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The genus *Bondarzewia* is a macrofungus that usually has a relatively large and imbricate globally distributed basidiocarp, and is believed to have originated in East Asia. In the present study, an attempt has been made to isolate the pure culture of wild *Bondarzewia* sp. from East Sikkim, taking into consideration its edibility among the local people. Further investigation is needed to determine its actual culinary value and optimum conditions for fruiting in *in vitro*. Mycelium was isolated through the tissue culture method. The identification of the mushroom was based on morphological and molecular characterization. The pure culture is deposited in the culture collection of the Aghakar Research Institute, Pune, India. The spawn production in a controlled environment in a lab was successful. An attempt to grow the fruiting bodies of mushrooms using the mother spawns on different substrates compost combinations containing both organic and inorganic compounds was partially fulfilled.

Key words: Sikkim, fungi, mushroom, *Bondarzewia* sp., spawn, pure culture.

INTRODUCTION

One of the forest resources that hasn't been fully utilised is edible wild mushrooms. Wild mushrooms are well known for being used only by certain local or ethnic tribes for food and medical purposes. Over exploitation and loss of wild mushrooms may occur from unsustainable activities such as forest fires and overharvesting. Understanding the ecology of mushrooms, defining cultivation parameters, domesticating them, and researching the relevance of each species are the best ways to preserve these macrofungi. Over the past decades several new species have been discovered, some of which are edible and others which have medicinal properties (Chang and Miles, 2004; Valverde *et al*.,2015). There are an estimated 2000 species that can be eaten without causing harm to one's health, with 700 having scientifically proved medicinal characteristics (Wasser 2002). The study of naturally occurring species could be beneficial for increasing yields, introducing new species to markets, and preserving fungal germplasm before their natural settings are damaged or destroyed by humans. Pure cultures of wild mushrooms isolated via *in vitro* have become important for the development of high-quality food, medicinal sources, and natural component utilised in the pharmaceutical and other industries. Taking into account the world's growing population, the challenge today is to develop new species and strains that can help boost mushroom output in the coming years. To discover new industrial mushrooms, the main criteria are to make sure if it is edible and secondly, establish if it can readily be cultivated.

Numerous factors influence mushroom production, including compost type, nutrients in the growth medium, pH of the growth medium, temperature, humidity, aeration, and various biotic factors (Boddy *et.al* 2014). Saprobic species can be grown using standard mushroom cultivation techniques (Stamets, 2000). In addition to the fruiting bodies, several recent trends indicated exploiting mycelia and culture media as possible sources of bioactive chemicals (Reis *et.al.,*2011). Not only in pericarps, but also in mycelium (pure culture), filtrates, and other biologically active polysaccharides with

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immunostimulatory and anticancer activities can be discovered (Cheung 2008). Within the saprophytic mushroom family also, there are two distinct subgroups: Humicolous mushrooms, such as *Agaricus* spp., require a very complex substrate known as compost, which is very selective, and xilophagous mushrooms, such as *Pleurotus* spp. or *Lentinula edodes*, do not require a composting process and can fruit on many sterilised or pasteurised lignocellulosic substrates. These species feature a diverse set of substrate-inducible hydrolytic enzymes that digest the substrate and release nutrients for growth and fruiting.

The genus *Bondarzewia* is distinguished by its annual growth habit, pileate basidiocarps with poroid hymenophores, and physical similarities to numerous Polyporales species. Its basidiospores are heavily amyloid and decorated, and recent phylogenetic analysis revealed that it belongs to the Russulales (Larsson and Larsson, 2003; Miller *et al.* 2006). *Bondarzewia* is a genus with a few species and a lot of records from all over the world (Sharma, 2012; Das et al.,2015; Singh and Das, 2019). On the basis of features mentioned by Lyngdoh and Dkhar (2014), it was recently *recognised as B. berkeleyi*. Because the species feature unusually big, imbricate basidiocarps, it is a remarkably prominent genus. Some species are edible and some are medicinal (Boa 2004, Dai *et al*. 2009), while others are pathogens to their host trees (Dai *et al.*2007), they are not mycorrhizal (Larsson and Larsson, 2003) and are saprobic, hence having the potential to be cultivated.

