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Polycyclic Aromatic Hydrocarbons (PAHs) Metabolism by White Rot Fungi *Agaricomycetes sp* AGAT and Its Microcosm Study

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Abstract Hydrocarbon pollution is a perennial problem not only in India but throughout the globe. A plethora of microorganisms have been reported to be efficient degraders of these recalcitrant pollutants. In the present study the Basidiomycetous a fungal isolate *Agaricomycetes sp.* AGAT was isolated from wood bark collected from Anand, Gujarat India. The isolate was screened for production of ligninolytic enzyme by primary and secondary screening. The isolate *Agaricomycetes sp.* AGAT is also able to degrade the polycyclic aromatic hydrocarbons (PAHs) present in the contaminated soil. The basidiomycetous fungal *Agaricomycetes sp.* AGAT was able to grow on 100 ppm Pyrene, Phenanthrene, Fluorene and Fluoranthene as a sole source of carbon in minimal medium. Maximum degradation of 78.53%, 85.05%, 20.5% and 70.49% of Phenanthrene, Pyrene, Fluoranthene and Fluorene respectively. The depletion in the residual PAHs in the culture medium was determined by HPLC. By GC-MS analysis metabolites were identified as 1-hydroxypyrene and phthalic acid from Pyrene, 9H- Fluoren-9-ol from Fluorene, Fluorene, 4-[1,2-dihydroxethyl] from Fluoranthene and 9,10-dihydro-9,10-dihydroxyphenanthrene from Phenanthrene. Further effect of surfactants, mediator were also studied. Phytotoxicity study of degraded metabolite was also studied.

Keywords: *Agaricomycetes sp. AGAT, polycyclic aromatic hydrocarbons (PAHs), phytotoxicity*

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1. Introduction

Basidiomycetes group are regarded as most interesting group of fungi with their ability to grow under detrimental environment condition constantly acting as natural degraders of lignocellulose [1]. They are further classified into brown rot fungi and white rot fungi according to the way they degrade wood. Brown rot fungi have ability to degrade wood polysaccharides while white rot fungi have ability to decompose all wood polymers including lignin. White rot fungi are known for actively degrading lignin because of presence of extracellular enzyme complex containing lignin peroxidase (LiP, E.C. 1.11.1.14), manganese peroxidase (MnP, E.C. 1.11.1.13) and laccase (Lac, E.C.1.10.3.2). These enzymes are involved in oxidation of lignin present in wood and in oxidation of xenobiotic compounds including synthetic dyes and polycyclic aromatic hydrocarbons (PAHs).

Contamination of environment by aromatic compounds has become one of the global problem. Polycyclic aromatic hydrocarbons (PAHs) contamination is serious and increasing problem for human and ecosystem because of spill in soil, water and other natural resources. Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compound having two or more fused benzene ring which have been identified as persistent organic pollutants and are of great concern due to the characteristics of toxicity, mutagenicity and carcinogenicity. The US Environmental Protection Agency (US EPA) has listed 16 PAHs as priority pollutants [2,3]. PAHs are formed due to incomplete pyrolysis of organic materials such as petroleum, coal and wood.

Various potential techniques are being developed now-a-days to overcome these problems. Microbiological process of degradation of organic pollutants is considered as a promising method for environmental problem. Use of microbial resources for remediation of PAH contaminant has been important 'green solution' [4,5,6]. Bioremediation is the technique using microbes (bacteria, fungi and algae) which degrade or transform and mineralize contaminants to carbon di oxide and water. Bioremediation is simple process that is economical, versatile, ecofriendly and efficient as compared to chemical technique that produce toxic intermediate and have lower bioavailability for contaminants. The extracellular ligninolytic enzymes secreted by white rot fungi oxidize PAHs via a non-specific, radical-based reaction with corresponding quinones [7]. Catalysis reaction by laccase depends on the monoelectronic oxidation transforming the substrate to reactive radicals with the help of mediator [8]. Use of various mediators (ABTS, HBT and Phenol) for enhancement of PAH degradation have being reported by [9,10,11]. PAHs bioavailability can be enhanced by

addition of surfactants which increase its surface tension and increase its solubilization through formation of micelles in solution. This phenomena is observed at its critical micellar condition (CMC) [12].

The main objective of the present work was to study degradation of Pyrene, Phenanthrene, Fluoranthene and Fluorene by white rot fungi *Agaricomycetes sp* AGAT. Attempts were made for enhancing the degradation of PAHs by addition of surfactants and mediators to the medium. The potential isolate *Agaricomycetes sp* AGAT was explored for its interaction with indigenous microflora present in the soil through microcosm study. Degraded metabolites were analyzed using HPLC and GC-MS analysis.

2. Materials and Method

2.1. Microorganisms and Culture Conditions

Agaricomycetes sp AGAT (Genbank accession no. KT153994) was grown on 2% Malt extract agar (MEA). MEA contained (g/l) Malt Extract 30.0, Peptone 5.0, and Agar 25.0 (pH: 5.4) at 30℃.

2.2. Chemicals

ABTS (2, 2-Azino-bis (3-ethylbenzthiozoline-6-sulphonic acid), Phenol and HBT (1-hydroxybenzotriazole) was purchased from Sigma Aldrich (Sigma St. Louis, MO, USA). Malt Extract Agar (MEA), glucose, sodium acetate, KH_2PO_4, H_3BO_3, $NaNoO_4$, Alk $(SO_4)_2$, yeast extract, sodium dedecyl sulphate (SDS), sodium sulphate, thiamine were purchased from Hi-Media Labs (Mumbai, India). Ammonium tartrate, copper (II) sulphate pentahydrate, manganese (II) sulphate monohydrate, magnesium sulphate heptahydrate, calcium chloride dihydrate, zinc sulfate were procured from S D Fine chemicals (Mumbai, India). Tween 80, Triton X-100, Ethyl acetate, Acetone, Acetonitrile, HCl and HPLC grade water were purchased from Merck (Mumbai, India).

Polycyclic aromatic hydrocarbon (PAHs) Pyrene, Phenanthrene, Fluorene and Fluoranthene were procured from Sigma Aldrich (Sigma St. Louis, MO, USA).

2.3. Enzyme Assays

Laccase (E.C.1.1.03.2) activity was determined by measuring the oxidation of 2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulphoric acid (ABTS) as increase in absorbance for 3 minutes was measured spectrophotometrically at 420nm ($\varepsilon = 36000 cm^{-1} M^{-1}$) [13]. The reaction mixture contained 100µl of 50 mM ABTS and 800 µl of 20 mM Sodium Acetate buffer (pH 4.5) and 100 µl of appropriately diluted enzyme extract. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 µM of corresponding substrate per min.

2.4. Isolation, Screening and Identification of Isolated Fungal Strain

Various mushroom samples, decayed wood and barks of trees were collected from the nearby regions of Vadodara (22°19'18.64" N 73°09'19.19" E) and Anand (22°33'08.87" N 72°55'38.68" E) Districts, Gujarat, India. Samples collected were screened for the presence of ligninolytic enzymes. Surface sterilization of collected samples was performed by suspending in 0.01 %(v/v) mercuric chloride solution for 2-3 minutes followed by washing. The samples were placed on 2% (w/v) malt extract agar (MEA) plates containing streptomycin (25µg/ml) and the plates were further incubated at 28℃ for 8-10 days. Isolated cultures were repeatedly sub cultured to obtain a pure culture. Microscopic studies were performed to study the characteristic of white rot fungi. Further samples were screened for the phenol oxidase activity. The presence of laccase enzyme (primary screening) was studied using various chromogenic substrates like ortho-dianisidine; guaiacol and gallic acid at a concentration of 0.01% (w/v) supplemented in 2% (w/v) malt extract agar plates. The actively grown culture (agar plugs) were placed on the plates and incubated at 28℃ for 10 days. Fungal isolate showing positive Bevandamm's reactions were further explored for the quantitative detection of ligninolytic enzymes. Cultures were maintained on MEA plates at 30℃ and stored at 4℃.

Quantitative detection (secondary screening) of ligninolytic enzyme was performed using 5.0 gm of wheat bran as a solid substrate with Asther's medium as a moistening agent under solid state fermentation.

The molecular identification of the new isolate AGAT was done by using ITS4 (TCCTCCGCTTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) gene sequencing by Agarkhar Research Institute (ARI) Pune, India.

2.5. PAHs Degradation

Degradation studies were conducted in 250ml Erlenmeyer flasks containing 100 ml of degradation medium prepared according to Tien & Kirk [14] containing (g/l) glucose 10, ammonium Tartrate 0.2, sodium acetate 3.28, thiamine 2, KH_2PO_4 2; $MgSO_4.7\ H_2O$, 0.53; $CaCl_2$, 0.1; $CuSO_4$, 0.001, $MnSO_4$, 0.005; H_3BO_3, 0.001; $NaMoO_4.\ 2H_2O$, 0.0001; $ZnSO_4.\ 7H_2O$, 0.0001; $AlK\ (SO_4)_2.\ 12H_2O$, 0.0001; and yeast extract 0.5. The pH of the medium was adjusted to 5.0 with 2N HCl. Flasks were sterilized at 121℃ for 20 min. Thiamine was separately filter sterilized and added thereafter. All the flasks were inoculated with fungal agar disc of 9 mm diameter from the edge of the actively growing culture. All the flasks were incubated at 28℃ at 100 rpm for 6 days. Stock solution of PAHs were prepared in acetone, filter sterilized with help of 0.2 µm filter and added to 6 day old culture at a final concentration of 100 ppm. All the flasks were re-incubated at 28℃ at 100 rpm for 25 days.

2.5.1. Effect of Surfactants on PAHs Degradation

Surfactants plays an important role in degradation of PAHs in order to study the effect of surfactant the degradation medium was seeded with surfactants like SDS, Triton X-100 and Tween 80 at a concentration of 0.1,1 and 2mM. Control flask was without surfactant.

2.5.2. Effect of mediator on PAHs degradation

Mediator plays an important role in degradation of PAHs in order to study the effect of mediator the degradation medium was seeded with mediator like ABTS,

Phenol and HBT at a concentration of 0.1,1 and 2mM. Control flask was without mediator.

2.6. Extraction of Residual PAHs

The entire content of the flask (100ml) were extracted for determination of residual PAHs.

After addition of PAHs to six day old culture extraction was done on 5th, 10th, 15th, 20th and 25th day of incubation. The mycelia was filtered using Whatman filter paper No1. The liquid was extracted in double volume of ethyl acetate, kept at 30℃ at 150 rpm for 30 mins. The mixture was separated using separating funnel. The organic phase was collected and saved.

The extract obtained was dried using anhydrous sodium sulphate to remove aqueous phase.

2.7. Calculations for PAHs Degradation Rate

Degradation rate of PAHs was calculated by subtracting the amount of PAH extracted from the abiotic control from the other samples. The rate of degradation was calculated as:

PAH degradation rate (%) = Abiotic control extraction (100%) - Sample extraction

Sample extraction means filtrate obtained.

2.8. Microcosm Study

Microcosm preparation was planned in four different sets of soil model systems were prepared as:

- (Set A)-Sterile soil containing PAH which served as abiotic control
- (Set B)-Sterile soil supplemented with PAH (100ppm) was inoculated by *Agaricomycetes sp* AGAT to determine the PAH degradation efficiency by *Agaricomycetes sp* AGAT in absence of indigenous organisms
- (Set C)-Non sterile Soil with native or indigenous organisms and PAH, to evaluate the intrinsic ability of soil to degrade PAH
- (Set D)-Non sterile soil supplemented with PAH and inoculated with *Agaricomycetes sp* AGAT to check the degradation rate by *Agaricomycetes sp* AGAT in presence of indigenous flora.

2.9. Analytical Procedures

2.9.1. Determination of residual PAHs:

The residual PAHs were analyzed by reverse-phase HPLC (Shimadzu, Japan) equipped with Nuclosil 100-5 Column (C-18 PAH Column, 250mm X 4.6mm) series 200 pump. Detection was done using D2 detector set at 254 nm. Dried samples were re-extracted in 1 ml of mobile phase (Acetonitrile: Water, 70:30), filtered through 0.2µM filter and 20µl was injected to HPLC Column.

2.9.2. Detection of Metabolites

The residual metabolite was extracted from the sample with equal volume of ethyl acetate. Further 2ml of sample was transferred to GC vial for GC-MS analysis carried out by using Shimadzu Gas Chromatograph Mass Spectrophotometer GC-MS (Shimadzu). The total sample injected was 2µl.

2.9.3. Phytotoxicity Analysis

The method of testing of Phytotoxicity of PAHs and its metabolites was performed according to Kalme *et al.*, [15]. The test was carried out for standard and the extracted metabolites on the seeds of *Triticum aestivum*, common Indian agricultural crops. Ten seeds were supplied daily with 10ml of standard and extracted metabolites dissolved in water for 7-10 days in small pots, maintaining light and temperature (30℃) in a controlled environment. Toxicity effect was measured in terms of percent germination, lengths of root and shoot of the plant after 7-10 days.

2.9.4. Data Analysis

The data in subsequent sections represent arithmetic mean values of three experimental repetitions (each one was made in duplicate).

