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Original Research Article

A Study on bioactive fluorescent compounds from selected mushrooms

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Abstract

Mushrooms have long been appreciated for their flavor, texture, medicinal and nutraceutical attributes. The present study deals with fluorescent bioactive compounds from *Armillaria mellea* (MTCC 409) and *Omphalotus olearius* (MTCC 2790) were obtained from MTCC, Chandigarh and pure mycelial cultures were developed. Time scale studies for the production, extraction, and estimation of fluorescent dyes were carried out and the evaluated for their antioxidant and antibacterial activities. *A. mellea* and *O. olearius* grown in potato dextrose broth recorded the maximum mycelial dry weight of 5.90 ± 1.12g/50mL and 0.57 ± 0.025 g/50mL respectively on 28th day. *A. mellea* and *O. olearius* mycelium (1000 mg) recorded maximum crude fluorescent dyes in acetone (255 mg) and methanol (553mg) respectively. *A. mellea* recorded good antioxidant activity with water extract (47.5% ± 0.6), followed by ethyl acetate extract (46.4% ± 13.09). Acetone extracts of *A. mellea* and *O. olearius* recorded highest zone of inhibition (0.4 cm and 1cm) against *E. coli* and *P. aeruginosa* respectively.

Key words: *Armillaria mellea*, *Omphalotus olearius*, Antioxidant activity, Antimicrobial activity, Fluorescent dyes.

1. Introduction

Bioactive compounds are extra nutritional constituents that naturally occur in small quantities in plant and food products (Etherton *et al*., 2002). Secondary metabolites are the most commonly reported bioactive compounds such as antibiotics, mycotoxins, alkaloids, food grade pigments, plant growth factors, and phenolic compounds (Ferreira *et al*., 2010). Microorganisms are considered as sources of valuable bioactive metabolites are very well established for more than half a century and over 120 of the most important metabolites such as, penicillin, cyclosporine A, adriamycine antibacterial, antiviral, antitumor as well as anticoagulant properties were reported (Alanis, 2005). Fluorescent-labeled molecules have been used extensively for a wide range of applications in biological detection and diagnosis (Lian *et al*., 2004). Bioluminescence is defined as the metabolite that emits visible light by a living system. Bioluminescence has been widely exploited as marker systems for detection and tracking of cells in the environment and as biosensors for the detection of pollutants (Van, 2006). The fluorescent mushrooms are *Armillaria fuscipes*, *Armillaria gallica*, *Armillaria mellea*, *Armillaria tabescens*, *Dictyopanus foliicolus*, *Dictyopanus pusillus*, *Filoboletus pallescens*, *Filoboletus yunnanensis*, *Mycena chlorophos*, *Neonothopanus gardneri*, *Neonothopanus nimbi*, *O. illudens*, *O. japonicas*, *O. olearius*, *Panellus gloeocystidiatus*, and evaluated for their medicinal, nutraceutical and fluorescent properties (Chang *et al*., 1996). *A. mellea* contain the bioactive compounds polysaccharides, sesquiterpene aryl esters have anti oxidation, immunopotentiation, anti vertigo, anti aging, anti microbial, antibacterial activity (Zhang *et al*., 2001; Yu *et al*., 2006; Yang *et al*., 2007; Sun *et al*., 2009). *O. nidiformis* contain the bioactive compounds phenol, flavonoid, terpenoid, alkaloid and saponins have antioxidant activity (Jose *et al*., 2012). *O. illudens* contain the bioactive compounds illudinic acid, illudin S and M have antibacterial activity (Dufresne *et al*., 1997) and for treating cancer diseases (Van, 2006). *O. olearius*, *Lampteromyces japonicas* contain the bioactive compound cytotoxic, tricyclic sesquiterpene, illudin S have anticancer activity (Chatterjee *et al*., 2011). *Mycena sp.* contain

the bioactive compounds strobilurin M, tetrachloropyrolcatechol have antifungal, cytostatic, antibacterial activity (Daferner *et al*., 1998). Among the several fluorescent mushrooms, in this study *A. mellea* and *O. olearius* were utilized for the growth, production of fluorescent dyes, extracted, estimated and tested for its antimicrobial potential.

