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EXPLORATION OF THE UNIQUE FUNGAL ASSOCIATION AND PROTEIN PROFILE OF BODA FROM BASTAR, CHHATTISGARH, INDIA

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Bastar, Boda, *Shorea robusta*, Fungi, Protein, Spectrophotometer

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ABSTRACT: Bastar is endowed with a unique blend of traditional knowledge and rich floral diversity. The region is known as the island of Shorea robusta, which is a key source for white and black truffle called Boda. It is also known as the black gold of the sal forest. It sets underground with the onset of early monsoon and is an edible mushroom with high protein, vitamin, and fiber with the low calorific value used by the local inhabitants as delicious food and in the amelioration of heart disease, blood pressure, and stomach disorder. Owing to its tremendous health benefits, the present study deals with the assessment of the protein content, isolation, and identification of fungi associated with boda. The qualitative analysis revealed that black boda contains more protein content than white boda. The quantitative estimation revealed the presence of 0.97 mg/ml and 0.33 mg/ml of protein for 0.2 mg weight of black and white boda, respectively. Further, the boda sample were inoculated on potato dextrose agar and incubated at 28-30 ºC for 48 h. A total of ten fungi comprising of seven from white boda and three from black boda were isolated. The isolates were subjected to morphological and microscopic characterization. The moisture content and protein weight ratio analysis showed that black boda contains more moisture content than that of white boda. This is the pioneering research and first report of its kind from Bastar. However, the characterization of protein and molecular identification of fungi from boda is the future prospect of the research.

INTRODUCTION: Bastar is a district of Chhattisgarh state in central India with Jagdalpur as its district headquarter. It is the southernmost district of Chhattisgarh, which has high floral diversity and has escaped excessive human interference and exploitation of its forests [1-4].

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The entire region is a tilted peninsular plateau of about 40,000 km and varies in elevation from 284 m to1, 200 m above sea level. The track falls between the latitudes 18-30' N and 19-20' N and longitudes 81-10' E, and 82-15' E. Bastar comes under moist, sub-humid, agro-climatic region [5].

The climate is a dry moist tropical type with a mean rainfall of about 1100 mm, and has an average temperature varies from 25-27 ºC. Sal is a large deciduous tree with shining foliage. Conical, elongated crown in early-stage and rounded later is clean and straight with the bark having characteristic long and vertical fissures [6].

Bastar is covered with a dense sal forest, which is a key source of wild mushroom called as Bastar wild truffle (Boda). It is a small round fruiting body of a fungus that grows about 1-4 cm under the sal tree with the onset of the first shower of mansoon in Bastar. It is having a hard shell outside and a soft pulp inside and is basically of two types *viz.*, black pulp truffle (Jatha boda), and white pulp truffle (Rakhadi boda). The tribal community of bastar consumes this tuber traditionally as their nutritious dietary supplement.

Edible mushrooms are vital sources of food as they are highly nutritious and easily digestible. They are consumed not only for their innate flavor and taste but also for their tremendous medicinal values. Edible mushrooms have been found to be rich sources of protein, lipids, amino acids, glycogen, vitamins, and minerals [7]. On a fresh weight basis, mushrooms are superior in protein content to all vegetables and fruits [8]. Mushrooms have a high percentage of moisture 93-95% and are also a rich source of valuable minerals such as iron, potassium, phosphorus, calcium, and copper and essential biomolecules such as carbohydrate, protein, fat, and ash on dry weight basis along with vitamin B and vitamin D [9]. It is also enriched with essential amino acids required by an adult [10].

Mushrooms provide a high protein and low caloric diet and can thus be recommended to heart patients. The edible mushroom is a storehouse of valuable nutrients that confer it a huge medicinal potentiality as cardiovascular, anticancer, antiviral, antibacterial, anti-parasitic, anti-inflammatory, hepatoprotective and anti-diabetic activities [11]. Thus, in light of the vast potentiality of boda as a rich source of protein and essential nutrients for dietary sources among local tribal community of Bastar. An endeavor was made to systematically investigate and isolate the micro-flora associated with boda and to evaluate the protein content present in the boda sample collected from the Bastar region of Chhattisgarh, India.

MATERIALS AND METHODS:

Site of Study: Bastar district (19.1071ºN, 81.9535ºE) is located in the southern part of Chhattisgarh and has an area of 4029.98 km². Jagdalpur is both district and divisional headquarter of Bastar district **Fig. 1**. It is surrounded by Bijapur, Dantewada, Kondagaon, Narayanpur and Sukma districts of the state. The city lies on the southern bank of river Indravati with an average elevation of 562 meters. It has a total forest area of 292130 ha, which is more than 19% of the total land area of the district.