Given the ethnomycological uses of wild mushrooms found in Sikkim's forests, this is most likely the first attempt, with the primary aim of isolating a pure culture of *Bondarzewia* sp. and production of spawn and fruiting with the intention to explore its full biotechnological potential, since this mushroom is consumed for food and medicinal purposes in the study area.

MATERIALS AND METHODS

Sample Collection

East Sikkim, India, has the state's highest rainfall, and its temperate and subtropical climates encourage the growth of wood-decaying fungi. The sample was taken between June and August, during the wet season, in the month of August of 2020; the mushroom was found growing on the stump and root line of a dead *Prunus cerasoides* in the Pangthang region, and Amdo golai at around 1700-1650 metres above sea level, East Sikkim, India. *Quercus* sp. trees are also known to harbour this fungus. It has the potential to recur in the same location for years (2018–2021). It's considered a wild edible fungus in the region, and it is best when consumed young (local name: *Gedhe/Rakte/ Mirgechyau*). All of the key physical characteristics, growth habits, and environments were documented. The fresh samples were taken to the lab for additional examination.

Description and Identification

Morphological

The macroscopic and microscopic properties of the sample specimen were used to provide a partial identification. The macroscopic features were used for identification are: cap size, shape, colour, surface texture and surface moisture, pore colour, attachment, spacing, lamellules, stem size, shape, surface texture and surface moisture, flesh colour and texture by using standard books, taxonomic keys, and published research articles (Largent and Stuntz 1986; Iliffe, 1994; Lincoff, 1982). Standard microscopic procedures were used to examine microscopic features, which were then verified by comparing standard literatures.

Molecular

Due to overlapping features, a high degree of phenotypic plasticity, cryptic species, and the existence of different morphs for the same taxa, morphology-based taxonomy can occasionally fail to resolve species effectively (Hyde *et al*. 2016). Hence, genomic DNA was obtained from a pure isolated mushroom culture for molecular identification. For ITS-rDNA identification, the material was sent to Agharkar Research Institute in Pune, India. Using primers ITS4 and ITS5, the ITS-rDNA partial gene was effectively amplified. The ABI-BigDye® Terminatorv3.1 Cycle Sequencing Kit was used to set up the sequencing PCR. The ABI 3100 automated DNA sequencer's raw sequence was manually adjusted for inconsistencies. The sequence data was compared to publically accessible sequences (NCBI) and the identification was 99% determined and the phylogenetic tree was constructed using MEGA 10 software, neighbour joining method.

Pure culture Isolation

A tiny piece of tissue from the inner flesh of the pileus junction and base area was obtained aseptically for inoculation on PDA medium under sterile conditions (Stamets, 2000). For pure culture isolation, the mushroom mycelia were transferred to PDA medium and cultured at 27°C for 10-15 days. It is critical to examine the media in order to choose one that is suited for widespread mycelium growth (Wozniak, 2009). In order to know how different media affect mycelial growth, Malt Extract Agar (MEA), Czapek Dox Agar (CDA), Corn Meal Agar (CMA), and PDA at same temperature and pH were used and the growth diameter measured daily to establish growth rates (Nasim *et al.* 2001; Thongklang *et al*. 2010).

Preservation of culture

Mushroom production requires the preservation of strains and genetic traits of axenic cultures. The viability of strains can be maintained in a variety of ways, both short and long term. The preservation method was carried out in a sterile environment. The pure culture discs were made with a sterile cork borer (about 3 mm). Culture discs were placed in 10 mL screw cap tubes with paraffin liquid labeled with the strain Id. The tubes were subsequently stored in Myco-patho laboratory, PG Department of Botany, Nar Bahadur Bhandari Government College, Tadong, Gangtok, Sikkim, at 4°C for short-term preservation. For long-term preservation the pure culture was deposited at the Aghakar Research Institute's culture collection in Pune, India, with the accession number NFCCI- 5171.