2. Materials and Methods

2.1. Culture collection and maintenance

Fluorescent mushroom samples such as *A. mellea* (MTCC 409), and *O. olearius* (MTCC 2790) were received from Microbial Type Culture Collection (MTCC), Chandigarh, India. The pure culture mycelium was initiated from the point of inoculum and was sub cultured at every 15 days intervals (Mona *et al.*, 2014).

2.1.1. Composition of Media

The compositions of the media (g/L) used for the culture maintenance are malt based media. MTCC 409 maintained in the media consists of Malt extract 3g; Yeast extract 3g; Peptone 5g; Glucose 10g; Agar 20g; Distilled water (1L) and pH 7.0. MTCC 2790 maintained in the media consists of Malt extract 20g; Agar 20g; Distilled water (1L) and pH 6.5. Growth studies were done in PDB consists of Potato (Peeled and sliced) 260g; Dextrose (g) 20g; Distilled water (1L) and pH 5.6 and nutrient broth consists of Yeast extract 3g; Peptone 5g; Sodium Chloride 5g; Distilled water (1L) and pH 7.0 for the antibacterial activity.

2.2. Growth and production of fluorescent dyes from A. mellea and O. olearius

Mycelial cultures of *A. mellea* and *O. olearius* were inoculated in 250 mL of Erlenmeyer flask containing 50 mL of respective broth. Time scale studies were carried out for the period of 35 days and at every seven days interval, the mycelial growth in terms of wet weight and dry weight, followed by fluorescent dye were determined. Apart from the MTCC recommended media, the same growth and metabolites production was also tested in Potato Dextrose Broth.

2.2.1. Determination of mycelial wet weight

Mycelial mat recovered from Whatman no.1 filter paper was washed with distilled water and removed the excess moisture adhering to the mycelial mat using blotting paper and wet weight was calculated.

2.2.2. Determination of mycelial dry weight

Mycelial mat recovered from Whatman no.1 filter paper was washed with distilled water and dried at 80 ℃ for 24 hrs and the dry weight was recorded.

2.3. Intracellular extraction of fluorescent dyes from A. mellea and O. olearius

2.3.1. Extraction of fluorescent dyes from fluorescent mushrooms

The dried mycelium (1000mg) of *A. mellea* and *O. olearius* was taken, 10mL distilled water was added and grounded using mortar and pestle and left undisturbed for overnight. After the incubation, the extract was added with 100mL of distilled water and boiled in water bath for 4 hrs. The extract was transferred to a preweighed crucible and allowed to dry overnight in the hot air oven at 80℃. The crucible was kept inside the desiccator for 20 mins and final weight was recorded. To the extract, 5mL of distilled water was added, analyzed in UV - Visible spectrophotometer from 200 – 800 nm.

The dried mycelia (1000mg) of *A. mellea* and *O. olearius* was taken, 10mL acetone and methanol was individually added and grounded using mortar and pestle and left undisturbed for overnight. After the incubation, the extract was added with 120mL of acetone and methanol extraction was carried out with soxhlet apparatus for 4 hrs. The extract was transferred to a preweighed crucible and allowed to dry overnight. The crucible was kept inside the desiccator for 20 mins and final weight was recorded. To the extract, 5mL of acetone was added, analyzed in UV - Visible spectrophotometer from 200 – 800 nm.

2.4. Extracellular separation of fluorescent dyes from A. mellea and O. olearius

The solvents used for the separation were Chloroform, Ethyl acetate and Hexane. Separating funnel containing the 30 days old growth media (200 mL and 10 mL for *A. mellea* and *O. olearius* respectively) were individually mixed with the 50 mL and 10mL of above mentioned solvents and kept at still condition for 15 mins and observed for formation of layers. The separated sample was visualized under UV and compared its fluorescent property in comparison with control media. The extract was kept for drying in the hot air oven at 80℃ and final weight was recorded. To the dried sample 5mL of the

respective solvent was added and analyzed in UV - Visible spectrophotometer from 200 – 800 nm.