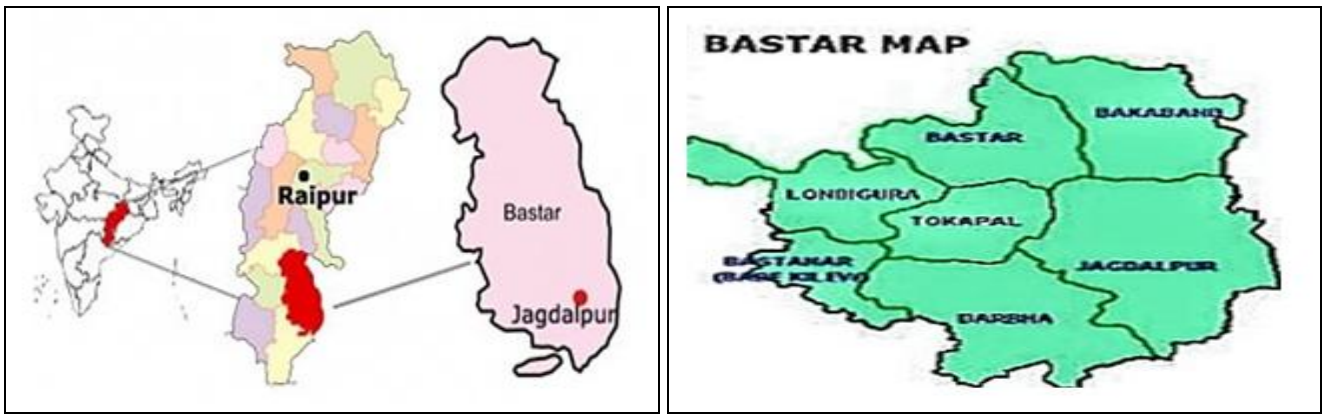

FIG. 1: MAP OF BASTAR DISTRICT SHOWING SAMPLE COLLECTION AREA

Sample Collection: The sample of boda was collected from the sal forest area behind the campus of Krishi Vigyan Kendra (KVK), Kumhrawand, Jagdalpur, Chhattisgarh, India **Fig. 2**.

The sample of boda was collected in a clean and dry polythene bag with the help of a clean and sterilized spatula and was brought to the laboratory for further study.

Sample Processing: The boda sample was rinsed thoroughly under running tap water to remove excess dirt and other suspended soil debris from its surface. The rinsed sample was then dried with the help of filter paper to remove excess moisture from the surface of boda sample **Fig. 3**. The sample was then subjected to surface sterilization, and finally, the above sample was rinsed thoroughly three to four times with sterile water for further use.

FIG. 2: SAL FOREST OF BASTAR, CHHATTISGARH

FIG. 3: SAMPLE OF BODA AFTER WASHING

Surface Sterilization: The sample was surface sterilized by subjecting it to 70% ethanol solution for a few seconds. Finally, the sample was rinsed thoroughly three to four times with sterile water. The rinsed sample was dried with the help of filter paper, and the transverse section of the boda sample was performed for the detailed investigation of its internal structures **Fig. 4**.

FIG. 4: T.S OF BODA AFTER SURFACE STERILIZATION

Media Preparation:

Potato Dextrose Agar: Peel off the potato and weight 250 g. cut it into small pieces and put in a beaker containing 500 ml distilled water. Boil it for about 30 min and collect the extract. Add the required amount of glucose and agar and mixed thoroughly. Raise the volume to 1000 ml. The pH was adjusted to 5.6 ± 0.2 with dilute acid or alkali. The media was transferred in 250 ml conical flasks, corked, and sterilized by autoclaving at 15 lb pressure (121ºC) for 15 min.

Potato Dextrose Broth: Peel off the potato and weight 250 g. cut it into small pieces and put in a beaker containing 500 ml distilled water. Boil it for about 30 min and collect the extract. Add the required amount of glucose and mixed thoroughly. Raise the volume to 1000 ml. The pH was adjusted to 5.6 ± 0.2 with dilute acid or alkali. The media was transferred in 250 ml conical flasks, corked and sterilized by autoclaving at 15 lb pressure (121 ºC) for 15 min.

Qualitative Analysis of Protein:

Biuret Test: To 2 ml of boda sample solution, add 5-6 drops of dilute $CuSO_4$. Then add 3 ml 40% NaOH solution and observe the color change and appearance of deep blue or purple precipitate.

Xanthoproteic Test: To 2 ml of boda sample solution, add an equal volume of concentrated HNO_3. Heat over a flame for 2 min and observe the color. Now cool the solution under the tap water and cautiously run in sufficient 40% NaOH to make the solution strongly alkaline. Observe the color change and appearance of yellow precipitate.

Quantitative Estimation of Protein:

Biuret Reagent: Dissolve 3 g of copper sulphate ($CuSO_4.5H_2O$) and 9 g of sodium-potassium tartrate in 500 ml of 0.2 mol/liter NaOH, add 5 g of potassium iodide and make up the volume to 1 liter with 0.2 mol/liter NaOH.

Procedure: Pipette out 0.0, 0.2, 0.4, 0.6, 0.8, and 1 ml of working standard into the series of labeled test tubes. Make up the volume to 1 ml in all the test tubes with distilled water. Now add 3 ml of Biuret reagent to all the test tubes, including the test tubes labeled 'blank' and 'unknown'. Mix the reaction mixture of all the tubes by vortexing and were incubated in a water bath at 37 ºC for 10 min. Now cool the reaction mixture in the test tubes to

the room temperature and record the absorbance at 540 nm against blank using spectrophotometer. Then the standard curve was plotted by taking absorbance along the X-axis and concentration of protein along the Y-axis. Finally, from this standard curve, the unknown concentration of protein in the boda sample was determined.

