamellae of 3-4 types.

The present study involved ITS sequenced based characterization of two newly identified strains belonging to Pleurotus himalayensis i.e. DL27 and DL-501 that were submitted at NCBI and were found to be close hit to morphologically different Pleurotus eryngii and P. nebrodensis.

In addition to this, studies on standardization and evaluation of various parameters viz., temperature, pH, substrates with solo objective to develop cultivation technology for newly identified delicious species of Pleurotus was carried out. The attempts were successful in artificially cultivation of newly described species but further investigation involving refinement to increase the yield has to be carried out.

REFERENCES


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