2.5. Anti-bacterial activity of bioactive compounds

2.5.1. Bacterial culture preparation

Nutrient broth was prepared and loopful of each culture was inoculated in the prepared broth and incubated at 37℃ for 12-14 hrs to get the log phase, which resulted in the best result.

2.5.2. Agar diffusion method

The efficacy of fluorescent dyes were extracted from selected mushrooms was tested for its efficacy towards selected bacteria by agar diffusion method which was performed by following the method as described by (Erturk *et al*., 2003). Bacterial strains such as *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Proteus vulgaris* and *Pseudomonas aeruginosa* were used for the anti bacterial assays which were obtained from the culture collection unit of Shri AMM Murugappa Chettiar Research Centre, Taramani, Chennai. Nutrient agar plates were prepared, after solidification, bacterial cultures grown on nutrient broth was aseptically taken using a sterilized cotton swab and swabbed on the surface of the NA plates and allowed to dry for 1h. Wells of size 0.5 cm in diameter was cut in the agar plates using a sterile cork borer. The extracts from fluorescent mushrooms (*A. mellea* and *O. olearius*) were added at concentrations of 25µl, 50µL, 75µL and 100µL into the wells of the inoculated plates. After 24 - 48 hrs of incubation at 37℃, plates were observed for the development of zone of inhibition. Size of the inhibited zone were measured and recorded. All the assays were carried out in triplicates. Petriplates containing respective solvents (100 µL) in the wells with bacteria were maintained as blank. The bacteriogenecity of the extracts was defined as the clear zone of no bacterial growth around the extracts loaded wells.

2.6. Anti-oxidant activity of fluorescent dyes

2.6.1. DPPH radical scavenging activity (Zhao *et al*., 2006)

DPPH (2, 2-diphenyl-1-picrylhydrazyl) (0.0004g) was added to 100ml of solvents (acetone and methanol). Mushroom extracts (1.5ml) was taken in tubes in the concentration of 1000µg/ml. To that 3ml of DPPH was added. It was kept in the dark room for 30min. OD values were measured at 517nm using UV spectrophotometer. DPPH and solvents (acetone and methanol) without mushroom extract was used as the control. DPPH free radicals were calculated as follows.

I (%) = (A blank –A sample / A blank) x 100

Where I is inhibition (%), A blank is the absorbance of control reaction and A sample is the absorbance of the test compound.

2.6.2. Hydrogen peroxide radical scavenging activity:

Mushroom extracts (1.5ml) was taken in tubes in the concentration of 1000µg/ml. To that 3ml of Hydrogen peroxide solution was added. It was kept in the dark room for 10min. OD values were measured at 230nm using UV spectrophotometer. Phosphate buffered saline without mushroom and bacterial extract was used as the control.

Hydrogen peroxide radical scavenging activity was calculated as follows.

I (%) = (A blank –A sample / A blank) x 100

Where I is inhibition (%), A blank is the absorbance of control reaction and A sample is the absorbance of the test compound.

3. Results and Discussion

3.1. Procurement and Maintenance of fluorescent mushrooms from MTCC

Pure culture was initiated and maintained in the malt extract media (Plate 1a).

3.1.1. Growth of A. mellea

A. mellea grown in malt extract broth recorded for the maximum mycelial fresh weight of 4.819 ± 0.557 g/50mL and dry weight of 1.294 ± 0.193 g/50mL on 28th day (Fig.1; Plate 1b). *A. mellea* grown in potato dextrose broth (PDB) recorded for the maximum mycelial wet weight of 10.81 ± 1.09 g/50mL and dry weight of 5.905 ± 1.125 g/50mL on 28th day (Fig. 2). Among the two media, potato dextrose broth has resulted for highest amount mycelia growth of *A. mellea*.

3.1.2. Growth of O. olearius

O. olearius grown in malt extract broth recorded the maximum mycelial wet weight of 0.334 ± 0.063 g/50mL and dry weight of 0.045 ± 0.001 g/50mL on 28th day (Fig. 3). *O. olearius* grown in potato dextrose broth recorded the maximum mycelial wet weight of 0.726 ± 0.059 g/50mL and dry weight of 0.57 ± 0.025 g/50mL on 28th day (Fig. 4; Plate 1 c). Among the two media, potato dextrose broth has influenced for maximum mycelia growth of *O. olearius*.