Estimation of Moisture Content: The pulp from boda sample was isolated for the moisture content analysis. Weigh the pulp of boda sample and its pre-weight was record. Place the pulp sample in the hot air oven at 100 ºC for 2 h. Now record the post-weight of the pulp sample. Finally, the difference of pre-weight and post-weight was recorded. The experiments were repeated in triplicates.

Preservation of Isolated Fungi: The potato dextrose agar slants were prepared and labeled. Sterilize the inoculating needle by holding it in the hottest portion of the spirit lamp flame. Flame until the entire wire becomes red hot and allows the needle to cool for a few seconds. Touch the tip of the needle to the surface of isolated discrete fungal colonies to be preserved by lifting the lid of the agar mother plate at 45 ºC. Now, inoculate the potato dextrose agar slant towards the center over the solidified agar surface with the help of inoculating needle and recap the cotton plug of the slant. Inoculate the isolated fungi from boda sample *viz.*, BKS-W1, BKS-W2, BKS-W3, BKS-W4, BK-W5, BKS-W6, BKS-W7, BKS-B1, BKS-B2, BKS-B3 and incubate all the fungal cultures in the incubator at 28-30 ºC for 48 h.

RESULTS: In the present study, an endeavor was made to collect boda samples from Jagdalpur, Bastar, Chhattisgarh, India. The sample was collected, and the fungus associated with boda *viz.*, white boda, and black boda were isolated and their morphological and microscopic characterization was performed. The boda sample was further subjected to the qualitative and quantitative protein estimation, and the moisture content in the boda sample was also determined. Finally, the isolated fungi were stored and preserved as pure culture in the laboratory of the School of Studies in Biotechnology, Bastar Vishwavidyalaya, Jagdalpur (Chhattisgarh) for further investigation.