3. Results

3.1. Isolation and Primary Screening

Table 1. Response of different fungal isolates on MEA plates containing different chromogenic substrates

Strain No	Isolate code	Primary screening Chromogenic substrates (0.01%w/v) ODA	Guaiacol	Gallic Acid	Secondary screening Laccase (U/gm)
1	AGAT	++++	++++	++++	117653.8
2	AGAT - 1	+++	+++	+++	60166.6
3	AGAT - 2	ND	ND	ND	ND
4	AGAT - 3	+	+	+	10077
5	AGAT - 4	++	++	++	52250.7
6	AGAT - 5	ND	ND	ND	ND
7	AGAT - 6	ND	ND	ND	ND
8	AGAT - 7	ND	ND	ND	ND
9	AGAT - 8	+	+	+	5145.8
10	AGAT - 9	++	++	++	20250.6
11	AGAT - 10	ND	ND	ND	ND
12	AGAT - 11	ND	ND	ND	ND
13	AGAT - 12	++	++	++	28520
14	AGAT - 13	+	+	+	2053
15	AGAT - 14	ND	ND	ND	ND

++++ - Excellent producer, +++ - Good producer, ++ - Moderate producer, + - poor producer, ND- No detection of colored zone on MEA plates containing chromogenic substrates

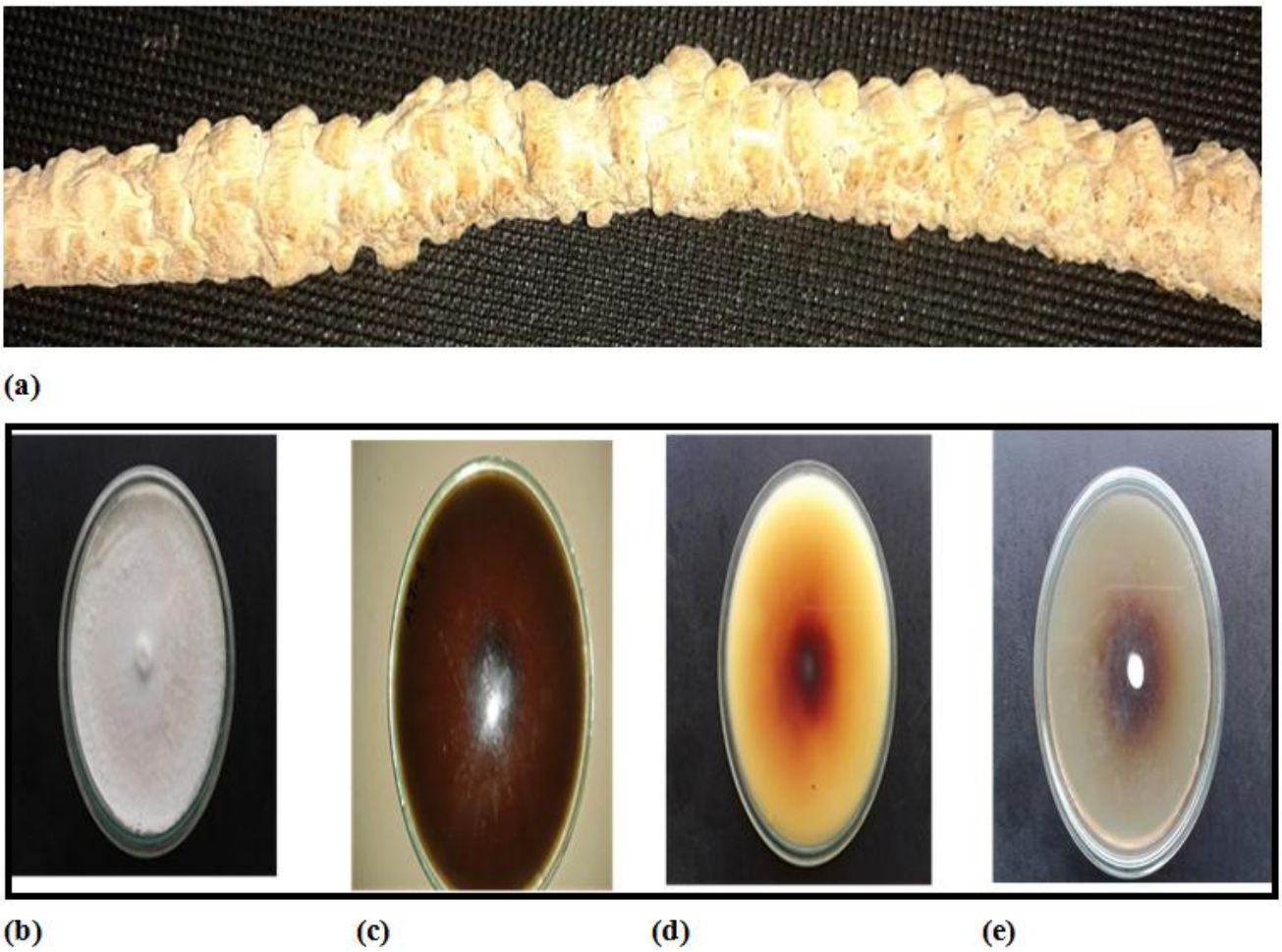

Figure 1. (a) Fruiting Body of AGAT. (b) Growth pattern of AGAT on 2%MEA, (c) - (e) Plate assay for Laccase on MEA plate containing different chromogenic substrates viz. orth-dianisidine, Guaiacol and Gallic acid

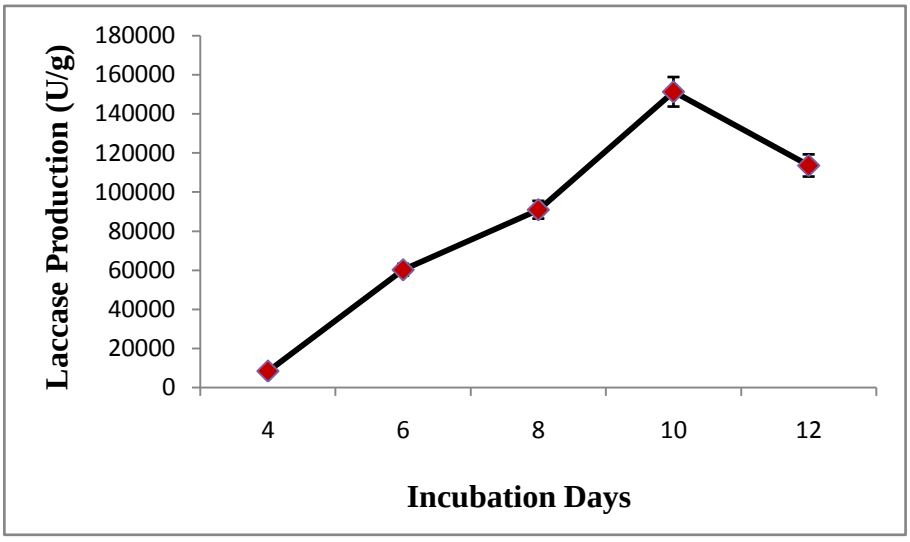

Figure 2. Secondary screening for laccase production

In the present study out of 15 isolates only 8 isolates were potential to produce ligninolytic enzyme by plate assay method. Dark brown zone surrounding the growth of mycelial disc on MEA plate containing o-dianisidine and gallic acid. Reddish brown colour zone was observed with plate containing guaiacol at concentration of 0.01% w/v (Figure 1). This is a characteristic of phenol oxidase which indicates of positive bevandamm's reaction for laccase activity. Further the isolate were classified as poor, moderate, good and excellent producer of laccase (Table 1).

Secondary screening of laccase was done using wheat bran as solid substrate and Asther's medium as moistening agent under solid state fermentation (SSF) (Figure 2). Maximum production of 1.65 X 10^5 U/g of dry substrate was obtained on 10^{th} day of incubation for wheat bran. Hence, on the basis of primary and secondary screening isolate AGAT was selected and explored for PAHs degradation.

3.2. Identification of the Fungal Isolate

3.2.1. Macroscopic Characteristics

Isolate AGAT fruiting body was found to be grown on bark of wood of mulberry tree with fleshy white mat like growth. Strain grew well on MEA covering the entire plate in 8-10 days incubated at 28℃.

3.2.2. Microscopic Characteristics

Microscopic examination of fruiting body revealed the presence of Basidiospores and gleopleros hyphae and binding hyphae as shown in Fig 3.sp Spore observed were size of around 12.56 X 7.75 µm. The molecular identification of the new isolate AGAT was confirmed by isolation of genomic DNA followed by amplification of ITS region of rDNA using universal fungal primers ITS4 and ITS5 (Agharkar Research Institute, Pune). Similarity of the DNA sequence in Genbank Database was found to be same with *Agaricomycetes sp.* with 99% similarity. The isolate was identified as *Agaricomycetes sp.* AGAT with Genbank Accession No KT153994 (Figure 4).

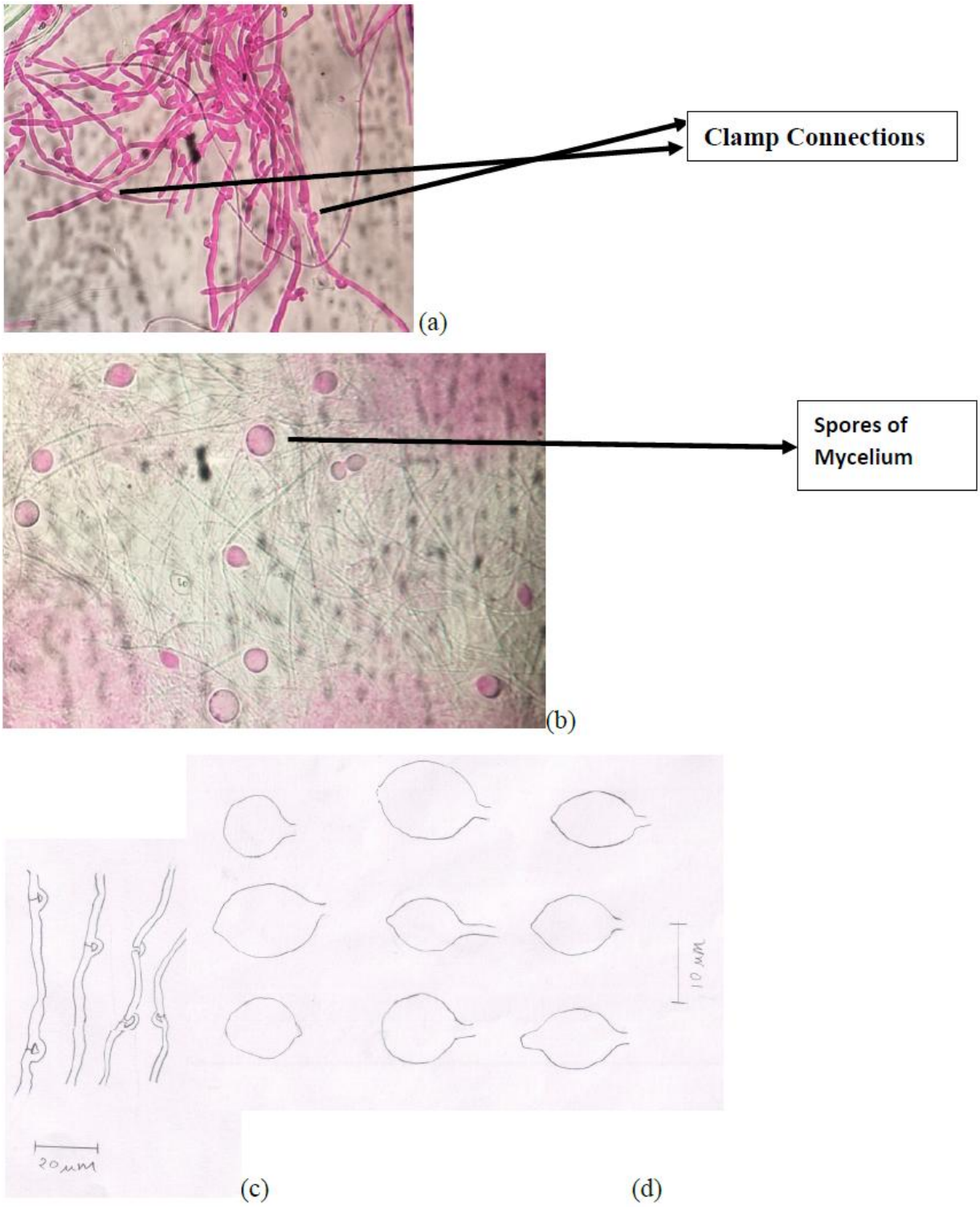

Figure 3. (a) Clamp connection observed in mycelium (b) Spores of mycelium (c) generative hyphae with clamp connection (d) Spores of mycelium

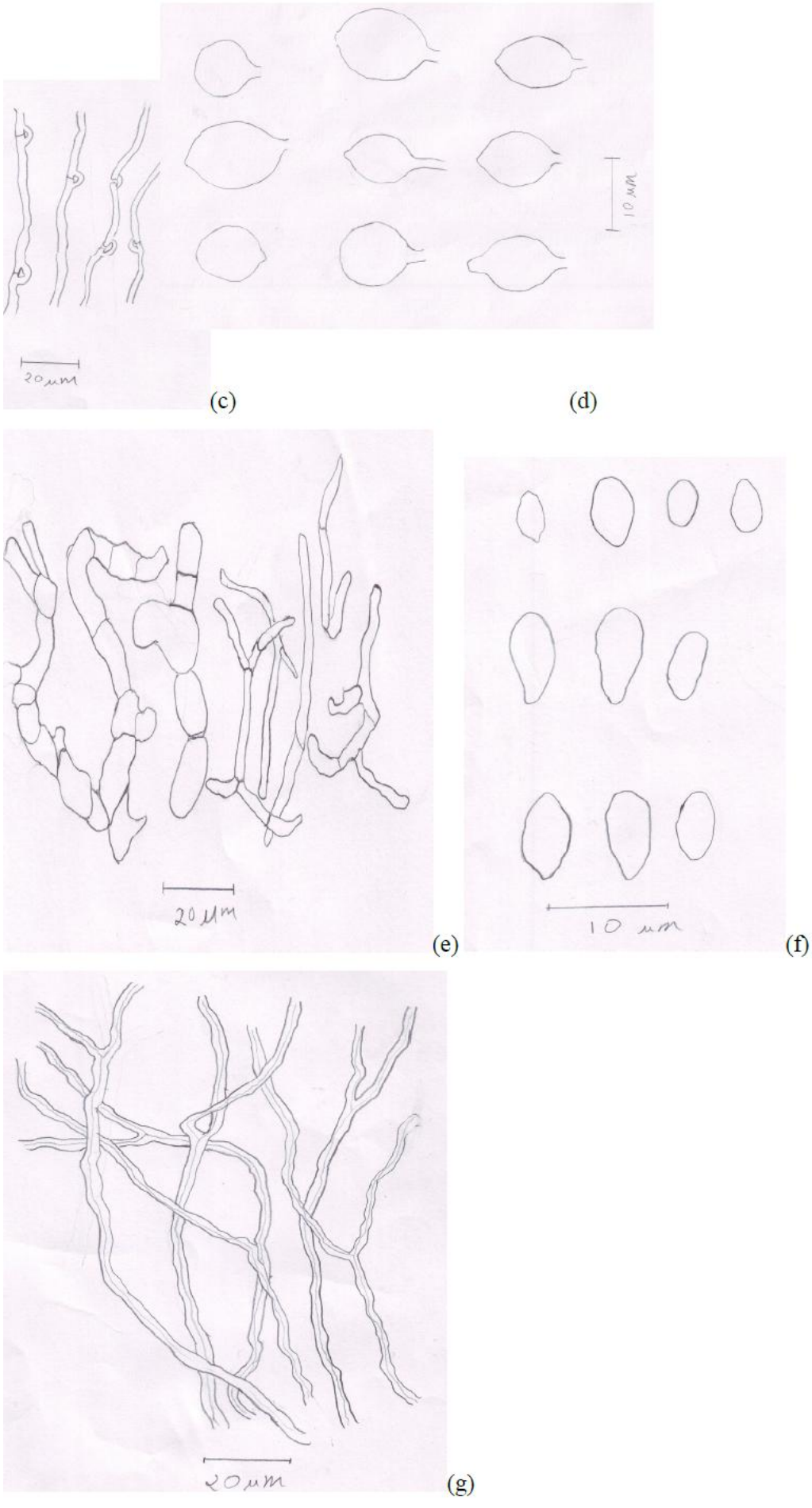

Figure 3. (e) Gloeopleros hyphae (f) Basidiospores observed in fruiting body (g) Binding hyphae.