Van (2006) successfully identified the basic parameter of physiological and nutritious characteristics in cultivating a tropical luminescent mushroom *O. af.illudent* for studying their luminescent and medical properties. Fruit-bodies were formed in artificial conditions, able to emit greenish light continuously in the whole fruit-body, mycelia, spore. This mushroom produces some useful compounds such as illudin S and M for treating cancer disease.

3.2 Extraction of fluorescent dyes

3.2.1. Intracellular extraction of fluorescent dyes from A. mellea

Maximum contents of crude fluorescent dyes extracted from the mycelium (1000 mg) of *A. mellea* recorded in acetone (255 mg), followed by water (89 mg) and methanol (33mg). These extracts were processed for the determination of antibacterial and antioxidant activities.

3.2.2. Extracellular extraction of fluorescent dyes from A. mellea

Maximum quantity of extracellular crude fluorescent dyes (200 mL) of *A. mellea* recorded in ethyl acetate (1.553g), followed by chloroform (1.279g) and hexane (1.125g). These extracts were taken for antibacterial and antioxidant activity.

3.2.3. Intracellular extraction of fluorescent dyes from O. olearius

Maximum quantity of crude fluorescent dyes was extracted from the mycelium (1000mg) of *O. olearius* in methanol (553mg), followed by water (487mg) and acetone (280mg). These extracts were taken for antibacterial and antioxidant activity.

3.2.4. Extracellular extraction of fluorescent dyes from O. olearius

Extraction of extracellular fluorescent dyes by liquid: liquid partition technique was followed. In that extracellular medium of 10 mL was added with 10 mL of solvents like hexane, chloroform and ethyl acetate. The high content of extracellular (10 mL) crude bioactive compounds was extracted utilizing chloroform (0.214g), followed by hexane (0.086g) and ethyl acetate (0.04g). These extracts were taken for antibacterial and antioxidant activity (Plate 1 d).

3.3. Antibacterial activity:

3.3.1. Antibacterial activity for A. mellea and O. olearius

A. mellea recorded zone of inhibition against both gram positive (*S. aureus*) and gram negative bacteria (*E. coli, S. typhi, P. aeruginosa and P. vulgaris*).

Acetone of *A. mellea* recorded the zone of inhibition against *E. coli* (0.0066 ± 0.0033cm), *S. typhi* (0 ± 0.0066 cm), *S. aureus* (0.0057 ± 0.0033 cm), *P. aeruginosa* (0 ± 0.006 cm) and *P. vulgaris* (0.0057 ± 0.003 cm) and methanol extracts (Mycelia) of *A. mellea* recorded the zone of inhibition against *E. coli* (0.4 ± 0.003 cm), *S. typhi* (0.3 ± 0.003cm), *S. aureus* (0.3 ± 0.003 cm), *P. aeruginosa* (0.3 ± 0 cm) and *P. vulgaris* (0.3 ± 0 cm). Water extracts doesn't recorded any anti-bacterial activity (Table 1; Plate 1 e - h). Chloroform, Ethyl acetate and Hexane extracts of *A. mellea* (Extracellular) doesn't shows activity against (*E. coli, S. typhi, P. aeruginosa, P. vulgaris* and *S. aureus*).