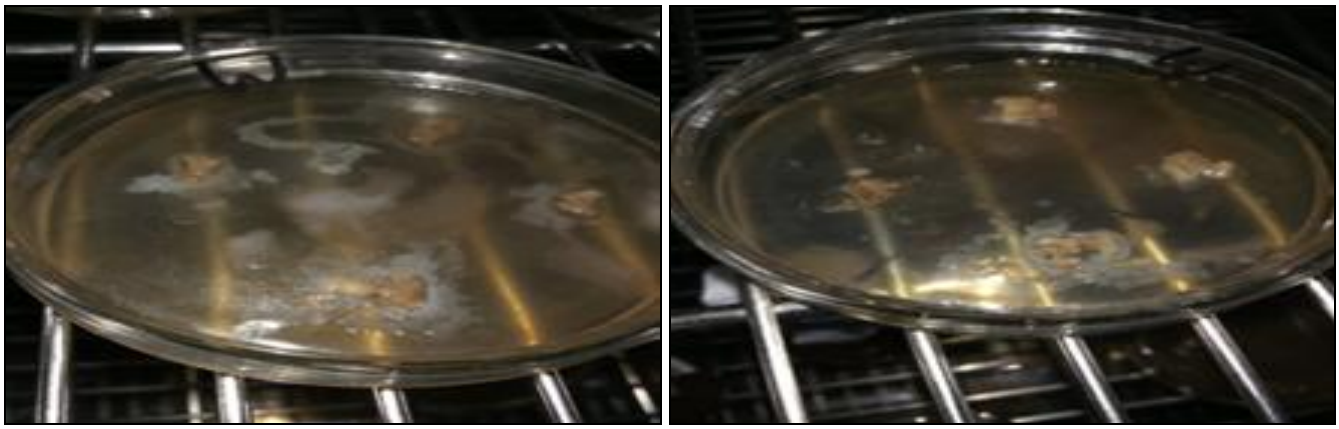

FIG. 5: ISOLATION OF FUNGI FROM WHITE BODA

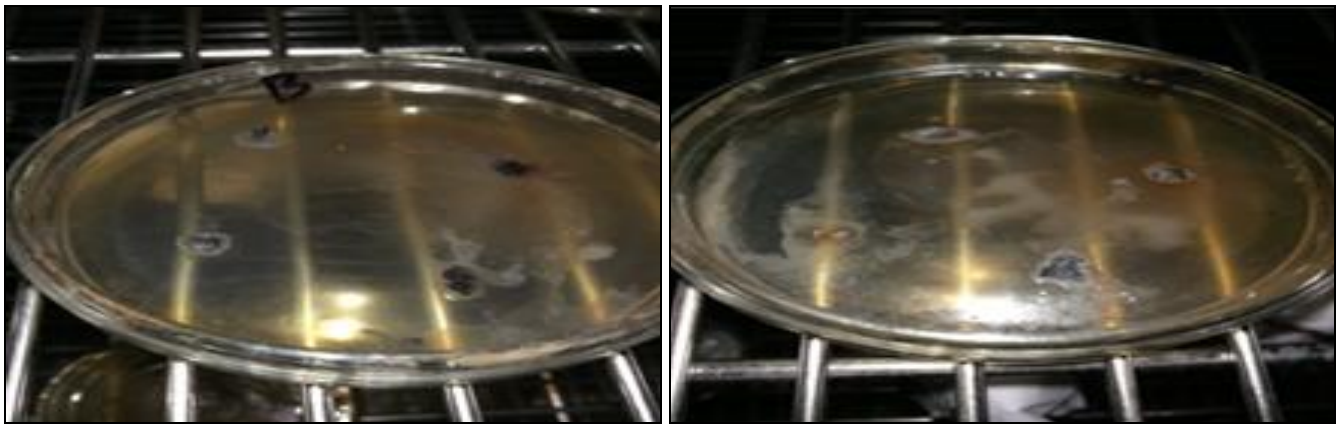

FIG. 6: ISOLATION OF FUNGI FROM BLACK BODA

After washing and surface sterilization of the sample with distilled water. The sample was dissected and the pulp of boda, which is spongy, dense, moist, and covered by a thin outer membrane inside a hard outer shell. The outer shell of boda was removed and the inner pulp *viz.*, black and white, was used for the isolation of fungus on to the PDA plate. A dense white mycelium was visible around the inoculated pulp. The PDA plate was divided into four sections, and in each section, white boda pulp was inoculated by a sterilized needle in the laminar airflow and finally incubated at 28-30 ºC for 48 h **Fig. 5-6**.

The plates were observed after incubation, and the mother plates were visible with different fungal colonies. The fungal colonies which originated from the surface of the boda sample *viz.*, white and black, were inoculated on another PDA plates as a pure culture for the complete growth of fungal colonies and their detailed morphological and microscopic characterization **Fig. 7**.

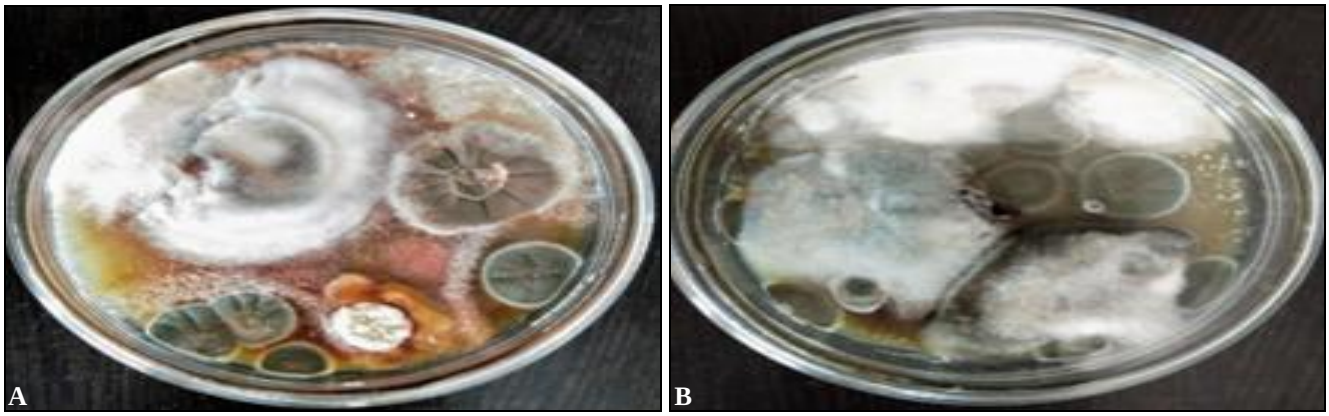

FIG. 7: ISOLATION OF FUNGI FROM (A) WHITE BODA (B) BLACK BODA

The boda sample *viz.*, white and black, were washed thoroughly, and their pulp was isolated and are crushed in the mortar and pistal with the help of 10 ml sterilized water and the stock extract of the boda sample so obtained were subjected to qualitative analysis of protein by Biuret test.

Added 1 ml of the boda extract in the test tube and 3 ml of biuret reagent. The appearance of deep blue precipitate confirms the presence of protein in the given sample **Fig. 8**.

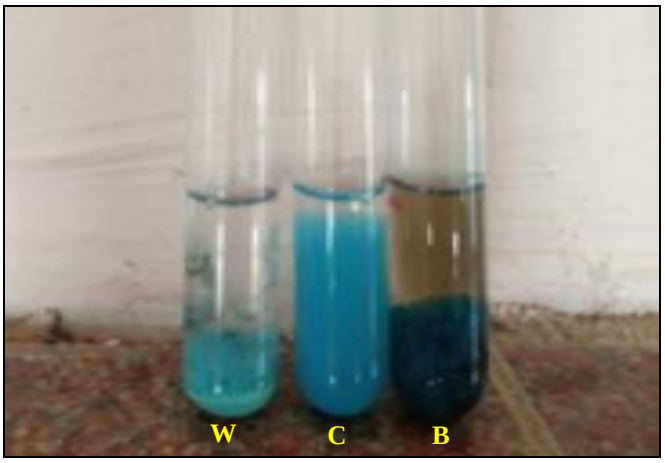

FIG. 8: QUALITATIVE TEST FOR PROTEIN OF WHITE AND BLACK BODA SAMPLE (W-WHITE BODA EXTRACT, C-CONTROL, B-BLACK BODA EXTRACT)

