Biodegradation and detoxification of phenanthrene in in-vitro and in-vivo conditions by a newly isolated ligninolytic fungus *Coriolopsis byrsina* strain APC5 and characterization of their metabolites for environmental safety

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant organic pollutants generated from agricultural, industrial, and municipal sources, and their strong toxic, carcinogenic and teratogenic properties pose a harmful threat to human beings. The present study deals with the bioremediation of phenanthrene by a ligninolytic fungus, *Coriolopsis byrsina* strain APC5, isolated from the fruiting body of decayed wood surface. During the experiment, *Coriolopsis byrsina* strain APC5 was found as a promising organisms for the degradation and detoxification of phenanthrene (PHE) in *in-vitro* and *in-vivo* conditions. HPLC analysis showed that the *C. byrsina* strain degraded 99.90% of 20 mg/L PHE in *in-vitro* condition whereas 77.48% degradation of 50 mg/L PHE was reported in *in-vivo* condition. The maximum degradation of PHE was noted at pH 6.0 at 25 °C temperature under shaking flask conditions. Further GC-MS analysis of fungal treated samples showed detection of 9, 10-Dihydroxy phenanthrene, 2, 2-Diphenic acid, phthalic acid, 4-heptyloxy phenol, benzene octyl and acetic acid anhydride as a metabolic products of degraded PHE. Furthermore, the phytotoxicity evaluation of degraded PHE was observed through the seed germination method using *Vigna radiata* and *Cicer arietinum* seeds. The phytotoxicity results showed that the seed germination index and vegetative growth parameters of plants were increased in the degraded PHE soil. As a result, *C. byrsina* strain APC5 was found to be a potential organism for the degradation and detoxification of PHE without showing any adverse effect of their metabolites.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are major environmental concern organic pollutants with toxic, mutagenic and carcinogenic properties to human and animals (Abdel-shafy and Mansour, 2015; Ainerua et al., 2021; Idowu et al., 2019). Atmospheric PAHs are released into soils, water, sediments, and vegetation through incomplete combustion, oil spills, incineration processes, forest fires, volcanic eruptions, and vehicular exhausts, by the transformation of PAHs via biological process and abiotic reactions (Bandowe et al., 2019; Ghosal et al., 2016; Xu et al., 2021). The United States Environment Protection Agency (USEPA) and European Commission (EC) have identified 16 PAHs compounds as priority pollutants including phenanthrene (PHE) on the basis of their toxicity (Kalugina et al., 2018; Wu et al., 2016). PHE is found most abundantly in the aquatic environment, including tap water, surface water, and wastewater; it has also been recognized in the sea food collected from contaminated water (IARC, 1983; USEPA, 1988). It is considered by scientific community as a model compound for the PAHs degradation study because it contains K-region and bay-region in their structure, which are found in the structure of higher molecular weight PAHs (Garcia-uitz et al., 2016; Luo et al., 2020). Due to the presence of K-region and bay-region in their structure, PHE is structurally stable and recalcitrant in nature. PHE showed toxic effect on the human respiratory system and caused photosensitization of the skin (Budavari et al., 1989; Xu et al., 2006), therefore, globally it is necessary to the remediation of PAHs from the environment.

A wide array of various physical, chemical, and biological methods has been applied for the degradation and remediation of PAHs from the contaminated environment. The physico-chemical method includes incineration, photodegradation (Nguyen et al., 2020), nano-filtration (Li et al., 2019), electrokinetic remediation (Pourfadakari et al., 2020), thermal desorption (Falciglia et al., 2020; Li et al., 2020), soil washing (Gitipour et al., 2018), radiocolloid treatment, electron beam irradiation (Xu et al., 2020), and chemical immobilization has been found efficient for degradation of PAHs. However these methods of remediation are expensive, in many cases they only transfer the toxic compounds from one phase to another phase and cause a secondary pollution and unable to degrade high molecular weight (HMW) PAHs (Antosova et al., 2020; Li et al., 2014). Thus, cost effective, efficient, and sustainable biological method involving microorganisms for the degradation of PAHs have been developed (Zhao et al., 2009).