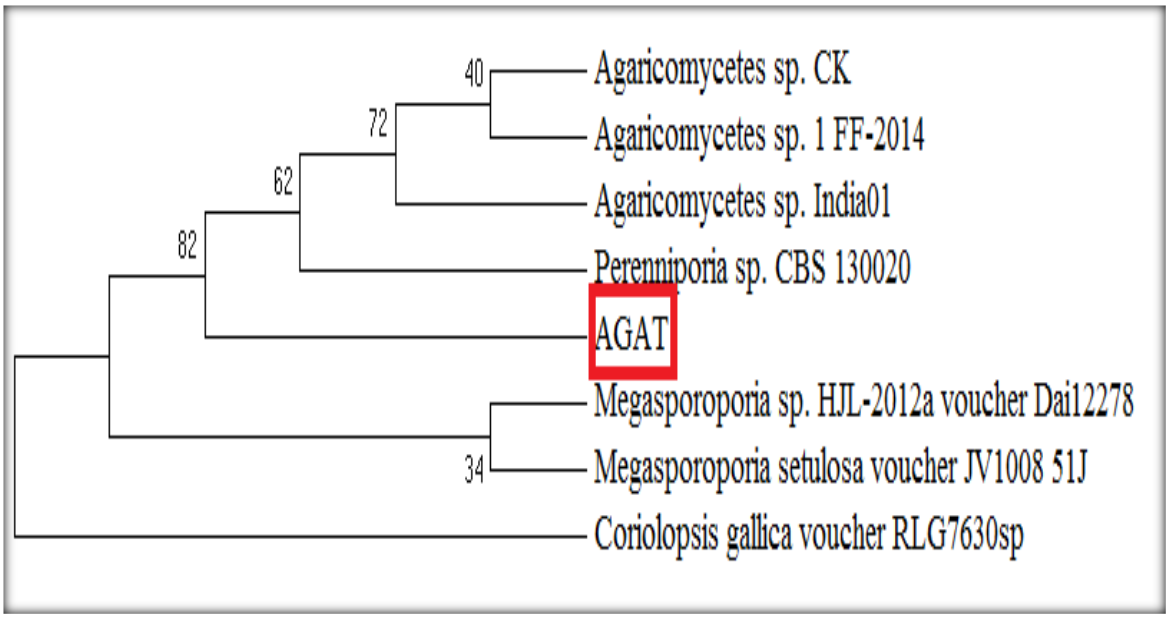

Figure 4. The bootstrapped unrooted tree of AGAT by NJ analysis

3.3. Growth of Fungal Culture *Agaricomycetes sp* AGAT on PAHs

The isolate *Agaricomycetes sp.* AGAT was grown on MEA plate containing 100 ppm of each PAH (Benzo (a) Anthracene, Fluorene, Pyrene, Acenaphthene, Anthracene and Phenanthrene).the isolate *Agaricomycetes sp.* AGAT was able to grow and utilize PAH as a sole source of carbon and energy as shown in Figure 5. Table 2 shows the growth in terms of zone of proliferation of isolate *Agaricomycetes sp.* AGAT on 8th day of incubation.

3.4. Degradation of PAHs by *Agaricomycetes sp* AGAT

The isolate *Agaricomycetes sp.* AGAT was grown in degradation medium (Minimal Salt medium) by spiking with different PAHs as sole source of carbon and energy. Degradation study using LMW PAH like Phenanthrene and Fluorene while HMW PAH like Pyrene and Fluoranthene was done. *Agaricomycetes sp.* AGAT was able to degrade 30.5% Phenanthrene in 20 days with 1250 U/ml of enzyme activity. Degradation of PAHs was dome more efficiently in presence of co-substrate such as glucose (Figure 6).

Table 2. Growth of Fungal Culture on MEA Plate with different PAHs (100 ppm) on 8th day of incubation period

PAHs	Diameter (cms)
Malt Extract (Control)	9.0
Pyrene	8.25
Anthracene	8.5
Fluorene	7.25
Fluoranthene	9.0
Phenanthrene	3.9
Acenaphthene	4.0

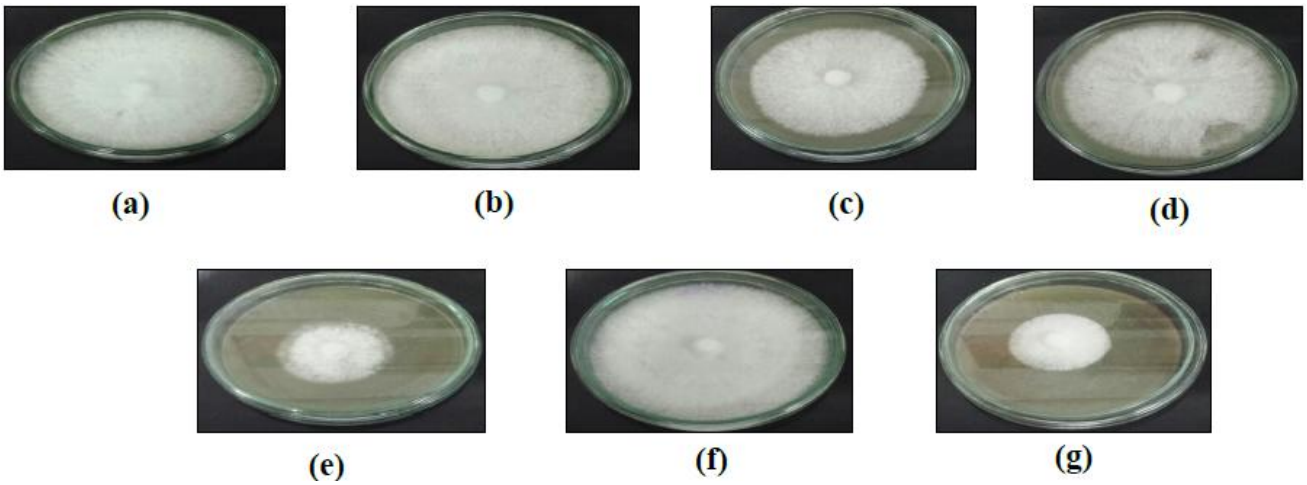

Figure 5. Fungal culture grown on different PAH used as sole source of carbon: (a). Control (b) Benzo(a) Fluoranthene (c) Fluorene (d) Pyrene (e) Acenaphthene(f) Anthracene (g) Phenanthrene

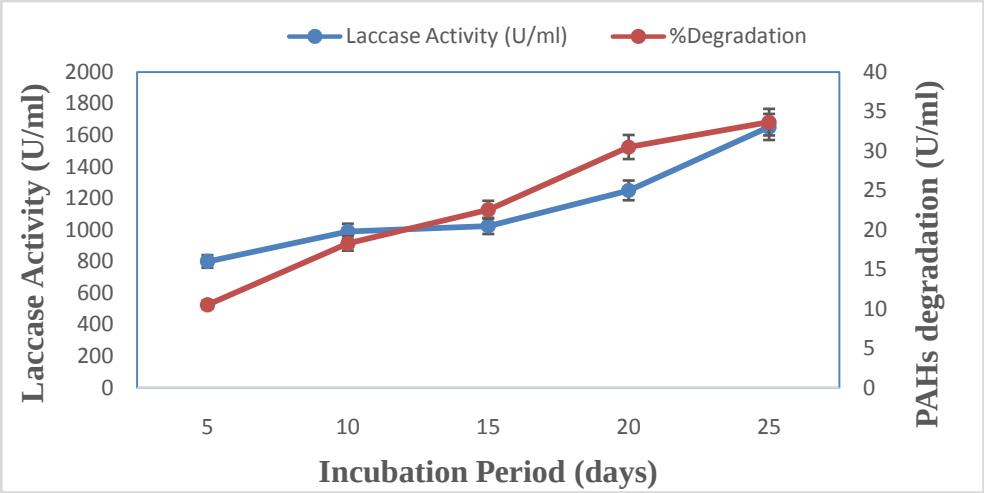

Figure 6. Phenanthrene degradation by *Agaricomycetes sp* AGAT as a sole source of carbon and energy

With addition of glucose as co-substrate in degradation medium the degradation of Phenanthrene increased to 78.53% with 3394.16U/ml on 20th day of incubation. Amongst the various co-substrates glucose was found to be best. Therefore, glucose was used in our further studies for the degradation of PAHs (Figure 7).

The degradation profile of various PAHs were studied with respect to laccase activity, incubation period and % degradation. In the present study 100 ppm of different PAHs were used for degradation study by fungal culture *Agaricomycetes sp* AGAT. Pyrene showed maximum degradation of 85.05% on 15th day of incubation with 2650.2 U/ml of enzyme activity and Fluoranthene reported 20.5% degradation on 25th day of incubation with 3580.2 U/ml enzyme activity (Figure 8 - Figure 11).

No further significant PAHs degradation was observed. The growth of fungus in liquid medium with PAHs can be observed by comparing it with control flask (Figure 12).

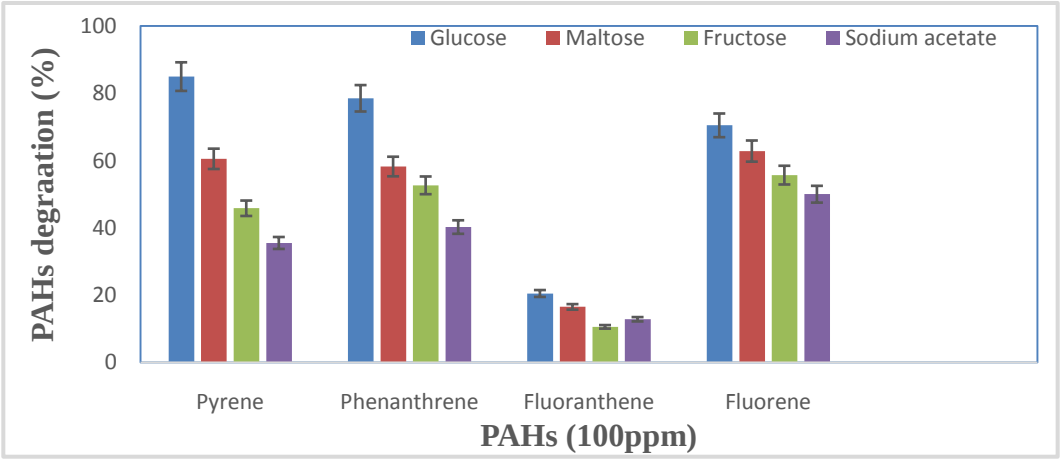

Figure 7. Different co-substrate used for PAH degradation

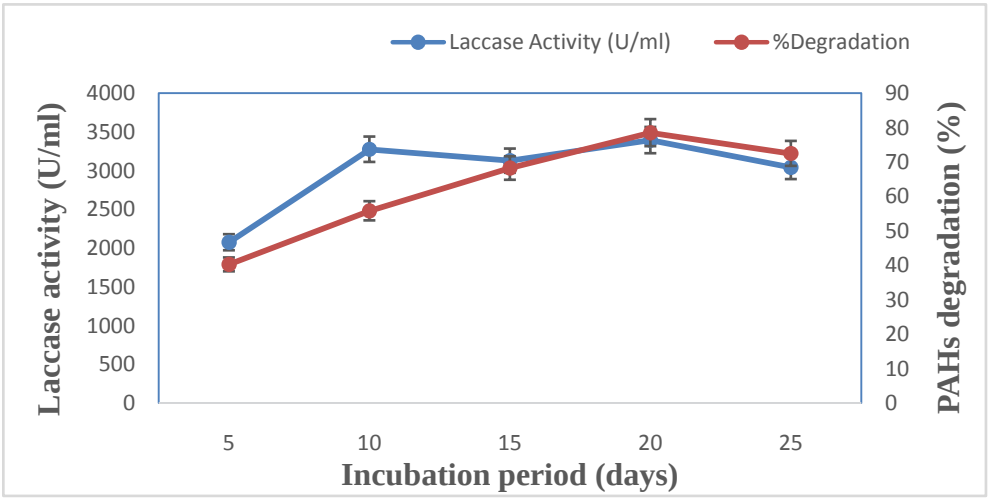

Figure 8. The co-metabolic degradation of Phenanthrene by *Agaricomycetes sp* AGAT