TABLE 1: QUALITATIVE TEST FOR PROTEIN OF WHITE AND BLACK BODA

S. no.	Qualitative Test	White Boda	Black Boda
1	Biuret Test	++	+++
2	Xanthoprotic Test	+	++

+++ Strongly positive; ++ moderately positive; + positive

The boda sample was further subjected to a qualitative analysis of protein by the Xanthoprotic test. To 1 ml of boda sample extract in the test tube and add 2 ml of NaOH and few drops of 2 drops of nitric acid, the appearance of yellow-colored precipitate confirms the presence of protein in the given boda sample **Fig. 9**.

FIG. 9: QUALITATIVE TEST FOR PROTEIN OF WHITE AND BLACK BODA SAMPLE (W-WHITE BODA EXTRACT, C-CONTROL, B-BLACK BODA EXTRACT)

The qualitative estimation of the protein was performed by the Biuret method; the standard graph was plotted with the standard protein as albumin. The series of test tubes were prepared. The complete reaction mixture in all the test tubes of the series is incubated at 37 ºC for 10 min in the water bath. Finally, the optical density (OD) and percentage of transmittance (%T) were recorded. The standard curve was prepared by taking optical density along the X-axis and concentration of the protein mg/ml along Y-axis. Finally, from the standard graph the unknown concentration of the protein in the boda sample *viz.*, white and black were determined in **Fig. 10** and **Table 2**.

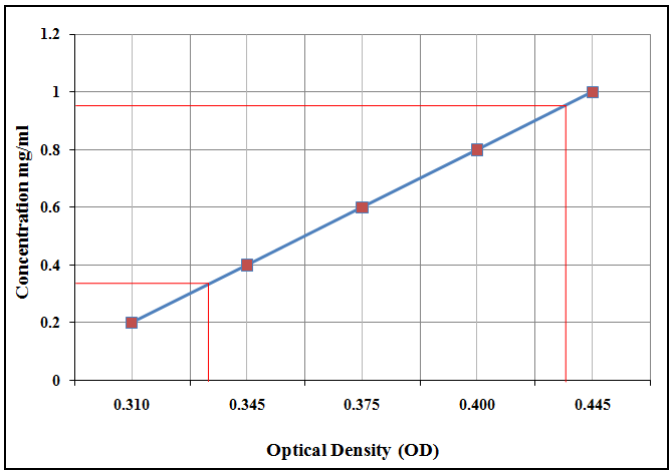

FIG. 10: STANDARD CURVE OF PROTEIN BY BIURET METHOD

TABLE 2: ESTIMATION OF UNKNOWN PROTEIN CONCENTRATION OF THE BODA

Conc. of the Boda Sample	Optical Density (O.D)
White 0.2 ml	0.338
Black 0.2 ml	0.442

Black Boda: 0.2 mg = 0.97 mg/ml

Weight of black boda = 0.3872 gm

0.3872 gm = 387.2 mg

387.2 mg × 0.97 mg/ml = 375.58 mg/ml

White Boda: 0.2 mg = 0.33 mg/ml

Weight of black boda = 0.3872 gm

0.3872 gm = 387.2 mg

387.2 mg × 0.33mg/ml = 127.776 mg/ml

The quantitative estimation of protein in boda sample *viz.*, white and black were compared with respect to their optical density (OD) at four different concentrations as 5.0, 10, 15 and 20 mg/ml. The results revealed that black boda is possessing higher concentration of protein as compared to that of white boda at a particular sample concentration **Fig. 11**.

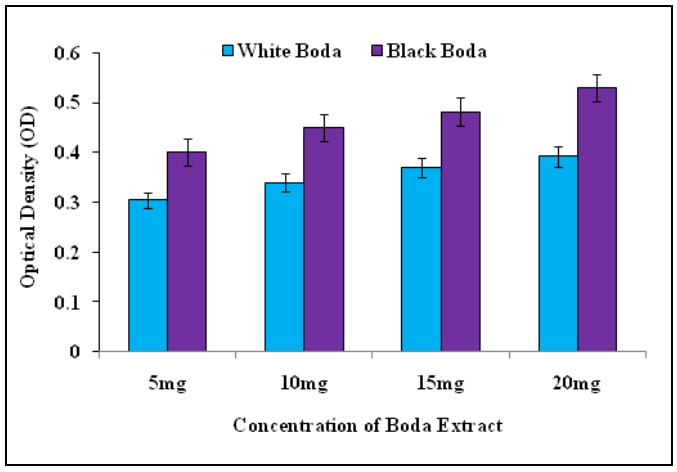

FIG. 11: COMPARATIVE ASSESSMENT OF PROTEIN IN BODA SAMPLE EXTRACT

Estimation of Protein Weight Ratio: The protein weight ratio was determined for the boda sample *viz.*, white and black the ratio of total protein concentration / weight of the sample was determined. The results revealed that the black boda with PWR value 0.99 and white boda with PWR value 0.33, which depicts that black boda possesses more protein than that of white boda sample **Table 3**.