Spawn preparation

For this investigation, wheat and paddy grain were used as substrate for spawning. The cleansed grains were properly rinsed and immersed in tap water overnight. Following that, the water was drained and the substrate was heated for 15-30 minutes before being dried for half an hour or two. Finally, 10 g of $CaCO_3$ was combined with half kg of wheat grain and paddy, respectively. The processed grains were placed in the conical flasks nearly half-filled with substrate. The flask's mouth was closed with a cotton plugs, thereafter autoclaved twice at 121°C for 15 minutes. After sterilization, the flasks were allowed to cool for 1 h before mycelia from pure mushroom cultures with a fraction of PDA were inoculated into the individual grain media in sterile condition and incubated for 15- 20 days at 23°C – 24°C (Borah *et.al.*2019; Nwanze *et al*. 2005; Stamets 2000).

Compost preparation for fruiting

Wood-inhabiting mushrooms can be grown in a variety of lignocellulosic substrates, including straw, sawdust, and rice husk (Thawthong *et al*. 2014). Rice straw, sawdust, and corn bran were the main compost substrates used in this study. All the substrates were soaked overnight in different containers to create different compost mixture types such as A, B, and C (Table 1**)**. After draining the water, the varied substrates were boiled for half an hour and then dried for half an hour for the moisture to evaporate. Finally both the organic compound $CaCO_3$ (calcium carbonate) and inorganic compound $CaSO_4$ (gypsum) were added to the various substrate combinations. Different compost combinations were then placed into the plastic bags filled it to half. With the help of loose thread/rubber band, the mouths of the bags were closed with cotton plugs. The filled plastic bags were then sterilized for 1 h at 121°C in an autoclave and allowed to cool for another 1 h. Different compost mixtures were injected with mother spawns and incubated at 23°C - 24°C in sterilized

Table 1: Different types of compost substrate mixture combinations.

Item No.	Substrate 1	Substrate 2		Substrate 3	Inorganic compound 1	Inorganic compound 2
Compost mixture A	Paddy (250g)	Sawdust (100g)	Mixture	Straw (100g)	$CaCO_3$ (9g)	$CaSO_4$ (9g)
Compost mixture B	Paddy (250g)	Sawdust (100g)	Mixture	Corn bran(100g)	$CaCO_3$ (9g)	$CaSO_4$ (9g)
Compost mixture C	Wheat grain (250g)	Corn bran(100g)		Straw (100g)	$CaCO_3$ (9g)	$CaSO_4$ (9g)

conditions; protocols of (Borah *et.al.*2019; Nwanze *et al*. 2005; Stamets 2000) were followed with slight alteration.

RESULTS AND DISCUSSION

Morphological description and identification

Bondarzewia Singer (Bondarzewiaceae, Russulales) is a globally distributed genus of mushroom forming fungi,grows in one or more large clusters and form rosettes, on the ground near the bases of deciduous trees. It also frequently occurs as a saprophyte on dead trees in forests or stumps left remaining in managed areas (Chen *et. al.*2016). In the study area it was usually found to be associated as saprophyte especially in *Prunus cerasoides*. The fungus produces annual mushrooms consisting of one to five overlapping, cream-colored caps; the mushrooms typically grows from infected roots a few feet away from the main trunk, but can also grow directly from the main stump at the soil line. The mushrooms can appear anytime from late May onward till September. Fruiting body up to 50- 60 cm wide, several cap emerging from short central stem, Cap is 8 to 25 cm wide, cap's central area thickness 3-6 cm, margin 1-1.5 cm, fan shaped to irregular form, smooth, velvety, fleshy, pale orange to tan or dull brown, colour fading with maturity, does not bruise when pressed or cut. Gills were absent, pores cream coloured/off white; circular to irregular more prominent towards the base, the pore surface runs partway down the stem, pore size 0.5 – 1 mm, spore print white, tube length;1-2 mm. Spores are white, round with ridges and spines. Stems were short, gnarled. The interior flesh is white, thick, and does not bruise on maturity it gets tough and woody. The spores are released from pores on the underside of the caps. Morphologically characters are matching with *Bondarzewia berleleyi* (Figure 1. a-c).