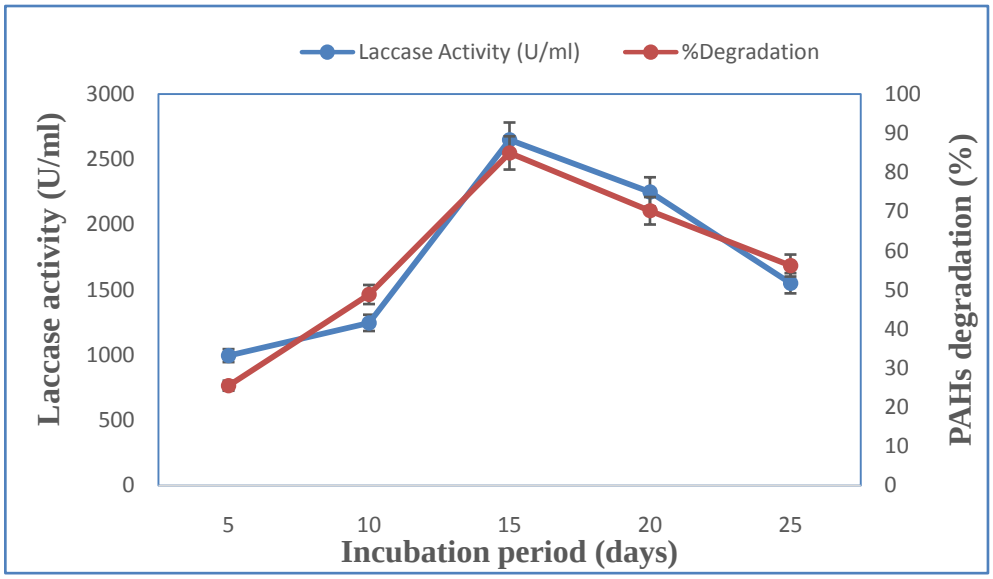

Figure 9. The Pyrene (100ppm) degradation profile by *Agaricomycetes sp* AGAT

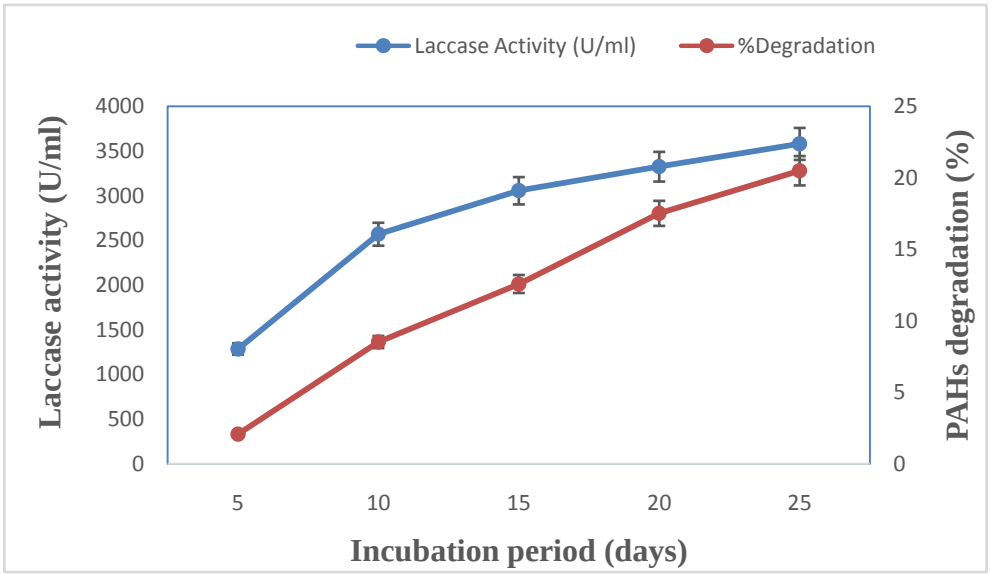

Figure 10. The Fluoranthene (100ppm) degradation profile by *Agaricomycetes sp* AGAT

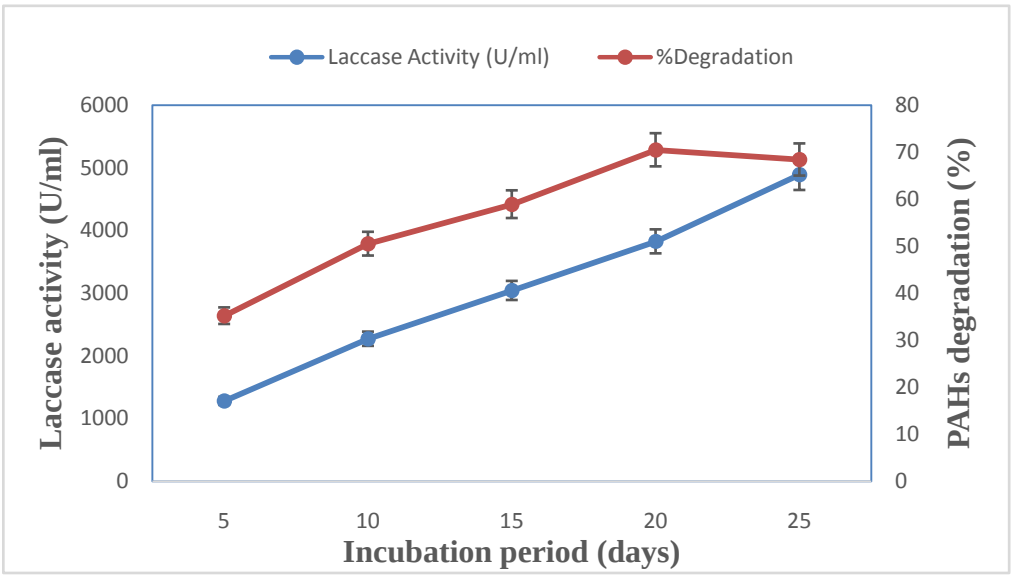

Figure 11. The Fluorene (100ppm) degradation profile by *Agaricomycetes sp* AGAT

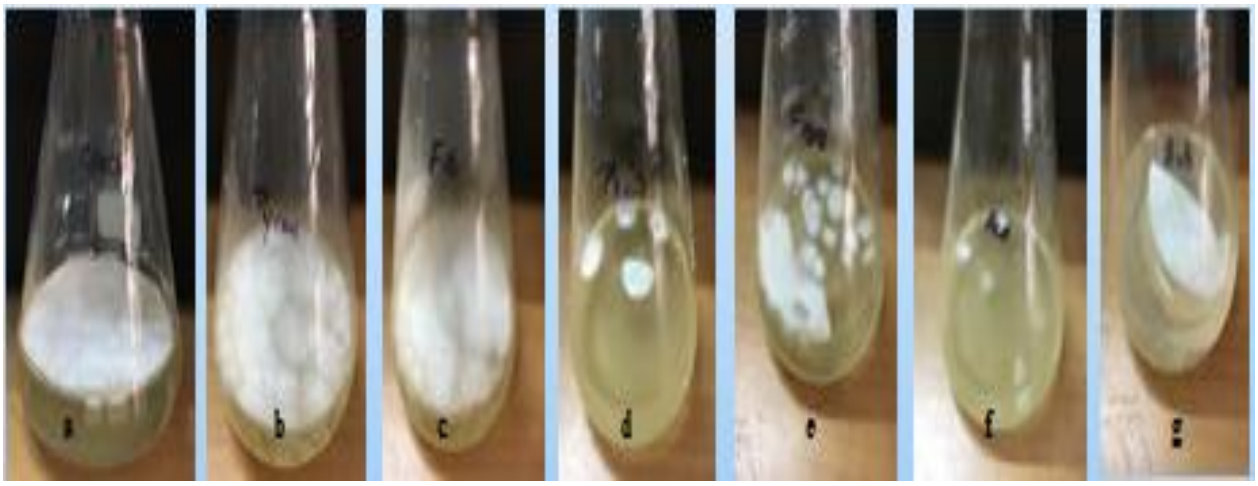

Figure 12. Growth of *Agaricomycetes sp.* on different PAHs. (100ppm): (a) Control (b) Pyrene (c) Fluoranthene (d) Phenanthrene (e) Fluorene (f) Acenaphthene (g) Anthracene

3.5. Effect of Different Surfactants on Degradation of PAHs

All the surfactants (SDS, Tween 80 and Triton X-100) were incorporated in medium. At concentration of 1mM were found to enhance the degradation of PAH as compared to control and other two concentration i.e. 0.1mM and 2 mM. When the surfactants used at different concentration then considerable difference in degradation was observed (Figure 13 - Figure 15).

Whereas Figure 13 shows a significant increase in degradation of Fluoranthene from 20.35% to 88.09%. Further increase in concentration of Tween 80 reduced the degradation as high concentration of surfactant may affect the growth of fungi in medium.

Similarly SDS was also found to be effective in increasing PAHs degradation as Tween 80. Maximum degradation of around 99% of Pyrene, Fluoranthene, Phenanthrene and Fluorene at concentration of 0.1mM, 1mM and 2mM was obtained.

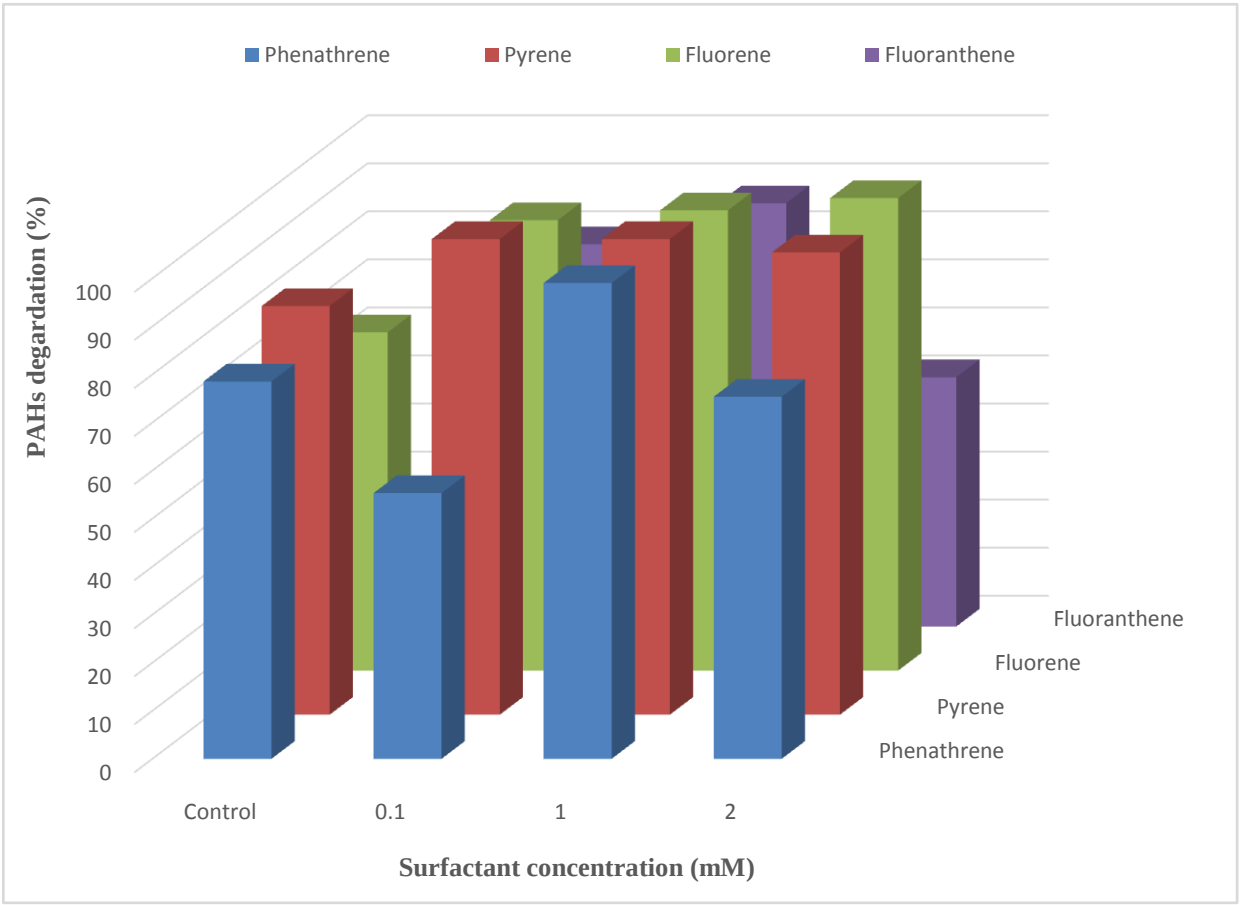

Figure 13. Degradation of PAHs in the presence of different concentrations of Tween 80 on 15th day of Pyrene, 20th day of Phenanthrene and Fluorene and 25th day of Fluoranthene