Bioremediation has gained much attention in recent past for the remediation of hazardous and toxic environmental pollutants from contaminated sites (Asgher et al., 2008; Haritash and Kaushik, 2009; Romantschuk et al., 2002). Various microbes such as bacterial species, *Pseudomonas aeruginosa* PFL-P1 (Mahto and Das, 2020), *Paracoccus aminovorans* HPD-2 (Teng et al., 2010), *Burkholderia cepacia* (Chen et al., 2013), *Roseobacter clade* (Zhou et al., 2020) *Sphingomonas* sp. GY2B (Liu et al., 2017) and fungal species, *Trametes hirsuta* D7 (Hidayat and Yanto, 2018), *Ganoderma lucidum* (Agrawal et al., 2018), *Pleurotus ostreatus* (Pozdnyakova et al., 2018), *Leucoagaricus gongylophorus* (Ike et al., 2019), and *Armillaria* sp. FO22 (Hadibarata and Kristanti, 2012) have been reported for the remediation of PAHs contaminated soil, however as compared with bacteria, fungi is a promising alternative for the remediation of PAHs. Bacteria are able to degrade maximum four ring containing PAHs and secretes intracellular substrate specific enzymes, fungi secreted non-specific, non-selective ligninolytic enzymes viz., lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (LAC) that are associated with ligninolytic activities., other than this fungi degrade more than four ring containing HMW PAHs. Fungi secretes extracellular broad substrate specific ligninolytic enzymes which penetrate the polluted soil, sludge or matrix and then remove the pollutants, they involves less energy, time, and chemicals and grow on a broad range of substrates (Balaji and Ebenezer, 2008; Bankole et al., 2021; Czaplicki et al., 2018). However, white rot fungi are considered as the most favourable fungi for the investigation of PAHs degradation and their efficiency depends on the microbial strain and the structure of organic pollutants (Agrawal and Shahi, 2017; Jia et al., 2019; Llorens-Blanch et al., 2018). White rot LAC belongs to the multicopper containing phenol oxidase (EC 1.10.3.2: benzenediol, oxygen oxidoreductase or p-diphenol oxidase), which catalyze the oxidation of aromatic ring containing phenols, polyphenols, and non-aromatic substrates through the radical catalyzed mechanism (Moon et al., 2018; Shafiei et al., 2019; Wulandari et al., 2021), LiP (EC 1.11.1.14, 1, 2-bis (3, 4-dimethoxyphenyl) propane-1, 3-diol: hydrogen-peroxide oxidoreductase) catalyze the hydrogen peroxidase dependent cleavage of C α -C β and aryl C α bond, oxidize phenolic and non-phenolic lignin model compounds (Biko et al., 2020; Naghdi et al., 2018). MnP (EC 1.11.1.13; Mn (II) hydrogen-peroxide oxidoreductase) catalyze the manganese (Mn) dependent oxidation of phenolic compounds in presence of hydrogen peroxide (Emami et al., 2020; Nowak et al., 2020; Wong, 2009). The rate of the PAHs degradation depends on the culture conditions like pH, temperature, oxygen, concentration of PAHs, accessibility of nutrients and agitated culture (Kadri et al., 2017; Ting et al., 2011). Several authors have been reported the degradation of PHE by white rot fungi such as *Polyporus* sp. S133 (Hadibarata and Tachibana, 2010), *Pleurotus eryngii* (Wu et al., 2016), *P. ostreatus* D1 (Pozdnyakova et al., 2018). However, few reports are available on the *in-vitro* and *in-vivo* degradation and detoxification of PHE by white rot fungi.