Acetone extracts of *O. olearius* recorded the zone of inhibition against *E. coli* (0.4 ± 0.06cm), *S. typhi* (0.5 ± 0.05 cm), *S. aureus* (0.6 ± 0.05cm), *P. aeruginosa* (1 ± 0cm) and *P. vulgaris* (0.6 ± 0.17cm), methanol extracts shows activity against *E. coli* (0.5 ± 0.17 cm), *S. typhi* (0.3 ± 0.03cm) and *P. aeruginosa* (0.3 ± 0 cm). Water extracts recorded the zone of inhibition against *S. typhi* (0.3 ± 0.03cm) (Mona *et al*., 2014). Extracellular fluorescent dyes extracted from Chloroform, Hexane and Ethyl acetate extracts of *O. olearius* doesn't record any zone of inhibition against (*E. coli, S. typhi, S. aureus, P. vulgaris* and *P. aeruginosa*) (Table 2; Plate 1 i - l). Donnelly *et al*., (1985) isolated two new sesquiterpene aryl esters, 4-*O*-methylmelleolide and judeol, both of strong antibacterial activity against gram positive bacteria. Armillaric acid also exhibited marked inhibitory activity against gram positive bacteria and yeast (Obuchi *et al*., 1990). Momose *et al*., (2000) isolated three compounds, melleolides K, L and M. Melleolides K were of antimicrobial activity against

gram positive bacteria, yeast and fungi. In addition, three antibacterial sesquiterpenoids, melleolides B-D, were yielded from *A. mellea* (Arnone *et al*., 1996). *O. olearius* no reports has been reported for antibacterial activity.

3.4. Antioxidant Properties of selected mushrooms

3.4.1. DPPH radical scavenging activity:

DPPH is a stable free radical, which can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to its odd electrons, the absorption is at 517 nm. DPPH radical reacts with suitable reducing agents and then electrons become paired off and the solution loses colour stoichiometrically with the number of electrons taken up. *A. mellea* recorded good antioxidant activity with water extract (47.5% ± 0.6), followed by ethyl acetate extract (46.35% ± 13.09), methanol extract (36.72% ± 1.27), hexane extract (25.32% ± 0.93), acetone extract (15.89% ± 1.32) and the minimum in chloroform extract (12.92% ± 1.67). *A. mellea* shown good activity (52 ± 3.5) with hot water extract at concentration 3 mg/mL (Mikhiel, 2007). EPS from *A. mellea*, extracted with hot water recorded strong DPPH radical scavenging activity (81.43%) at the concentration 20mg/mL (Lung and Chang, 2011a). *O. olearius* recorded high antioxidant activity with Ethyl acetate extract (51.88% ± 0.77), followed by methanol extract (41.91% ± 0.43), chloroform extract (26.73% ± 0.52), Acetone extract (19.52% ± 0.95), water extract (8.01% ± 2.36) and the minimum in hexane extract (6.31% ± 0.97). Ethanol extracts of *O. olearius* showed the strongest radical scavenging effect (60.25%) at 1 mg/ml. Mikhiel, 2007 reported scavenging activity (59 ± 6.5%) with hot water extract at 1.5mg/1mL. Ascorbic acid has scavenging effect of 92.1% at 1 mg/ mL. The scavenging DPPH radical ability (83.2%) of the methanol extract at 10 mg/mL from *A. mellea* submerged cultures is comparable to those of *T. albuminosus* and *G. frondosa* mycelia but less effective than that of *M. esculenta* mycelia (Mau *et al*., 2004). Hot water extracts from mycelia of *A. mellea* submerged cultures are stronger in scavenging DPPH radical capacity (62.7%) than *P. igniarius*, but weaker than *H. marmoreus*. These results suggest that extracts from *A. mellea* submerged cultures reveal a considerably high scavenging ability on DPPH radicals (Lung and Chang, 2011b). Future our work focused on to examine the bioluminescent characteristic and medicinal properties of this mushroom and apply into gene biotechnology and environment technology.

4. Conclusion

The present study recorded encouraging results towards anti bacterial and antioxidant activities. Further our research focused on continuous production and purification of the specific compounds for microbial bioproducts development.