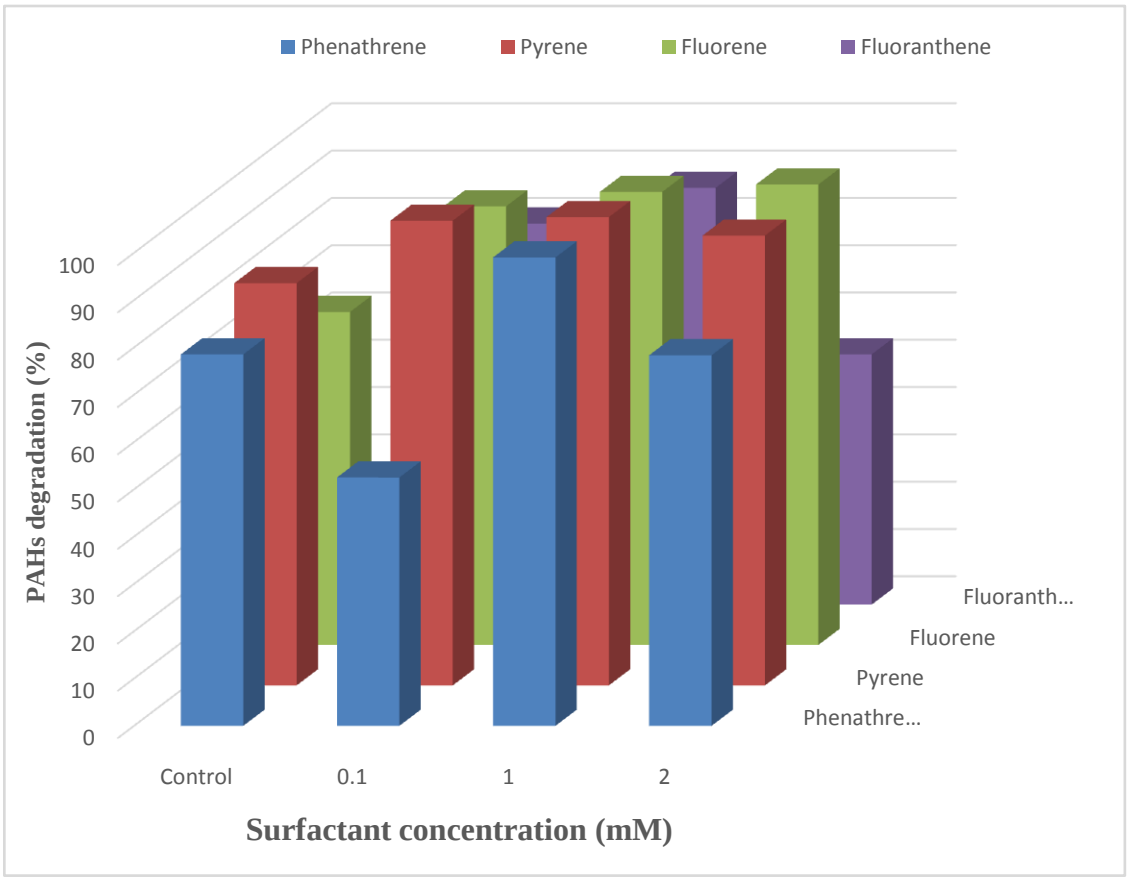

Figure 14. Degradation of PAHs in the presence of different concentrations of SDS on 15th day of Pyrene, 20th day of Phenanthrene and Fluorene and 25th day of Fluoranthene

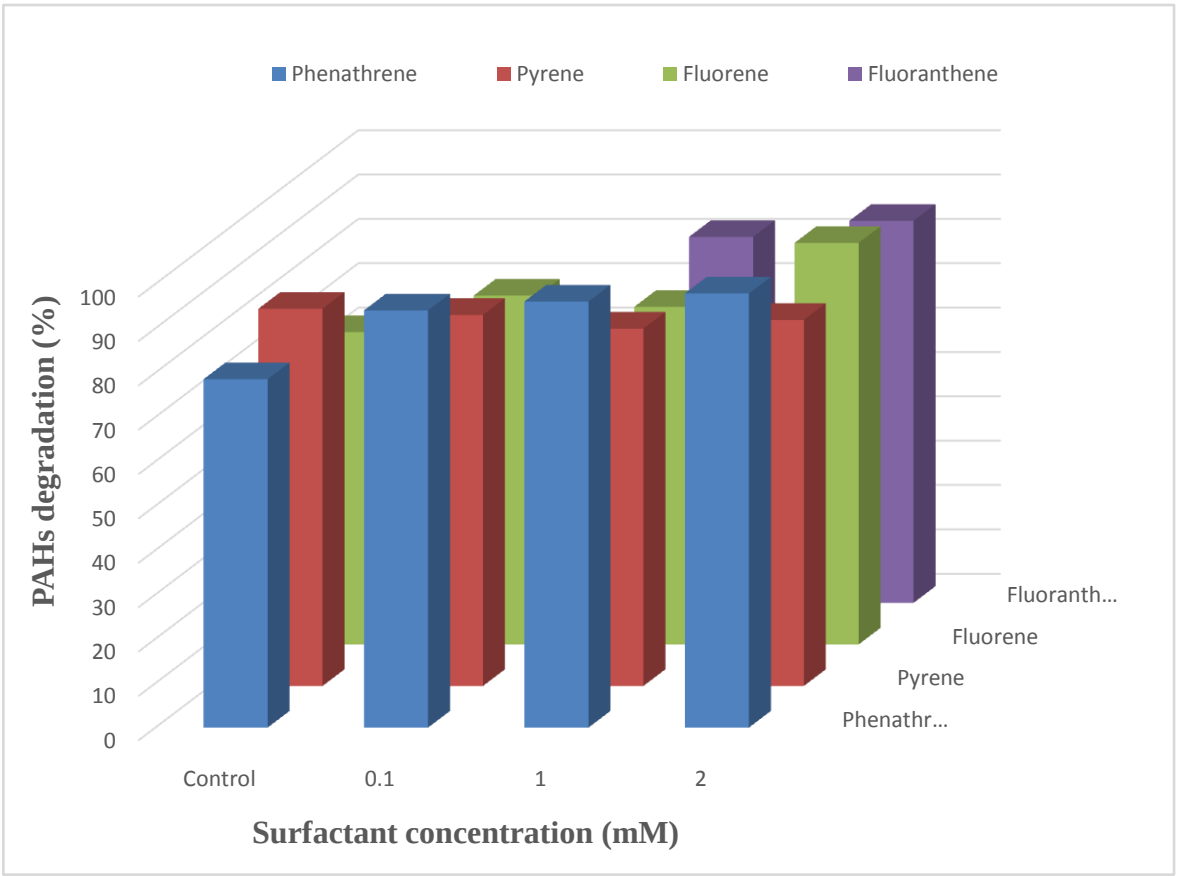

Figure 15. Degradation of PAHs in the presence of different concentrations of Triton X-100 on 15th day of Pyrene, 20th day of Phenanthrene and Fluorene and 25th day of Fluoranthene.

3.6. Effect of Different Mediator Compounds on Degradation of PAHs

In the present study 3 mediator used were ABTS, HBT and Phenol at concentration of 0.1mM, 1mM and 2mM. Amongst all HBT was found to be best and powerful mediator in PAH degradation. Around 99.1% of Pyrene removal was observed on 15th day of incubation. HBT showed 95.25%, 94.74% and 75.25% removal of Phenanthrene, Fluorene and Fluoranthene on 20th and 25th day of incubation respectively at 2mM concentration. Even at low concentration of 0.1mM HBT showed effective enhancement in PAH degradation (Fig 16).ABTS showed 98% elimination of Pyrene and Phenanthrene at concentration of 2mM. Around 90.21% degradation of Fluorene and 86.98% degradation of Fluoranthene were obtained at 2mM concentration of ABTS (Figure 17). Phenol also used mediator showed almost 90% removal of all PAHs in system at concentration of 2mM (Figure 18).

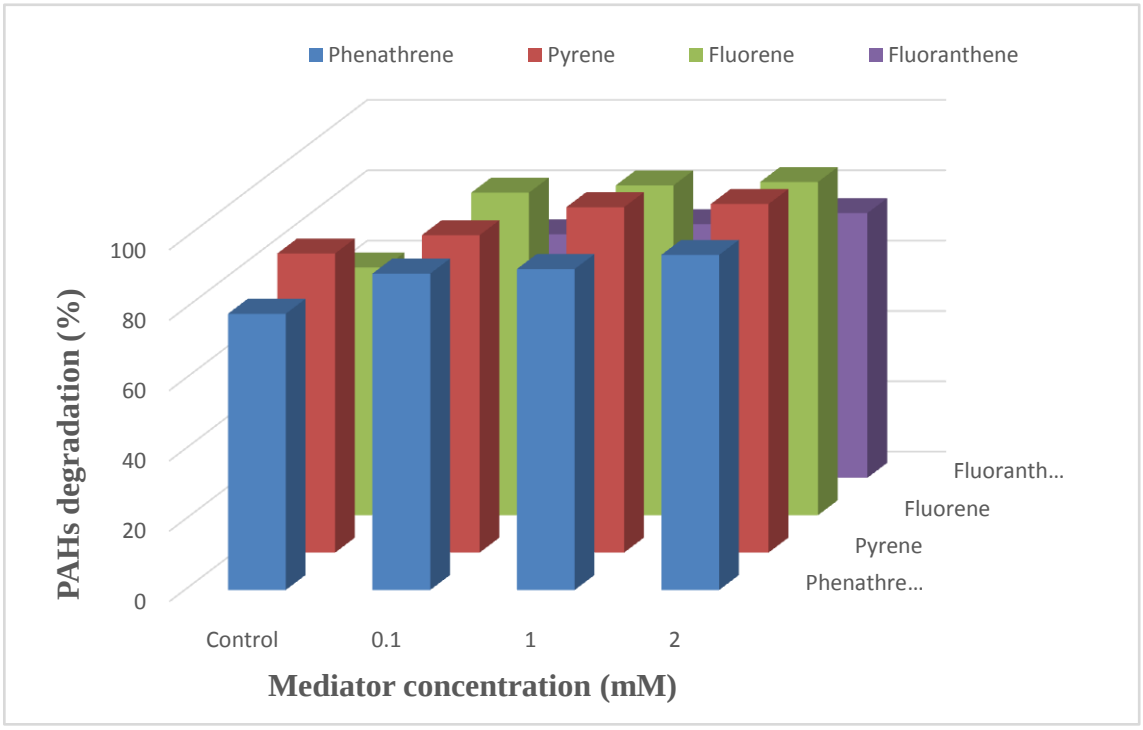

Figure 16. Effect of different concentrations of mediator compound HBT on degradation of PAHs

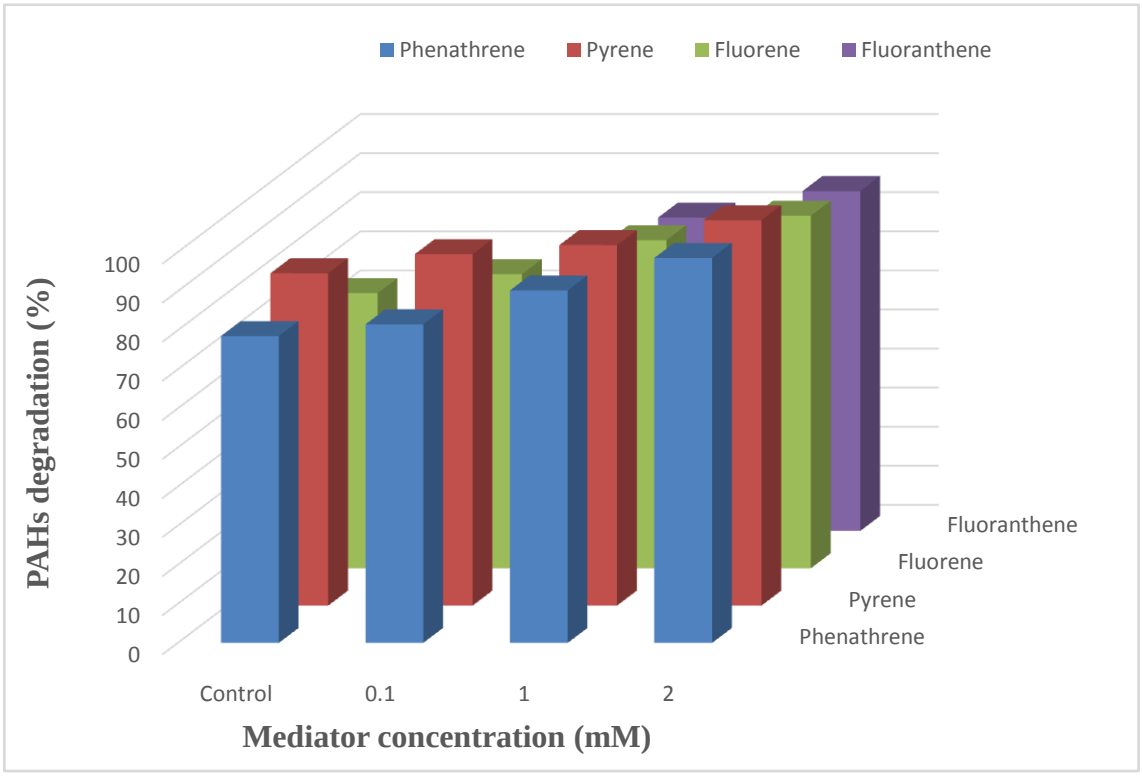

Figure 17. Effect of different concentrations of mediator compound ABTS on degradation of PAHs