In the present study, PHE degradation by *Corioloopsis byrsina* strain APC5 was assessed in the *in-vitro* and *in-vivo* conditions. Further, degradation of PHE was evaluated by HPLC, FT-IR and GC-MS techniques. Furthermore, ligninolytic enzyme activity and PHE degradation were optimized under different concentration of PHE and physical parameters like pH, temperature, salinity condition. After that formulation of *C. byrsina* fungus was prepared in different substrate to check the PHE degradation efficiency of fungus *in-vivo* condition. The phytotoxicity of PHE metabolites on the growth of *Vigna radiata* and *Cicer arietinum* was also investigated.

2. Material And Methods

2.1 Chemicals and reagents

All the chemicals, solvents and reagents were used in the present investigation, were of analytical grade and used without further purification. PHE (> 98.0% analytical standard), Sabouraud dextrose agar (SDA), Azure B, guaiacol, 2, 6-dimethoxy phenol, and ethyl acetate (HPLC grade) were purchased from Merck Life Science, Mumbai, India. Bovine serum albumin (BSA) used as a protein standard was supplied from Sigma–Aldrich Chemical Co. Ltd. (St. Louis,

USA). All the starad media and working solutions were prepared by using ultrapure water generated by the Milli-Q system (ELIX, Merck Millipore, India)

2.2 Fungi culture and medium for PHE degrdation

C. byrsina strain APC5 (KY418163.1) isolated from the fruiting body of decayed wood surface in our laboratory, was used for the investigation of PHE degradation (Agrawal and Shahi, 2017). Sabouraud dextrose agar (SDA) medium containing seven days old culture of strain APC5 (one disc of 8 mm diameter) was used as the inoculum for the degradation and enzyme analysis. The Mineral salt broth (MSB) medium used for PHE degradation and ligninolytic enzyme activity investigation was contains (g/L): Glucose – 10, KH_2PO_4 – 2.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.5, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ – 0.1, Ammonium tartrate – 0.2, trace element solution – 10 (mL) (Arora and Gill, 2001). MSB medium was supplemented with antibacterial compound (to inhibit the bacterial growth) and 0.45 μm membrane filter sterilize 20 mg/L concentration of PHE. The pH of the MSB medium was adjusted to 7.0 with the help of 0.1 N NaOH or 0.1N HCl.

2.3 Analysis of PHE degradation by plate assay

Preliminary Bushnell Haas Agar (BHA) medium (composition, g/L: MgSO_4 (0.2), CaCl_2 (0.02), KH_2PO_4 (1), K_2HPO_4 (1.0), NH_4NO_3 (1.0), FeCl_3 (0.05), agar (20) supplimented with 20 mg/L PHE (dissolved in acetonitrile) was (Fig. 1a) used for the qualitative analysis of PHE degradation. In this methods, mycelial disc (8.0 mm in diameter) of strain APC5 was transferred to BHA medium, then incubated at 27 °C for 10 days. After the incubation, clearing zone formation was observed around the inoculated mycelia of *C. byrsina* strain APC5.

2.4 Measurement of PHE degradation efficiency

The PHE degradation experiment was performed in Erlenmeyer flasks (250 mL) containing 20 mL of previously prepared sterile MSB medium supplimented with PHE. Triplicate flasks were inoculated with culture mycelium of strain APC5 and incubated at 27 °C under rotary shaker (120 rpm) for 15 days. The sample were collected at regular 2 days of time interval during inubation period and residual PHE in the MSB medium and the ligninolytic enzyme production were investigated. At the same time, the control (without fungus culture) was incubated. The PHE degradation efficiency was measured as percent (%) of degradation.

$$\text{Degradation (\%)} = \{(C_i - C_t) / C_i\} \times 100$$

Where C_i – initial concentration of PHE (mg/L), C_t - remaining concentration of PHE (mg/L) (Bishnoi et al., 2008). After the extraction, filtrate culture mycelium of strain APC5 were kept in a hot air oven at 50 °C, dried weight of mycelium (biomass) was observed.

2.4.1 Extraction of PHE

PHE and other existing organic compounds present in treated and untreated samples of PHE were extracted by liquid-liquid extraction procedure with ethyl acetate as described previously by Agrawal and Shahi (2017). An equal volume of PHE degraded medium and ethyl acetate were taken for the extraction of metabolites. Obtained organic solvent phase of metabolites were dried under vaccum using rotary evaporator. Then obtained residues weredissolved in HPLC grade 2.0 mL acetonitrile and used for HPLC, FTIR, and GC-MS analysis.