Isolation and Culture Characterization

Pure culture isolation through tissue culture technique was achieved on PDA medium, which is very significant for culture characterization, accurate identification and preparation of spawn for cultivation purposes. Four different media including MEA, CDA, CMA and PDA were used to observe the effect of different media on mycelia growth and its growth rate at same temperature and pH. Mycelial growths in PDA medium were recorded with 3.5 cm and in MEA medium it was 3 cm after seven days of inoculation, but mycelial growth was more consistent and uniform in PDA and grows faster during later stage as compared to MEA medium. No proper growths were seen in CMA and CDA media (Fig. 1 d-f). Therefore PDA medium was found to be the most suitable media for mycelial growth of this mushroom strain.On PDA media mycelium was off white, reverse buff. Vegetative hyphae are branched, septate, hyaline, smooth walled, pigmented, thick and thin, ropy, intertwined, 2.0 – 4.7 µm diameter. Chlamydospores were produced intercalary or in terminal, globose, to subglobose, smooth walled, hyaline or fusoid, 11.8 × 11.8 µm. Clamp connection was absent (Fig. 1 g-i).

Molecular identification

The tested fungal strain of the isolated mushroom showed 99.33 % sequence similarity with *Bondarzewia* sp. and it shows 98.49% similarity with *Bondarzewia berkeleyi* (Fr.) Bondartsev & Singer as shown in the given BLASTn analysis (Altschul *et . al*., 1990).Phylogenetic tree based on ITS-rDNA suggestsour species is genetically closely related with *Bondarzewia berkeleyi,* with bootstrap value of 100 % which is corroborating with our morphological identification,but full taxonomic study may suggest it to be some other species or novel sp. For accurate identification, microscopic details from fresh samples will be carried out in coming season and molecular analysis LSU/ elongation factor 1-αgenes sequencing can be carried out (Table 2, Fig. 2).

Mother spawn production

The growth of mycelia was observed in both paddy and wheat substratesafter 4 days of inoculation. After 7 days we kept the spawn indirect natural light for few h and notice that mycelium grows fast in the presence of light as compare to the dark condition incubation. Density of mycelia was high in paddy spawn after 15 days of inoculation as compared to wheat substrate. In our studies one interesting observation was recorded; the initiation of fruiting bodies was seen in one of the paddy mother spawn, two month after inoculations as shown in Fig.1 (j).

Production of fruiting body

Mushroom mycelium grew successfully in the different compost mixture types after 5 days of

Fig. 1: **(a-c)** Fruiting bodies of *Bondarzewia sp.;* dorsal view, ventral view, pores close up; **(d-f)** Tissue section, mycelium growth from tissue culture;**(g-i)** Pure culture mycelia on PDA media, hyphae, chlamydospores;**(j)** Initiation of fruiting body in paddy spawn, **(k-l)** Mycelium establishment in different compost mixture after 1 month of inoculation.

Table 2: Result of top five hits upon BLASTn analysis.

Gene Bank Accession No.	Description	Max score	Query cover	Query coverage	E value	Identity (%)
MK713680.1	*Bondarzewia*sp. voucher US 0835	1054	1054	100%	0.0	99.33%
FJ644288.1	*Bondarzewia berkeleyi* voucher Li 1097	1024	1024	99%	0.0	98.49%
MK167432.1	*Bondarzewia berkeleyi* voucher MHHNU 857	1005	1005	97%	0.0	98.28%
MG253912.1	*Bondarzewia dickinsii* strain LF1827	968	968	96%	0.0	97.74%
MG253912.1	*Bondarzewia berkeleyi* voucher CLZhao 112	958	958	100%	0.0	95.97%