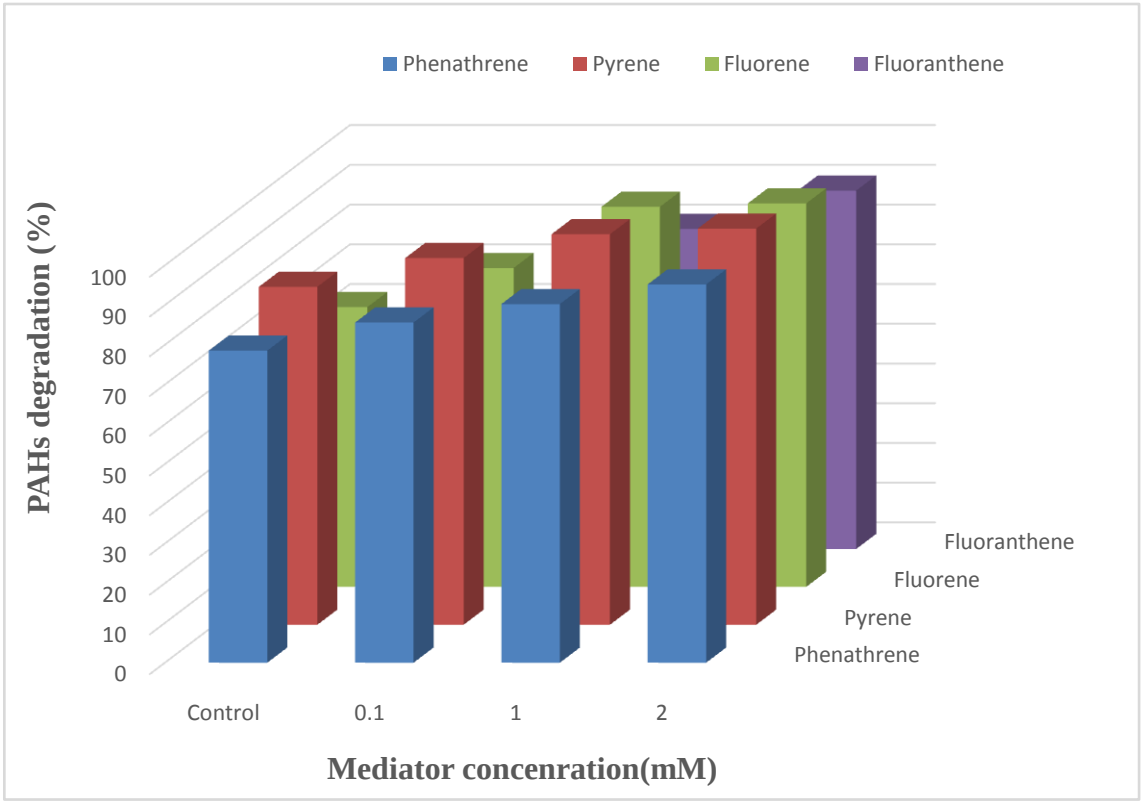

Figure 18. Effect of different concentrations of mediator compound Phenol on degradation of PAHs

3.7. Microcosm Study to Explore the Bioremediation Potential of *Agaricomycetes sp* AGAT

Microcosm study of PAHs such as Phenanthrene, Fluoranthene, Fluorene and Pyrene at concentration of 100 ppm using different sets of experiments were studied. The study shows the abiotic loss of Phenanthrene i.e. set A without microorganisms was about 25% i.e. control set (Set A). In set B 76.63% Phenanthrene degradation was observed by *Agaricomycetes sp* AGAT without the presence of indigenous flora. Presence of microbes in soil showed 45.50% (set c) degradation while set D showed maximum degradation of 96.66% with presence of both micro-flora and isolate AGAT (Figure 19). Present study showed that the maximum degradation in presence of Fluorene (93.88%) was achieved in set B i.e. having only presence of isolate *Agaricomycetes sp* AGAT as compared to set D which showed 46.20% degradation in presence of both indigenous flora along with isolate *Agaricomycetes sp* AGAT as shown in Figure 20. Pyrene and Fluoranthene also showed maximum degradation of 90.25% and 35.74% by *Agaricomycetes sp* AGAT present in sterile soil i.e. Set D. (Figure 21- Figure 22).

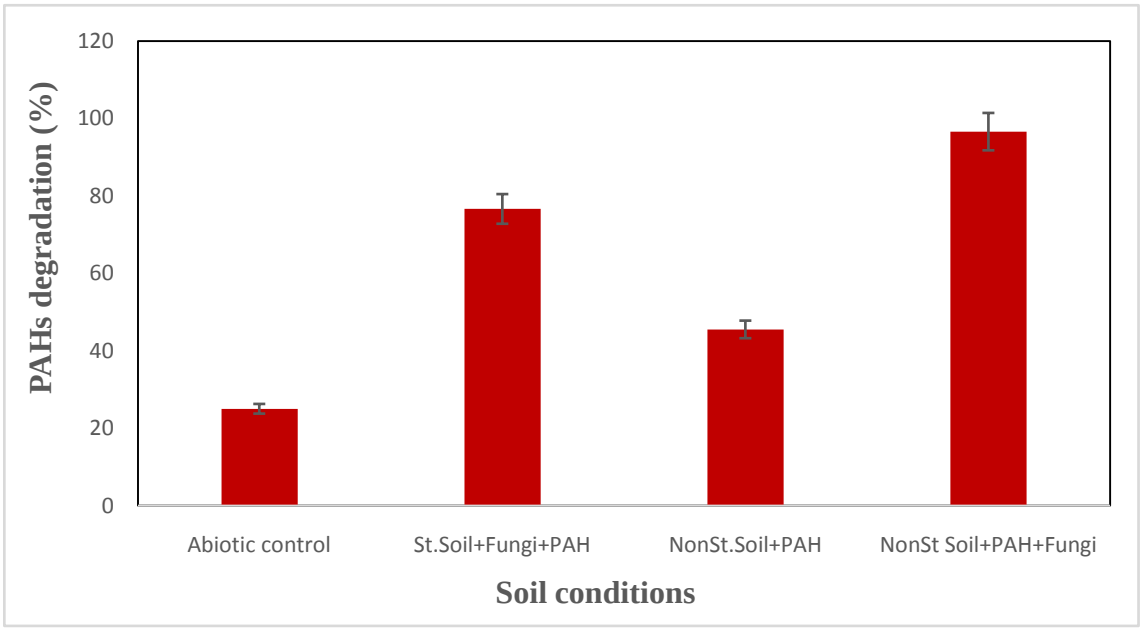

Figure 19. Microcosm study for Phenanthrene degradation

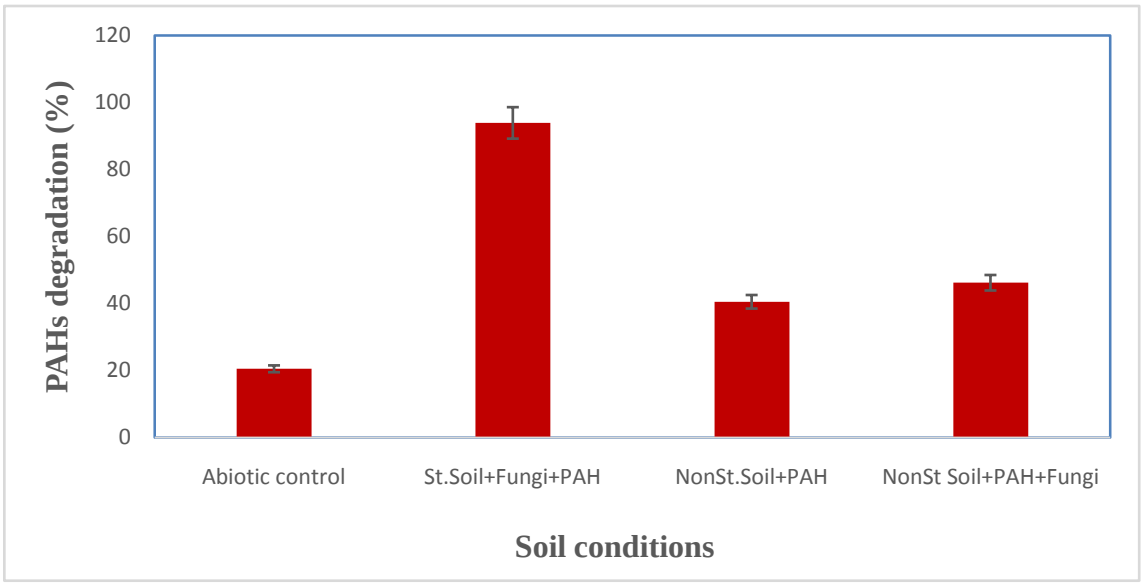

Figure 20. Microcosm study for Fluorene degradation

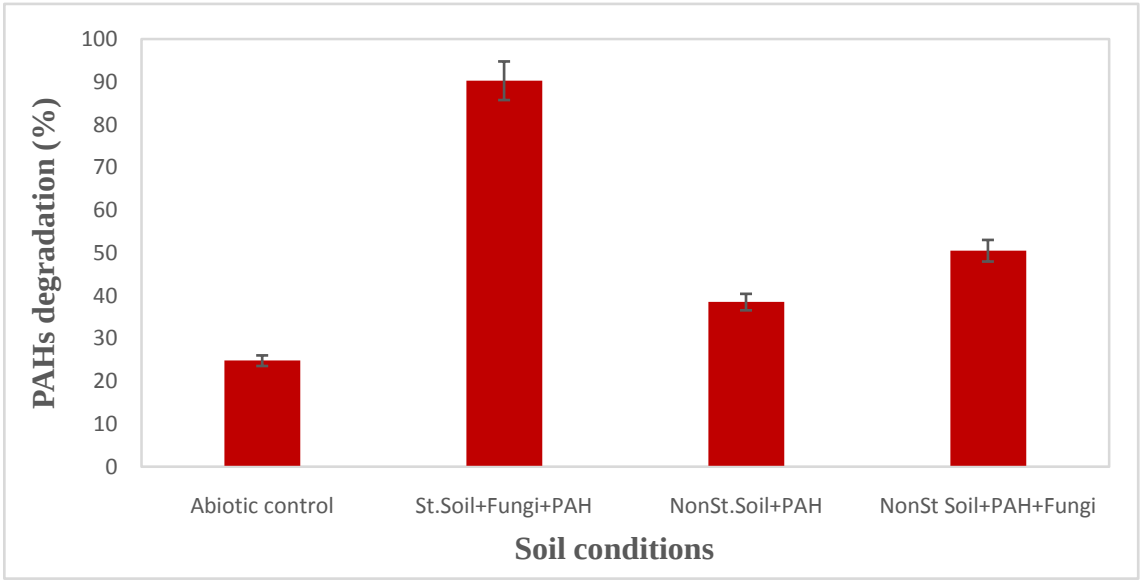

Figure 21. Microcosm study for Pyrene degradation

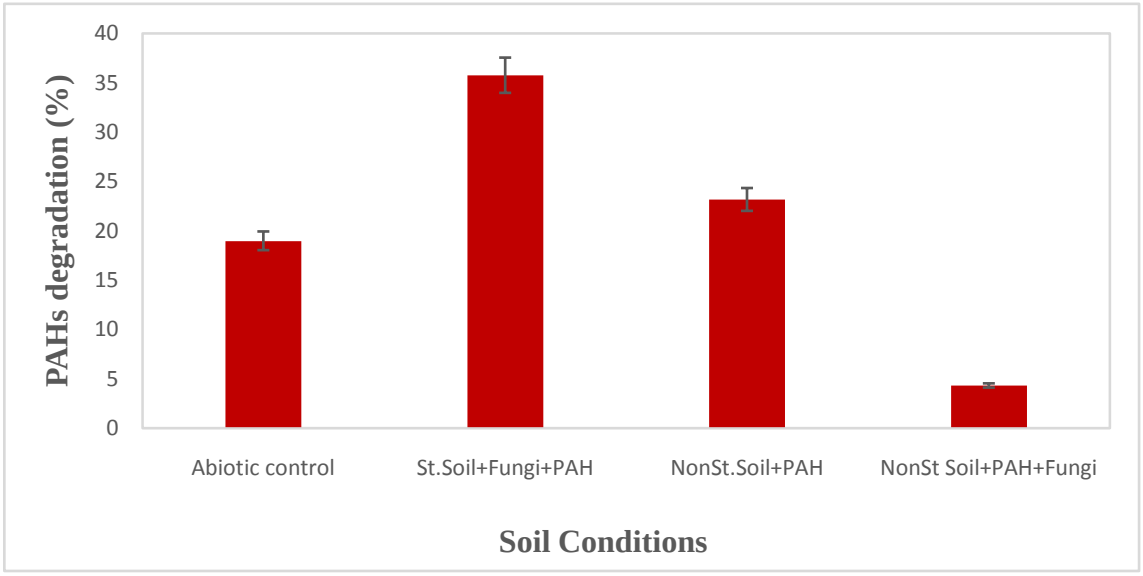

Figure 22. Microcosm study for Fluoranthene degradation

3.8. Detection of PAH Degradation Metabolites

For detection of degraded metabolite GC-MS analysis was performed. GC-MS analysis showed one peak as compared to control sample which was identified as 1-hydroxypyrene and phthalic acid with 218.5 m/z and 166.3m/z ratio respectively (Figure 23). Proposed pathway is shown in Figure 24.

GC-MS spectra of Phenanthrene showed presence of one peak as compared to control as shown in Figure 25. Peak corresponds to formation of 9,10-dihydroxy Phenanthrene it's an intermediate compound formed due to ring cleavage of cytochrome P-450 and epoxide hydrolase enzyme. Proposed pathway for degradation of Phenanthrene is shown as in Figure 26.

GC-MS analysis of Fluorene was Fluoren-9-ol as intermediate product after degradation on 20th day of incubation (Figure 27 - Figure 28). Fluorene 4- [1,2-dihydroethyl] is the intermediate detected by mass spectra of Fluoranthene (Figure 29 - Figure 30).

3.9. Phytotoxicity Study of Degraded Metabolite

The results of phytotoxicity studies using PAHs and its degraded metabolite on *Triticum aestivum* are shown in Table 3. Length of plumule and root length (in cms) of germinated seeds were recorded after 10 days. Maximum shoot length observed in degradation metabolite of Pyrene (21 cms) Figure 31.

% germination of seeds was affected after addition of PAH at concentration of 100 ppm to pot as compared to degraded metabolite as shown in Table 3. It is observed that Fluorene is most toxic for seed germination as compared to other PAHs.