2.4.2 HPLC analysis

The residual concentration of PHE was assessed with the help of HPLC chromatogram peak area. HPLC analysis was performed on UFLC model euquipped with a PDA detector system (Shimadzu prominence, Japan), C_{18} , 100 Å° column (Luna5u, 250 X 4.6 nm) and pump (LC – 20AT), detector (PDA) was used at 254 nm). A gradient of solvent A and solvent B acetonitrile and water (70:30) were applied at 1.0 mL/min flow rate for 20 min. The detection was

observed at wavelength of 254 nm to measure the degradation of PHE. Remaining concentration of PHE was measured by the following formula;

$$\text{Concentration of PHE in sample (mg/L)} = A_{\text{SAM}} / A_{\text{STD}} \times C_{\text{STD}}$$

Where A_{SAM} – peak area of chromatogram of sample, A_{STD} – peak area of standard PHE compound, C_{STD} – concentration of standard PHE compound (mg/L)

2.5 Identification of PHE degradation metabolies

Extracted metabolites were analyzed by FTIR spectroscopy and GCMS.

2.5.1 FTIR spectroscopy

To access the bond modification of metabolies after degradation, FTIR spectroscopy analysis was performed in the range of 375–4000/cm using spectrophotometer (IR affinity-1, Shimadzu, Japan). The separated extract of metabolites were mixed with spectroscopic grade potassium bromide to prepare the pellet for FTIR analysis. At the same time IR spectra of PHE (control) was examined.

2.5.2 GC-MS analysis

The metabolites of degraded PHE were identified by GC-MS (Thermo Fisher Scientific, USA, GC model - Trace GC ultra, MS model – Polaris Q) with DB-5 MS capillary column (30 m length, 0.25 mm internal diameter and 0.25 μm film thickness). The injection volume of sample was 1 mL. The ion source of GC-MS was kept at 230°C and transfer line temperature along with inlet temperature was maintained at 280°C. The temperature program kept for 2 min hold at 70°C, an increase to 200°C at 10°C per min, kept for 1 min in 200°C, then again increase temperature to 325°C at 5°C per min and hold for 15 min at 325°C. Helium gas (1 mL per min) was used as the carrier gas. The mass spectrometer was operated in an electron ionization mode of 70 eV energy ion trap detector. The detected metabolites were identified by compairing with the MS library NIST (National institute of standard technology, USA) attached with the GC-MS instrument (Ghosal et al., 2010).

2.6 Ligninolytic enzyme activity

To assess the ligninolytic enzyme activity during PHE degradation, the culture supernatant was taken as per the protocol describe earlier (Sect. 2.4). LAC enzyme assay of collected treated supernanat was performed according to method described by Sandhu and Arora (1985), take 3 mL of solution containing 1.5 mL sodium acetate buffer (10 mM, pH 5.0), 0.5 mL of the enzyme extract, 1 mL guaiacol (2 mM, $\epsilon_{450} = 12100/\text{M}/\text{cm}$), then incubate for 2 h and absorbance read at 450 nm using double beam UV-Visible spectrophotometer (ELICO SL 218, Andhra Pradesh, India). For the LiP assay, total 3 mL of reaction mixture containing 1 mL of 125 mM sodium tartrate buffer (pH 3.0), 1 mL of 0.16 mM azure B ($\epsilon_{651} = 48800/\text{M}/\text{cm}$), 0.5 mL of the culture filtrate and then add 0.5 mL of 2 mM hydrogen peroxide. Then absorbance was recored at 651 nm (Archibald, 1992). MnP activity was analyzed by the method reported by de Jong et al. (1992). Take 3 mL of reaction mixture containing 1 mL of sodium tartrate buffer (50 mM, pH 4.0), 0.5 mL culture filtrate and 1 mL of 2 mM 2, 6-DMP ($\epsilon_{468} = 49600/\text{M}/\text{cm}$). The reaction was started with the addition of 0.5 mL hydrogen peroxide (0.4 mM) by the oxidation of 2, 6-DMP. The enzyme activity was expressed in terms of international units per liter of enzyme extract (U/L).