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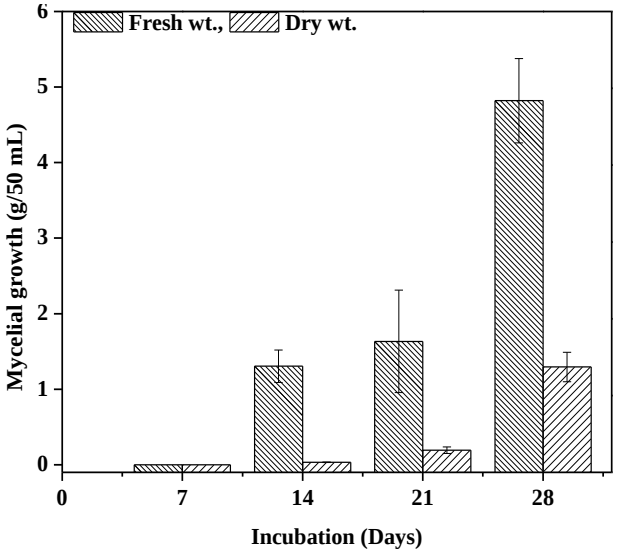


Fig. 1. Growth of MTCC 409 in Malt Extract Broth

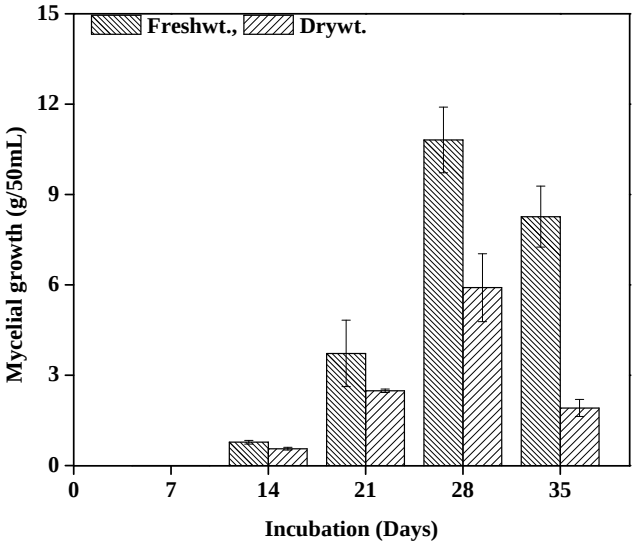