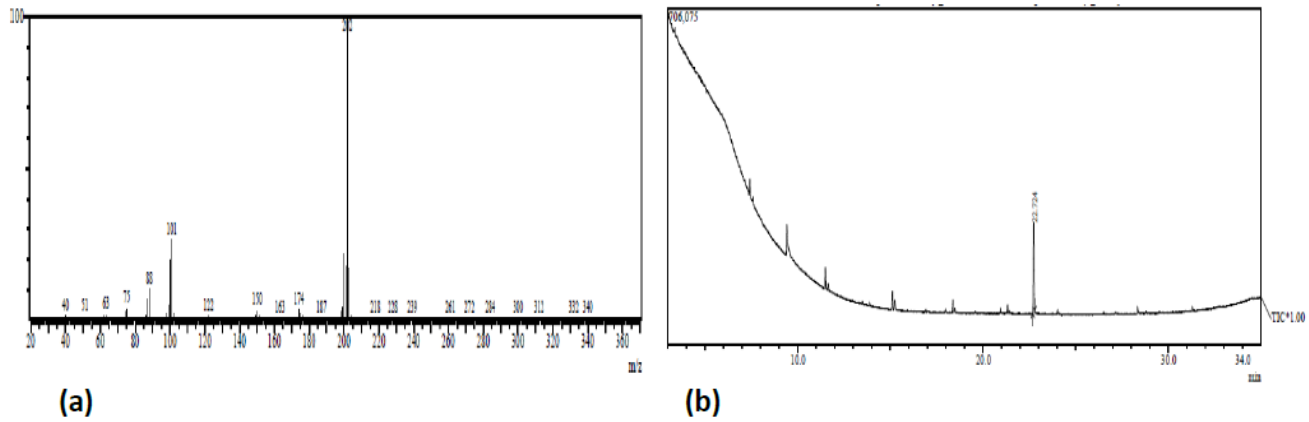

Figure 23. (a) & (b): Shows the mass spectra of control and experimental pyrene

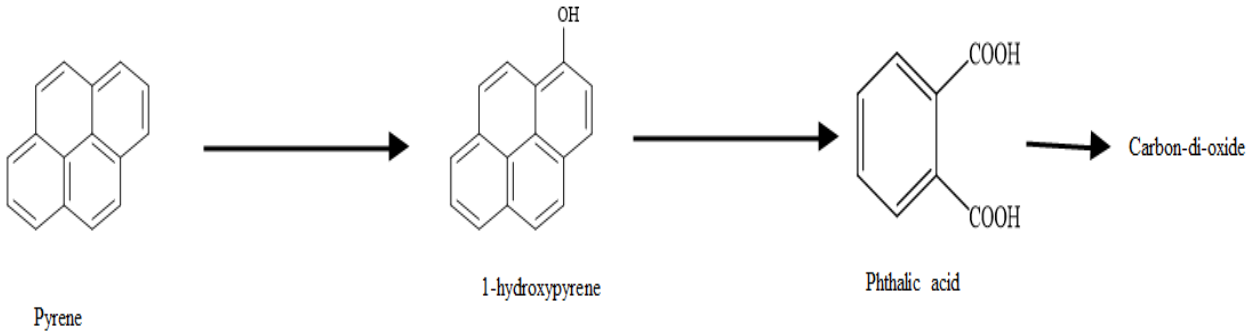

Figure 24. Proposed pathway of pyrene

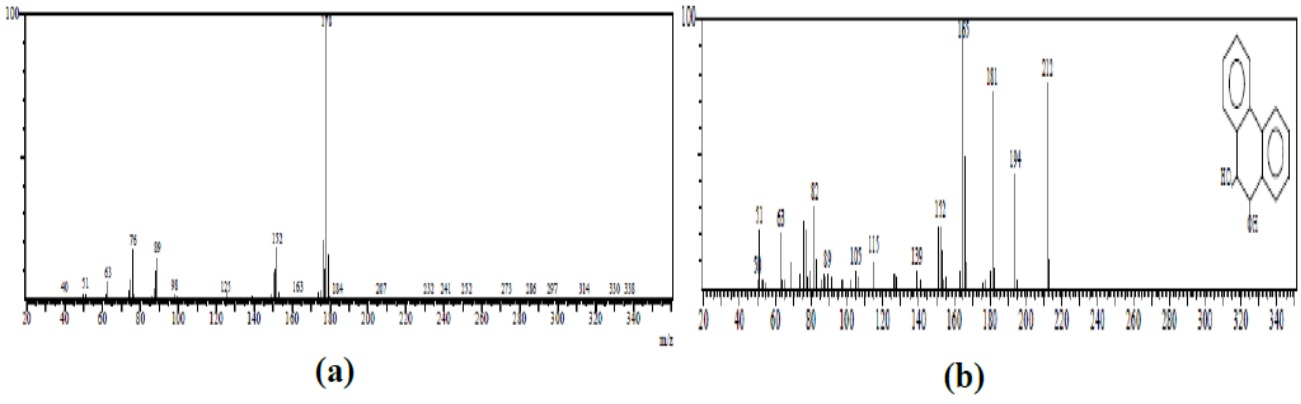

Figure 25. (a) & (b): Mass spectra of control and experimental Phenanthrene

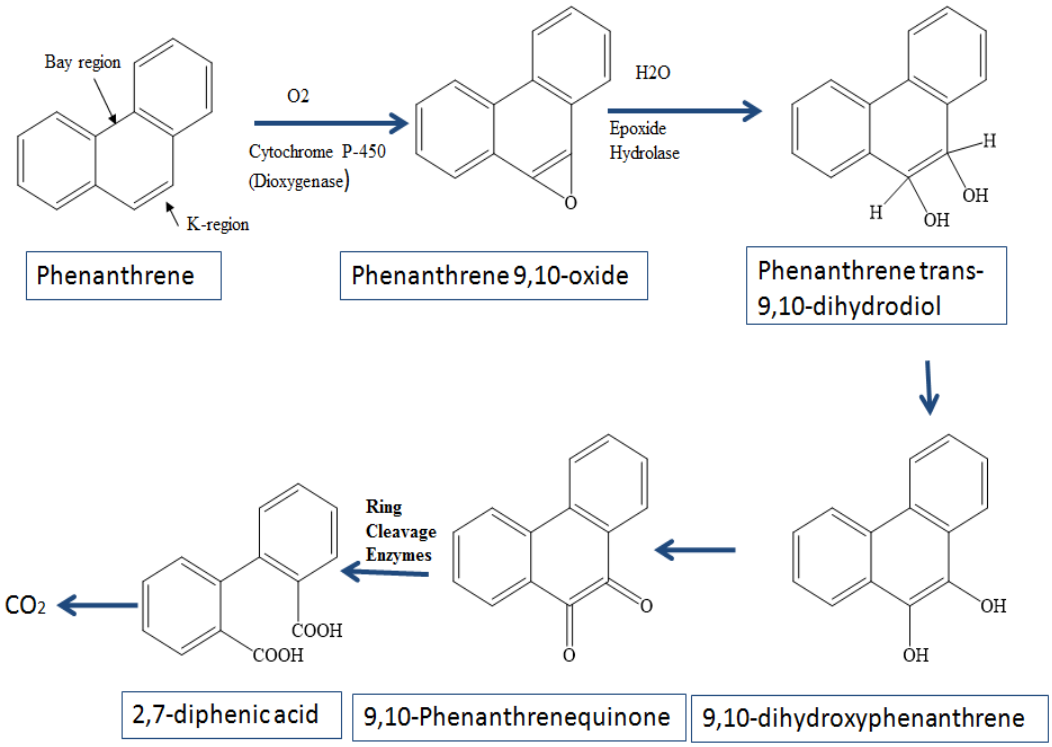

Figure 26. Proposed pathway for Phenanthrene degradation

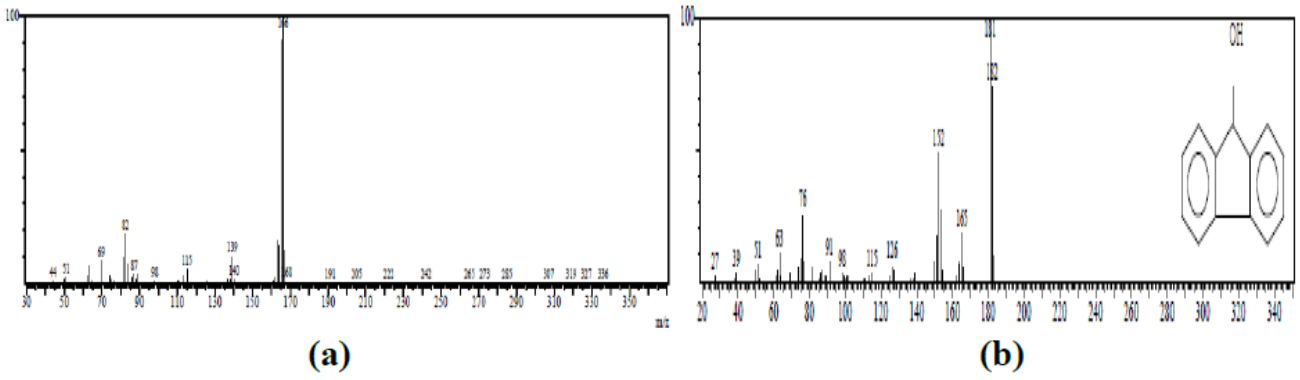

Figure 27. (a) & (b): Mass spectra of control and experimental Fluorene

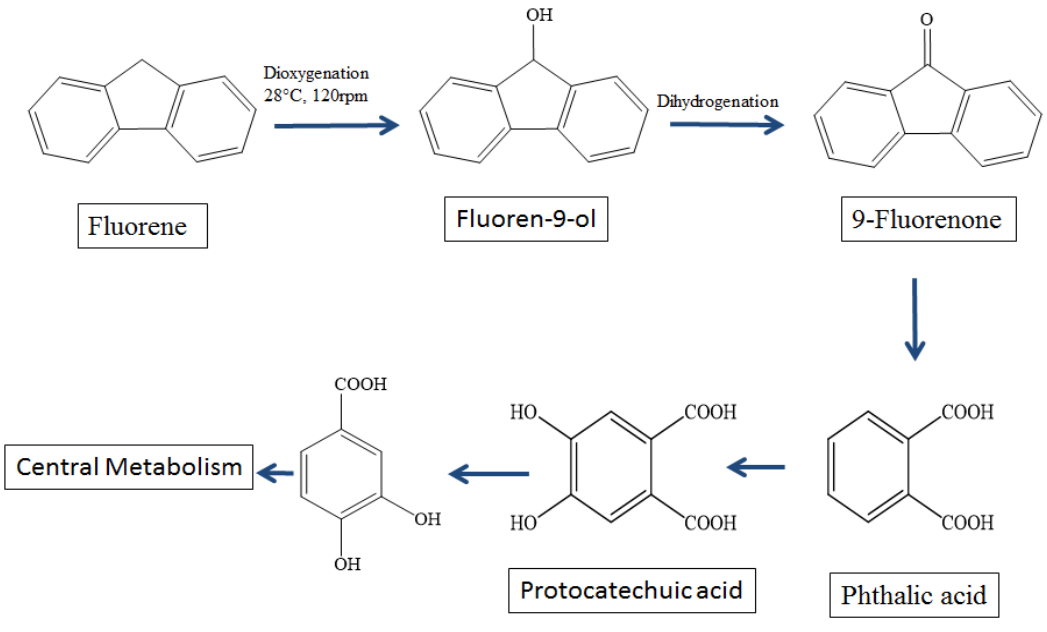

Figure 28. Proposed pathway for Fluorene

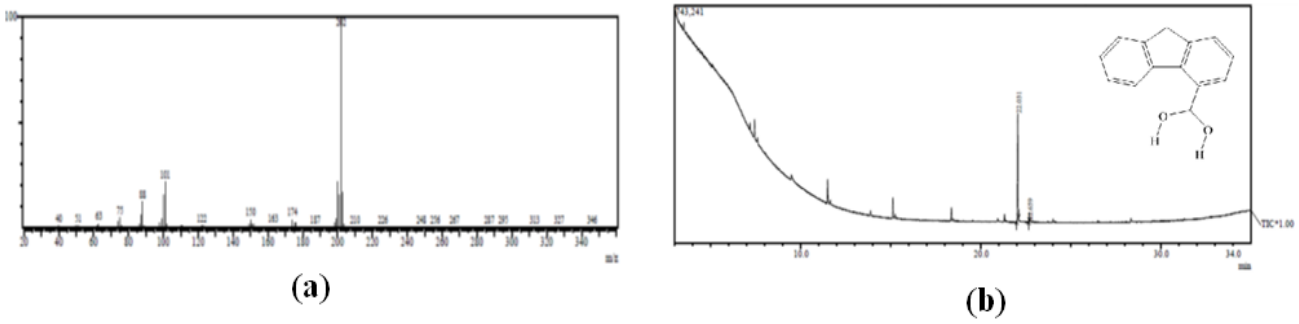

Figure 29. (a) & (b) Mass spectra of control and experimental Fluoranthene

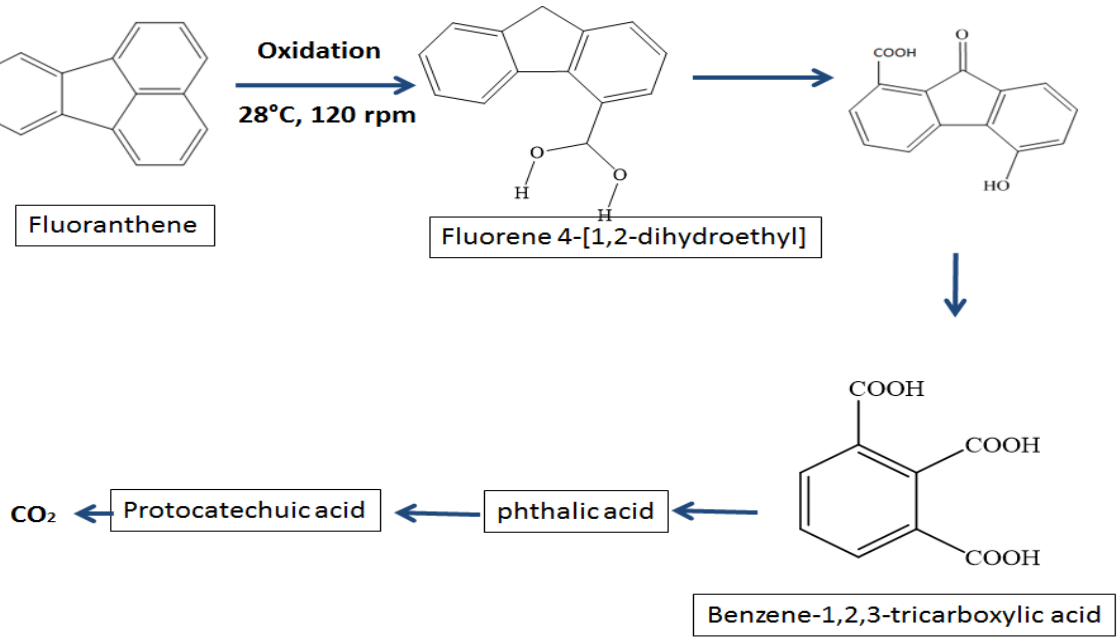

Figure 30. Proposed pathway for Fluoranthene

Table 3. Phytotoxicity study of different PAHs and its degraded metabolite on lengthe of plumule of *Triticum asetivum* seeds