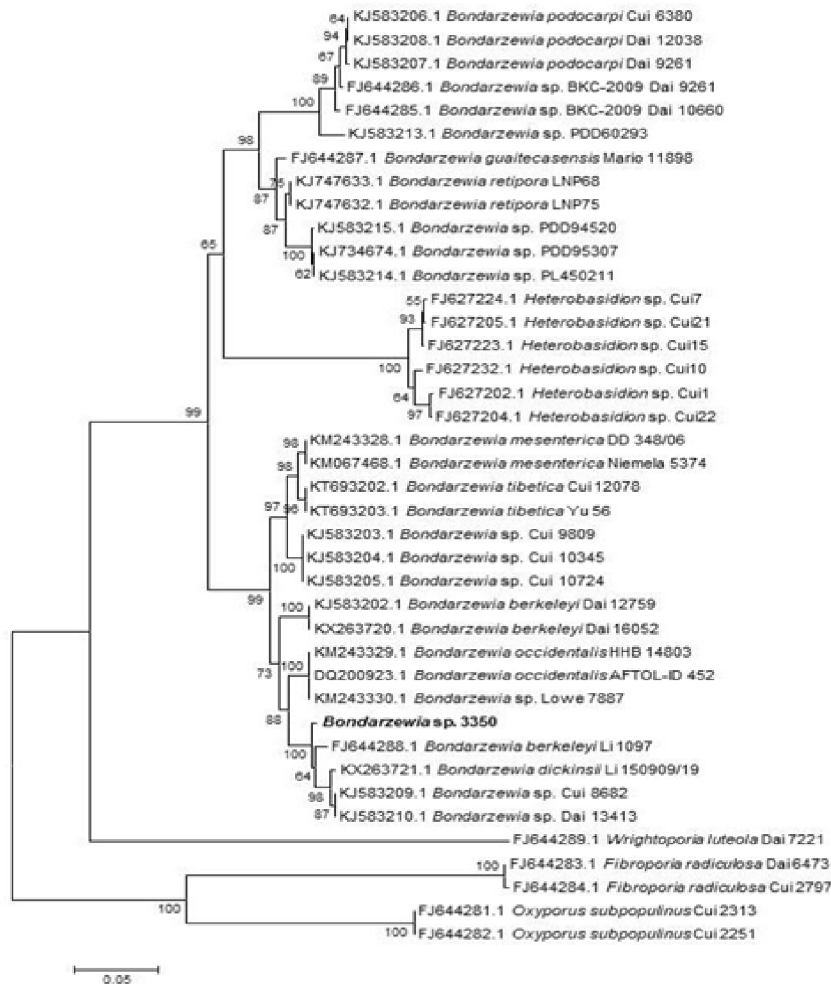

Fig.2: Phylogenetic tree of *Bondarzewia* sp. with multiple sequence alignment

inoculation. After one month, growth of mycelia almost covered the substrate in compost mixture type A and B as shown in Fig.1 (k,l). The growth rate of mycelium in compost mixture type C was slow and does not show proper growth. The compost mixture containing sawdust showed fast and proper growth of mycelia which was absent in substrate mixture type C. It was concluded that for the proper establishment of the mycelia, sawdust is an important substrate as it may nearly or fully fulfill the nutrients provided by decaying wood on which it grows naturally. Initiation of fruiting in mother spawn (paddy) was positive and but initiation of fruiting bodies in cultivation bags were not successful. There are different environmental and physiological factors affecting the fruiting body development of mushrooms, for e.g, CO_2 concentration has an intense impact on sporophore development, higher CO_2 concentrations may activate growth of mycelia but can prevent fruiting completely. Fruiting bodies might get deformed at lower CO_2 concentrations. Normal sporophore development usually occurs at about 0.2% CO_2 (Milkwood 2004). Temperature and pH affects sporophore development too, we set the fruiting during the winter temperature. Light positively affects both aggregation of hyphae and maturation of the fruiting body, but, on the contrary, the formation of the hyphal knot can be suppressed by the light. Too strong and too long lighting in the development stage of fruiting body are hampered too (Kues and Liu, 2000). Presence of some microorganisms or organisms also affects fruiting of some saprophytic species. The reason behind not forming of fruiting bodies in our cultivation experiment could be any possible reasons as discussed above.

CONCLUSION

Mushrooms can be found in forests all over the world; however unsustainable practices such as forest fires and over harvesting may lead to over exploitation and extinction of wild mushrooms. They grow under correct conditions and can provide an excellent source of natural vitamins and minerals, as well as other crucial bioactive compounds from the culture filtrates. In recent years the number of saprobes species being cultivated has steadily increased, although further research and refinement are needed. Since it was our first attempt to develop a pure culture of *Bondarzewia* sp. from Sikkim through tissue culture, spawn preparation, and cultivation, more research on its full biotechnological potential, such as nutritional value, biochemical properties, and medicinal properties, can be conducted.

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