Fig. 2. Growth of MTCC 409 in Potato Dextrose Broth

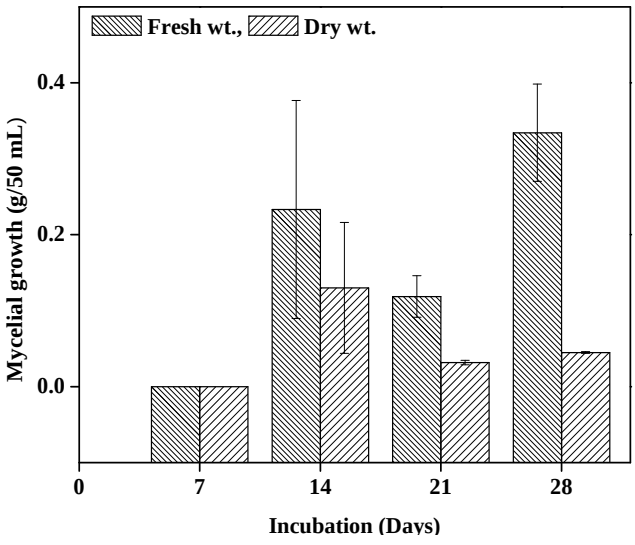


Fig. 3. Growth of MTCC 2790 in Malt Extract Broth

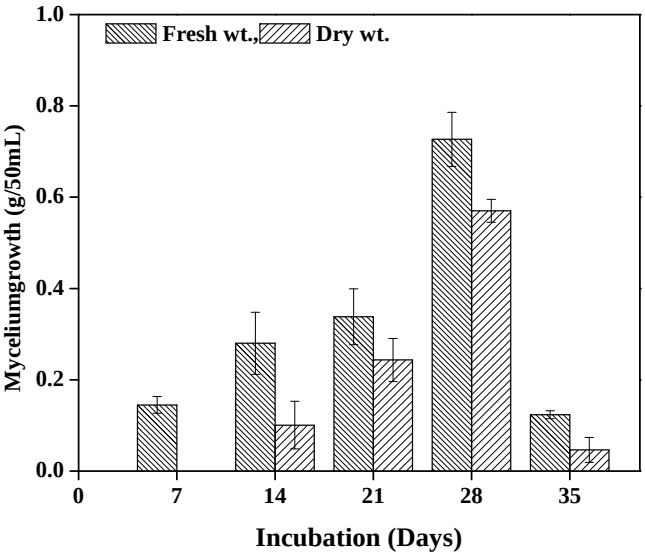
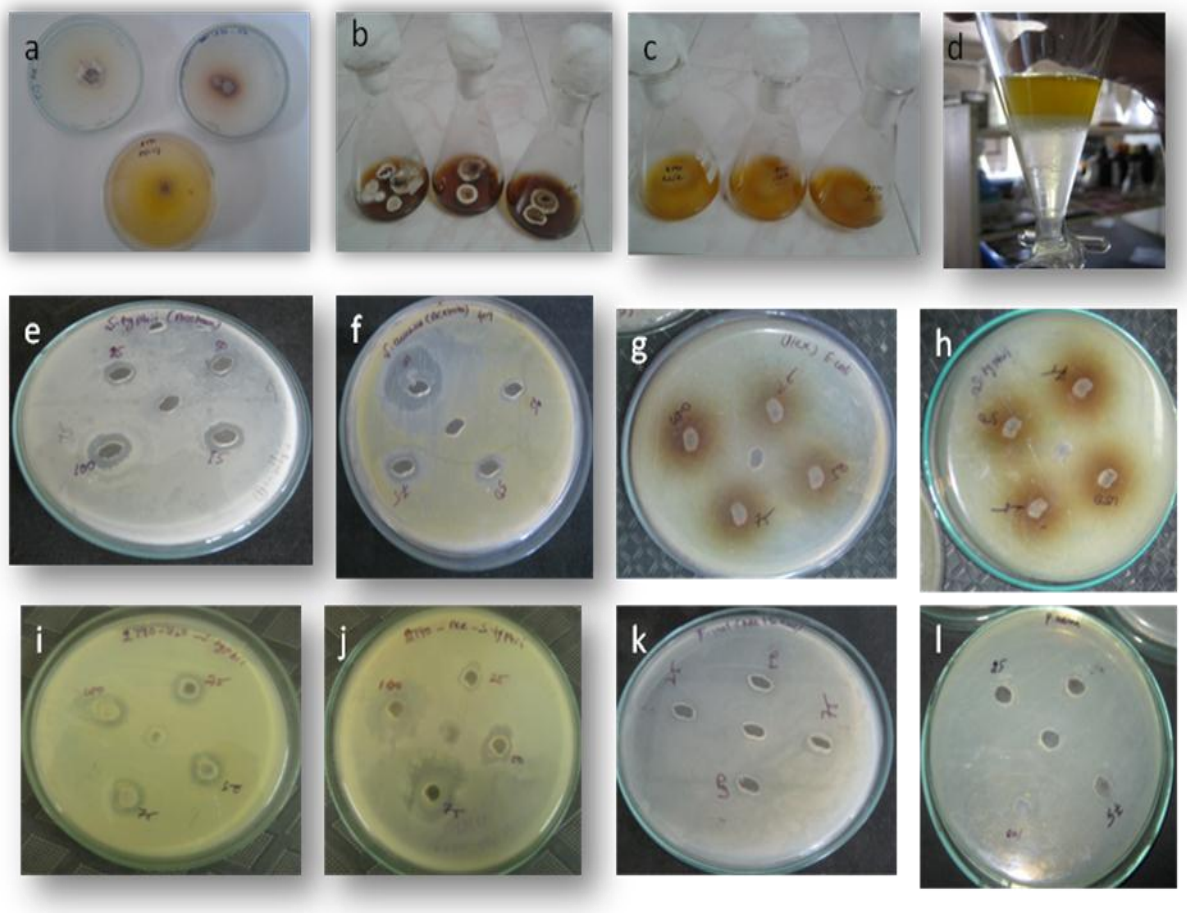