TABLE 3: ESTIMATION OF PROTEIN/ WEIGHT RATIO OF THE BODA

Sample	Weight (mg)	Total Protein Concentration (mg)
White Boda	387.2	127.776
Black Boda	387.2	375.58

Protein Weight Ratio (PWR) = Total protein concentration ÷ Weight of sample

White Boda: Weight of white boda =387.2mg

Total protein concentration= 127.776mg

PWR Ratio = 127.776 / 387.2 = 0.33

Black Boda: Weight of black boda = 387.2 mg

Total protein concentration = 375.58

PWR Ratio = 375.58 / 387.2 = 0.99

Estimation of Moisture Content: The moisture content analyses were performed in both the boda sample, and the results revealed that the black boda possess more moisture content than the white boda sample under investigation. The experiments were conducted in triplicates, and the mean and standard error Mean ± SE were determined, and the results were represented graphically **Fig. 12**.

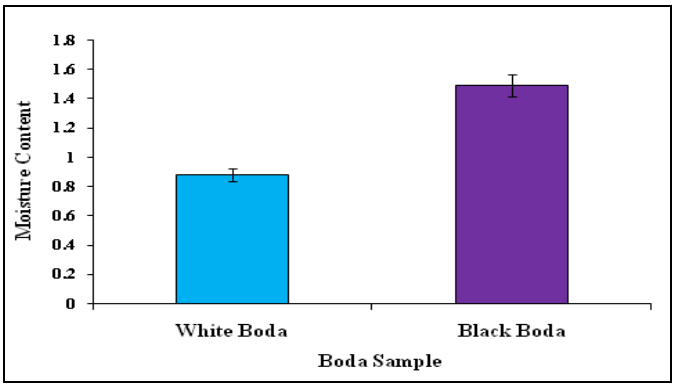

FIG. 12: COMPARATIVE MOISTURE CONTENT IN WHITE AND BLACK BODA SAMPLE

Morphological Characterization of Fungi: A total of ten fungus were isolated from the boda sample comprising of seven fungus isolated from white boda with the identification code as BKS-W1, BKS-W2, BKS-W3, BKS-W4, BKS-W5 BKS-W6, and BK-W7. From black boda three fungus were isolated with identification code as BKS-B1, BKS-B2, and BKS-B3 **Table 4**.

TABLE 4: MORPHOLOGICAL CHARACTERIZATION OF FUNGI ISOLATED FROM BODA

S. no.	Identification Code	Size	Colour	Form	Elevation	Margin	Pigment
1	BKS-W1	Large	White	Filamentous	Convex	Filiform	Yellow
2	BKS-W2	Small	Green	Irregular	Raised	Undulate	Yellow
3	BKS-W3	Large	White	Filamentous	Convex	Filiform	Yellow
4	BKS-W4	Small	White	Filamentous	Convex	Filiform	Yellow
5	BKS-W5	Small	Green	Irregular	Flat	Lobate	No
6	BKS-W6	Large	White	Irregular	Raised	Filiform	Yellow
7	BKS-W7	Small	Yellow	Irregular	Raised	Filiform	No
8	BKS-B1	Moderate	White	Filamentous	Nmbonate	Filiform	Yellow
9	BKS-B2	Large	White	Filamentous	Raised	Filiform	Yellow
10	BKS-B3	Small	Green	Irregular	Flat	Lobate	No

Microscopic Characterization of Fungi: A total of ten fungus were isolated from the boda sample with their identification code as BKS-W1, BKS-W2, BKS-W3, BKS-W4, BKS-W5 BKS-W6, BK-W7, BKS-B1, BKS-B2 and BKS-B3. All the fungal isolates were identified with the help of an inverted microscope, MICAM software version 1.4 **Table 6,** and **Fig. 13**.

TABLE 6: MICROSCOPIC CHARACTERIZATION OF FUNGI ISOLATED FROM BODA

S. no.	Identification Code	Mycelium	Spore	Rhizoids	Conidia
1	BKS-W1	Aseptate	Oval	Absent	Absent
2	BKS-W2	Aseptate	Oval	Present	Present
3	BKS-W3	Aseptate	Oval	Absent	Absent
4	BKS-W4	Aseptate	Oval	Absent	Present
5	BKS-W5	Aseptate	Branched	Absent	Present
6	BKS-W6	Aseptate	Round	Absent	Present
7	BKS-W7	Aseptate	Curved	Absent	Absent
8	BKS-B1	Aseptate	Branched	Present	Absent
9	BKS B2	Aseptate	Round	Present	Absent
10	BKS-B3	Aseptate	Branched	Absent	Present

Preservation of Isolated Fungi: A total of ten fungal isolates were isolated from the boda sample comprising of seven from white boda and three from black boda. All the fungal cultures were preserved in the form of PDA slants in the refrigerator for further investigation. However, the molecular characterization using 18s r-RNA sequencing of all the isolated fungi from boda sample and to study the molecular mechanism of boda nodule formation, its fungal association in natural habitat and its lab cultivation techniques along with the protein isolation from the boda sample its purification and characterization is the future prospect of the present investigation understudy **Fig. 14**.