Sr.No.	No.of seeds**(%)**		Root length (cms)		Shoot length (cms)	
	Control	Expt	Control	Expt	Control	Expt
Phenanthrene	50	70	± 0.75	9±1.7	18±0.6	18.5±1.1
Fluoranthene	40	80	5 ± 0.6	10±1.6	17 ± 0.35	19.5±0.9
Fluorene	20	60	4 ± 0.35	10±0.81	14.5 ± 0.45	20±1.2
Pyrene	50	90	6 ±0.45	8±0.9	15± 0.25	21±0.85

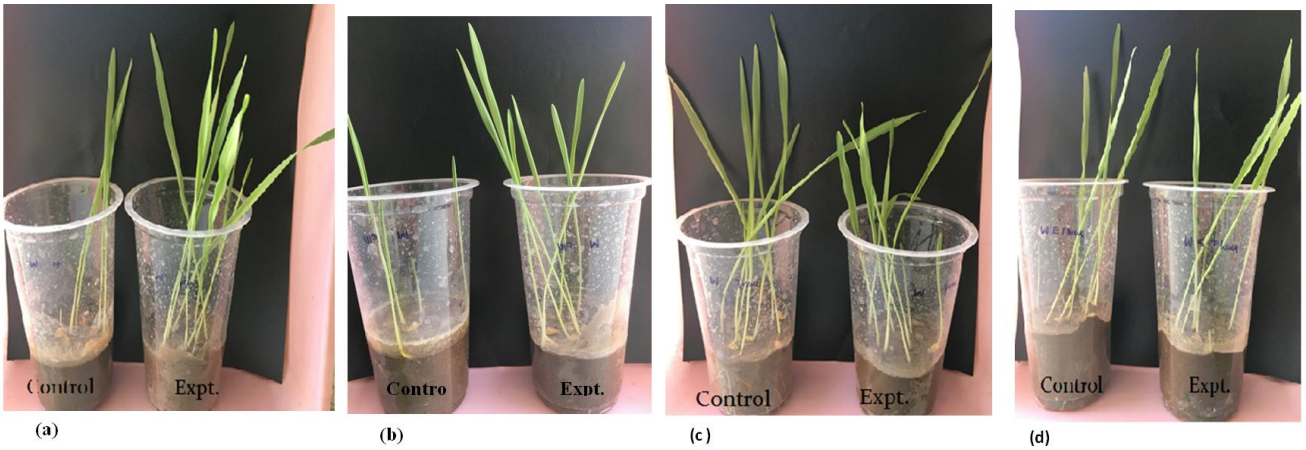

Figure 31. Phytotoxicity of degraded metabolite on *Triticum asetivum* (a) Fluoranthene (b) Fluorene (c) Pyrene and (d) Phenanthrene

4. Discussion

By plate assay method isolate were screened for ligninolytic enzyme production using o-dianisidine, guaiacol and gallic acid. Positive Bevandamm's reaction observed for laccase activity. Chhaya and Gupte [16] reported the similar results with litter dwelling fungi for screening of laccase producers using ABTS and guaiacol as a substrate. Gao *et al.,* [17], Patel *et al.,* [18] and Agrawal *et al.,* [19] reported the similar results for the screening of presence of laccase enzyme in the fungi by plate assay. Primary screening method is a very rapid for detection of the presence of the laccase enzyme by comparing the intensity of color and time required for development of colour due to oxidation of chromogens.

Solid state fermentation is one of the best process which have been used for ligninolytic enzyme production using agro-industrial residues. As agro-industrial residues are produced world-wide in abundance as a waste/ by product of crop cultivation and food processing industry. Secondary screening of laccase production was carried out using wheat bran as a solid substrate and Asther medium as a moistening agent under solid state fermentation. The Asther's medium [20] was used as a moistening agent to maintain the moisture level of the substrates. Maximum laccase production of 1.65 X 10^5 U/g of dry substrate for wheat bran was reported on 10^{th} day of incubation. Vares *et al.,* [21] and Gupte *et al.,* [22] reported higher laccase production with *Phlebia radiata* and *Pleurotus ostreatus* respectively under SSF using wheat straw as substrate. Kapoor *et al.,* [23] also reported maximum laccase production with *Lentinus edodes* from peanut meal supplemented wheat straw. On the basis of primary and secondary screening the isolate *Agaricomycetes sp.* AGAT was selected for further studies as it was considered as an excellent producer of laccase.

PAHs are considered to be environmental pollutants which having the ability to affect plants, animals and humans. PAH are mainly degraded by biological transformations thus the use of microbes represents the potential route for the removal of PAH from the environment. White rot fungi are the choice of organisms used widely in bioremediation. They are known to actively degrade wide range of recalcitrant and xenobiotic compounds. It has been assumed by many researchers that the degradation of these compounds is catalyzed by the enzymatic machinery of these fungi [24,25,26]. Fungi like Pleurotus *eryngii* [27], *Pleurotus pulmonaris F043* [25], *Scopulariopsis brevicaulis [28], Pycnoporus sanguineus H1* [2] and Phanerochaete chrysosporium [29] has been used for PAHs degradation.

The isolate *Agaricomycetes sp* AGAT showed degradation in degradation medium. The % degradation was enhanced in presence of co-substrate glucose present in medium. Mao and Guan [28] reported enhanced degradation of PAH by *Scopulariopsis brevicaulis.* using glucose as co-substrate. Hwang *et al*., [30] and Tekere *et al*., [31] also reported using other co-substrates enhanced the degradation. It has been reported by Wang *et al*., [32]; Swamy and Ramsay [33]; that the degradation of organic pollutants is faster when glucose is used as a co-substrate for growth.100% removal of Fluorene within 23 days was reported by Hadibarata and Kristanti [27] at a concentration of 10 ppm by fungal isolate *Pleurotus eryngii* F032 while 100% Fluoranthene was eliminated in 30 days at 10ppm concentration by *Pleurotus pulmonarius* F043 [25]. Mao and Guan [28] using *Scopulariopsis brevicaulis* reported removal of Pyrene (64.3%), Fluoranthene (61.9%) and Phenanthrene (60%) after 30 days. The decline in PAHs removal probably occurs in nitrogen limiting conditions. This can attributed as ligninolytic enzymes are also produced in nitrogen-limiting condition which decrease the PAHs available in the system. The present study shows the involvement of laccase in the degradation process.

Bioavailability for PAH degradation was enhanced by addition of surfactant to the medium. Surfactants have been known to increase surface area of the PAHs and the subsequent flux in an aqueous medium [34]. The solubility of the PAHs studied were as Phenanthrene 1.3mg/l; Fluoranthene 0.26 mg/l; Pyrene 0.14mg/l and Fluorene 1.992 mg/l [35]. The rate of degradation depends on the mass transfer rate of PAHs from solid phase to water phase [36]. Surfactants become intermediate between immiscible phases because of their hydrophobic and hydrophilic moieties. By addition of surfactants it increases the concentration of hydrophobic compounds in water phase by solubilization or emulsification. Solubilization occurs only above a specific threshold concentration, critical micellar concentration (CMC) after which surfactants molecules will aggregate to micelles. 72% degradation could be achieved by Hadibarata and Tachibana [37] with Tween 80 as surfactants after 30 days of incubation. Tween 80 enhance both uptake and release of compounds from the cells through modification of cell membrane permeability. Zhao *et al*., [38] found that Tween 80 is best for the degradation of Phenanthrene. Rate of degradation increased from 68.8% to 87.4% in the presence of AOT with Tween 80 within 10 days by *Pleurotus ostreatus* D1 [11]. Higher concentration of SDS may inhibit the growth of fungi. It may be attributed due to change in physico-chemical interactions between fungus and surfactants or limited bioavailability of micellar PAH due to low exit rates from the micelles [39]. Degradation of PAHs has been investigated by using Triton X-100 and SDS with *P.chrysogenum, M.racemosus, L.theobromae* [40]..

In the present study *in vivo* system is used where whole of fungal culture is used for degradation of PAHs. The PAHs selected for the present study is LMW-PAH and HMW -PAH with two or more benzene rings. The reaction catalyzed by laccase depends on monoelectric oxidation which transforms substrates to corresponding reactive radicals. The small molecule which act as single electron donor and activator of enzymes known as mediators enhances the rate of oxidation of the enzyme. The synthetic mediators like ABTS and HBT are mainly used in degradation studies. In our study three mediator used were ABTS, HBT and phenol at varying concentration of 0.1mM, 1mM and 2mM. ABTS when used as a mediator in a system the rate of degradation of Pyrene increased to 92% by *Pyconoporus sanguineus* H1 [2]. HBT has been used widely as mediator because of its high redox potential and its ability to stimulate PAHs oxidation by laccase [41]. Higher concentration resulted in complete removal of PAH due to production of coupling

products with oxidized mediators in a nonstoichiometric production of the quinone [42].

Bioaugmentation technique has been proposed as a strategy for enhancement of the bioremediation of contaminated soils. For *in-situ* bioremediation of the contaminated sites, addition of microorganisms has been a valuable tool in order to increase the rate and extent of biodegradation of organic pollutants. Various physico-chemical parameters affect the efficiency of organisms as compared to native microbial population present in contaminated sites. The use of organism depends on survival and performance in natural ecosystem. Therefore, to ensure the organism need to be studied for its survival and degradation efficiency in soil and also in the presence of the ingenious microbial populations [43,44]. Microcosm study provides an insight towards the competition between culture and indigenous microflora present in the environment. Bioaugmentation by *Paracoccus sp* increased Phenanthrene degradation as reported by Teng *et al*., [45]. Jain and Bajpai [46]; Singh and Ward [47] reported that in natural environment various processes have been studied in order to understand the role of microorganisms and microbial communities in bioremediation. The degradation of Fluorene, Fluoranthene and Pyrene in soil was 0, 6.2% and 7.5% respectively within 10 days [8]. Degradation of PAHs Fluorene, Fluoranthene, Phenanthrene and Pyrene was increased from 98 to 100%, 73 to 98%, 99 to 100% and 26 to 94% by inoculation of *P.velutina* to soil sample which shows that the degradation rate enhanced together by *P.velutina* together with indigenous microbes [48]. *Anthracophyllum discolor* degraded Phenanthrene (62%), Fluoranthene (54%) and Pyrene (60%) from soil sample after 60 days of incubation [49].

For detection of degraded metabolite GC-MS analysis were performed of all PAHs. 1-hydroxypyrene and phthalic acid with 218.5 m/z and 166.3 m/z ratio respectively were reported from degradation of Pyrene. Bezalel *et al*., [50] reported that initially pyrene was metabolized at the 4, 5 bond (K region) which forms an epoxide, which was hydrated to form pyrene trans-4, 5-dihydrodiol using *Pleurotus ostreatus.* Sack *et al*., [51] reported 1-pyrenol as degraded metabolite using wood decaying fungi.

It has been reported by various researchers that the ring metabolite 2, 2'-diphenic acid is from Phenanthrene via Phenanthrene trans9,10-dihydrol or Phenanthrene 9,10-quinone. This dead end product formed 2, 2'-diphenic acid is produced due to activity of ligninolytic enzymes from various white rot fungi [26,50,51,52].

Further decarboxylation reaction leads to formation of dead end product phthalic acid which leads to central metabolism pathway. The GC-MS of all four PAHs compound showed the presence of phthalic acid as an intermediate compound. Phthalic acid formation is one of the ring fission products formed after degradation of PAHs by white rot fungi, further derivatization of phthalic acid to carbon-di-oxide and highly polar metabolites takes place [53].

The toxicity of the degraded product can be defined as phytotoxicity assay. The PAH and its degraded metabolite were used for phytotoxicity study using *Triticum asetivum* seeds. The rate of germination can be used as an indicator for pollutant contamination because this process is very sensitive to various kinds of pollutants [54]. The toxic effects of PAHs depends on various factors. Chemical structure and chemical properties of the pollutants are main reason for affecting PAH toxicity. Phytotoxicity study of various PAHs has been reported by Somtrakoon and Chouychai [55] on economic crops.

In conclusion, large number of isolate were screened for ligninolytic enzyme production by plate assay method. The isolate showing positive Bevandamm's reactions by oxidation of phenolic compounds were screened for secondary screening. The isolate AGAT was identified as *Agaricomycetes sp AGAT* by ITS4 & ITS5 sequencing and further explored for PAHs degradation study. In present study a varied rate of degradation of various PAHs (Pyrene, Fluorene, Fluoranthene and Phenanthrene) were observed using *Agaricomycetes sp* AGAT. Rate of degradation depends upon their molecular weight and structure. There is correlation observed between ligninolytic enzyme and PAHs degradation. *In-situ* bioremediation techniques i.e. microcosm study has been performed which showed the importance of the indigenous flora present by increasing the degradation of PAH. Phytotoxicity study was performed on *Triticum asetivum* to check the effect of degraded metabolites obtained after degradation. GC-MS analysis was performed for identification of metabolites formed. A proposed pathways for PAHs degradation has been given depending upon the intermediate obtained in our study.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

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