Fig. 4. Growth of MTCC 2790 in Potato Dextrose Broth

Plate 1. Production of bioactive fluorescence compounds and testing its activity



a - Pure cultures of *Armillaria mellea* (MTCC 409) and *Omphalotus olearius* (MTCC 2790) on PDB. b. Growth of *Armillaria mellea* (MTCC 409) on malt media. c- Growth of *Omphalotus olearius* (MTCC 2790) on malt media, d- Separation of Bioactive compounds from *Omphalotus olearius* (MTCC 2790) using chloroform, e, f- (MTCC 409) Zone of inhibition against *s. typhi* and *s. aureus.* g, h- (MTCC 409) No zone of inhibition. i, j- (MTCC 2790)Zone of inhibition against *s. typhi.* k, l - No zone of inhibition for (MTCC 2790)

Table 1. Anti bacterial activity of bioactive compounds extracted from *Armillaria mellea* (MTCC 409)

Zone of inhibition (cm) of methanol extract of *Armillaria mellea* (MTCC 409)				
Organisms	**25 µl**	**50 µl**	**75 µl**	**100 µl**
Escherichia coli	0 ± 0	0.0966 ± 0.003	0.2 ± 0	0.3966 ± 0.0033
Staphylococcus aureus	0 ± 0	**0.2 ± 0.0**	**0.3 ± 0.0**	**0.3 ± 0.003**
Salmonella typhi	0 ± 0	**0.096 ± 0.003**	**0.1966 ± 0.0033**	**0.2966 ± 0.0033**
Proteus vulgaris	0 ± 0	**0.1 ± 0.0**	**0.1966 ± 0.0033**	**0.3 ± 0.00**
Pseudomonas aeruginosa	0 ± 0	0.0966± 0.0033	0.2 ± 0	0.3 ± 0
Zone of inhibition (cm) of acetone extract of *Armillaria mellea* (MTCC 409)				
Escherichia coli	0 ± 0	0 ± 0	0.393 ±0.0066	0.0066 ± 0.0033
Staphylococcus aureus	0 ± 0	0.1 ± 0	0.19 ± 0.0057	0.0057 ± 0.0033
Salmonella typhi	0.1 ± 0	0.196 ± 0.003	0.3 ± 0	0 ± 0.0066
Proteus vulgaris	0.1 ± 0	0.196 ± 0.003	0.29 ± 0.0057	0.0057 ± 0
Pseudomonas aeruginosa	0 ± 0	0 ± 0	0.4 ± 0	0 ± 0.006

Key: No zone of inhibition in aqueous extract; Experiments are carried out in triplicates. Data represented here is mean ± standard error

Table 2. Antibacterial activity of bioactive/fluorescence compounds from *Omphalotus olearius* (MTCC 2790).

Zone of inhibition (cm) of aqueous extract				
	25 **µ**l	50 **µ**l	75 **µ**l	100 **µ**l
Escherichia coli	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Staphylococcus aureus	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Salmonella typhi	0.133 ± 0.033	0.2 ± 0	0.233 ± 0.033	0.333 ± 0.033
Proteus vulgaris	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pseudomonas aeruginosa	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Methanol				
Escherichia coli	0 ± 0	0 ± 0	0.333 ± 0.088	0.566 ± 0.176
Staphylococcus aureus	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Salmonella typhi	0 ± 0	0.1 ± 0	0.233 ± 0.033	0.366 ± 0.033
Proteus vulgaris	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pseudomonas aeruginosa	0 ± 0	0.1 ± 0	0.2 ± 0	0.3 ± 0
Acetone				
Escherichia coli	0.1 ± 0	0.2 ± 0	0.266 ± 0.033	0.466± 0.066
Staphylococcus aureus	0.1 ± 0	0.233 ± 0.033	0.366 ± 0.033	0.6 ± 0.057
Salmonella typhi	0.1 ± 0	0.2 ± 0	0.333 ± 0.033	0.5 ± 0.0577
Proteus vulgaris	0 ± 0	0.166 ± 0.066	0.266 ± 0.120	0.666 ± 0.176
Pseudomonas aeruginosa	0.1 ± 0	0.266 ± 0.033	0.266 ± 0.033	1 ± 0

Key: Experiments are carried out in triplicates. Data represented here is mean ± standard error