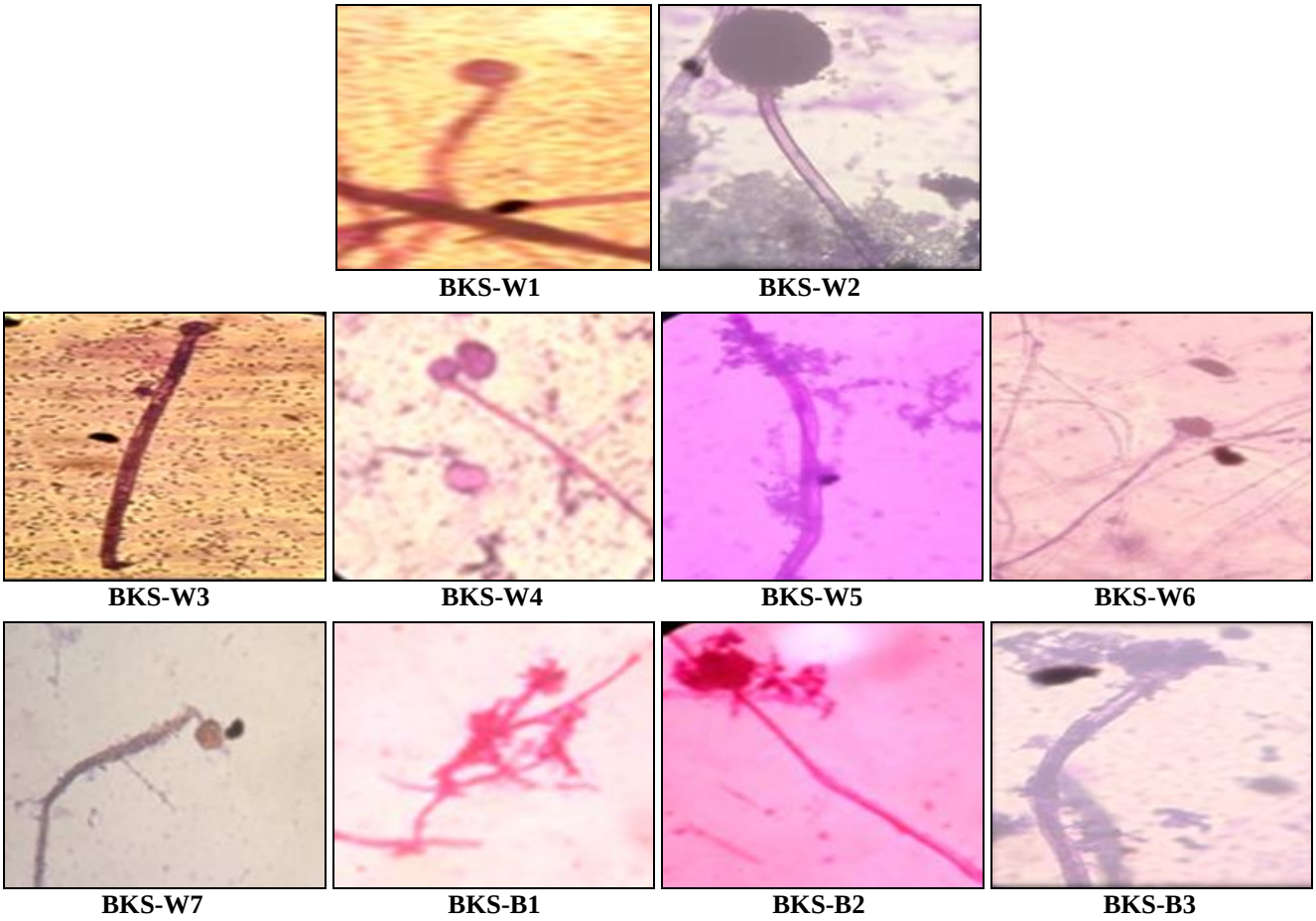

FIG. 13: MICROSCOPIC CHARACTERIZATION OF FUNGI ISOLATED FROM BODA

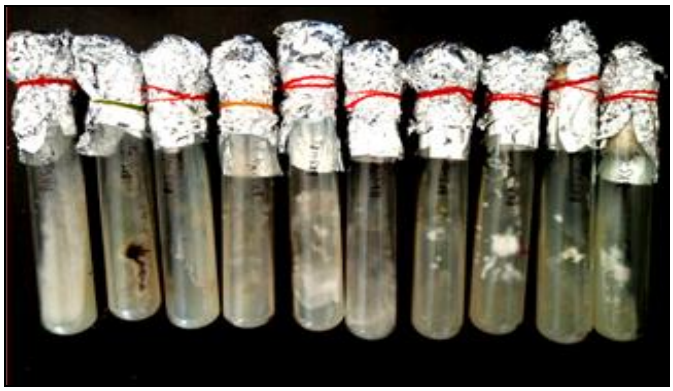

FIG. 14: PURE CULTURE AND PRESERVATION OF FUNGI ISOLATED FROM BODA

DISCUSSION: The present study was a focused endeavor on the exploration of fungi associated with boda sample and the protein profile analysis of boda sample *viz.,* white and black collected from the sal forest of Jagdalpur. In the present study, the fungus associated with the boda sample was isolated and their morphological and microscopic characterization was performed. The boda sample *viz.,* white and black was subjected to the qualitative and quantitative protein estimation, moisture content analysis and the protein weight ratio was also determined. Finally, the isolated fungi were stored and preserved as pure cultures in the laboratory of the School of Studies in Biotechnology, Bastar Vishwavidyalaya, Jagdalpur (Chhattisgarh) for further investigation. The qualitative test of protein in the boda sample *viz.*, white and black revealed a strong positive reaction for protein. The qualitative test for protein by precipitation reaction clearly demonstrated that black boda possess more protein as compared to that of white boda. However, protein as an important constituent of dry matter of mushrooms was reported by several researchers globally [12-15]. The biological activities of mushrooms include antifungal, antiviral, antibacterial, anti-cancer, anti-cholesterol, anti-tumor and antihypertensive [16]. The digestibility of mushroom protein is as high as 72 - 83% which makes it an ideal diet [17]. Due to the presence of high content of proteins, vitamins, fibres and minerals the mushrooms are also considered as "Poor man's Protein".

The quantitative estimation of protein in the boda sample *viz.,* white and black revealed the presence

of high concentration of protein. The quantitative analysis of protein justifies the qualitative test for protein and revealed that black boda possess higher concentration of protein 0.97 mg/ml as compared to that of white boda 0.33 mg/ml. The concentration of protein in different species of mushroom was investigated and the results revealed that highest amount of protein was recorded in *Cantharellus subcibarius* 1.567 mg/ml followed by *Russula virescens* 1.427 mg/ml, *Lactarius piperatus* 1.38 mg/ml and least amount of protein was found in *Cordycep sinemsis* 0.131 mg/ml [18]. The crude protein content in the fresh mushroom was found to be 5.27 g/100 g. Moreover, the detailed investigation and research on the protein content of mushroom by several investigators well justify that, boda possess a higher concentration of protein and can be used as a healthy protein diet. The moisture content analysis in the boda sample *viz.*, white and black revealed that black Boda contains more moisture content (1.492 ± 0.80) than white boda (0.882 ± 0.34). However, the moisture content in fresh mushroom was 89.69% and 12.27% in case of shade-dried mushroom.

Similar levels of moisture content (87.2 ± 0.5%) in *Pleurotus ostreatus* was also recorded [19]. The moisture content in shade-dried oyster mushroom was found to be 12.27%, which is in line with the results recorded by other investigators [20]. The above result well suggests that boda contains a higher percentage of moisture, which makes it an ideal food and dietary supplement.

The fungus isolated from the pulp of white boda sample was identified on the basis of its morphological and microscopic characterization as *Rhizopus* sp. and *Mucor* sp. belonging to the Division: Zygomycota, Order: Mucorales, Family: Mucoraceae, Class: Mucoromycotina. Similar findings depicting the presence of fungi *viz*., *Mucor circinelloides*, *Trichoderma koningiopsis*, *Phomopsis* sp., and *Cladosporium bruhnei* from king oyster mushroom was reported [21]. However, *Aspergillus* sp. was also isolated from the above sample belonging to the Division: Ascomycota, Order: Eurotiales, Family: Trichocomaceae, Class: Eurotiomycetes. The fungal isolates isolated from the pulp of black boda samples were identified on the basis of its morphological and microscopic characterization as *Penicillium* sp. belonging to the Division: Ascomycota, Order: Eurotiales, Family: Trichocomaceae, Class: Eurotiomycetes. The fungi such as *Penicillium* sp., *Rhizopus* sp., *Aspergillus flavus*, *Aspergillus niger*, and *Trichoderma harzianum* were isolated from paddy straw [22]. However *Trichoderma* sp. and *Penicillium* sp. were isolated from both substrate and fruiting bodies [23]. All the fungal isolates were stored as pure cultures in the slants for further research.

CONCLUSION: Boda is a rich source of protein and essential dietary nutrients for the local tribal inhabitants of Bastar. The present study was an endeavor to isolate the micro-flora associated with boda and to evaluate the protein content present in the boda sample collected from Bastar region of Chhattisgarh, India. A total of ten fungal isolates comprising of seven from white boda and three from black boda were isolated and characterized. The unique fungal association with boda and its protein profile was investigated. Moreover, this is the first report of its kind depicting the presence of novel array of fungal association and the unique nutritive value of boda from Bastar region. Thus, boda is not only nutritionally sound stuff but also provides a source of seasonal employment for boda collecting communities of this area due to its high market value. However, the molecular characterization using 18s r-RNA sequencing of all the isolated fungi and to study the molecular mechanism of boda nodule formation, its fungal association in natural habitat and its lab cultivation techniques along with the protein isolation, purification and characterization along with the identification of bioactive compound from boda for novel drug discovery can also be targeted for future research.

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