2.7 Optimization of culture conditions for the ligninolytic enzyme production and PHE degradation

2.7.1 Effect of physical parameters

Ligninolytic enzyme production and PHE degradation by strain APC5 was optimized in different physical parameters conditions like pH, temperature, and salinity. During optimization experiment, MSB (20 mL) supplemented with 20 mg/L PHE was inoculated by 8 mm diameter mycelial disc of strain APC5. Afterwards, inoculated flasks were incubated at varying pH (3.0–8.0), temperature (15–55 °C) and salinity (10 and 32 g/L) in the rotary shaker incubator (120 rpm) for 10 days. The PHE degradation and ligninolytic enzyme activity were determined as described earlier and protein concentration was analyzed according to the standard protocol of Lowry et al. (1951).

2.7.2 Effect of different concentration of PHE

To evaluate the effect of different concentration of PHE on the degradation by strain APC5, Erlenmeyer flasks containing different concentrations of PHE (10–100 mg/L) in MSB were inoculated by mycelial disc (8 mm diameter) of strain APC5 and flasks were incubated at 27°C in shaking flask condition (120 rpm) for 10 days. After incubation, the degradation of PHE was evaluated by HPLC analysis.

2.8 Degradation of PHE in soil

PHE degradation by *C. byrsina* strain APC5 was also investigated in the soil (*in-vivo* condition). For experiment, the soil was collected from village sendri, Bilaspur (Chhattisgarh) as prescribed earlier in Agrawal and Shahi (2017). The soil type was classified as loam soil, with 0.225 carbon (5%), 275 nitrogen (kg/ha), 11.25 sulphur (kg/ha), 168 potassium (kg/ha) and 5.8 pH, 0.14 electro conductivity analyzed by soil testing lab, Dept. of Agriculture Bilaspur, Chhattisgarh. Sterilized soil was treated with 50 mg/kg of PHE (dissolved in acetonitrile).

2.8.1 Inoculum preparation in wheat bran and talc powder substrate

Fungal inoculum was prepared in two types of substrates: wheat bran and talc powder. Wheat bran inoculum (100 g) was prepared in the sterilized spawn bags with 14000 CFU (colony forming unit)/g. To prepare talc powder inoculum, homogenized culture broths (100 mL, 13300 CFU/mL) were transferred and mixed with sterilized talc powder (1kg) containing carboxy methyl cellulose (20 g, as a binder) and then dried, after drying inoculum was ready for application.

2.8.2 Treatment of PHE incorporated soil with the inoculum

Wheat bran and talc powder inoculum were mixed in PHE incorporated soil separately. Wheat bran inoculum treated soil (100 g/kg) and control sets (soil containing PHE and without any fungal inoculum) were kept in sterilized polythene bags and incubated at 27 °C for 30 days and talc powder inoculum treated soil and control sets (soil containing PHE and without any fungal inoculum) were transferred in nursery bags. Nursery bags were incubated for thirty days in the outer environmental condition.

2.8.3 PHE degradation analysis by HPLC

PHE degraded metabolites were extracted from soil according to the method described by Zebulum et al. (2011). Briefly, control as well as treated soil samples were taken separately in Erlenmeyer flasks and mixed with 20 mL of acetonitrile and shake for 10 min. To extract large number of metabolites from treated sample, the extraction procedure was repeated three times. Obtained extract was concentrated by vacuum evaporator. Concentrated extract was investigated with the help of HPLC to calculate the PHE degradation percentage.

2.9 Phytotoxicity assessment of PHE degradation metabolites

The phytotoxicity assessment control (untreated) and bioremediated soil were studied in the plant of *Vigna radiata* and *Cicer arietinum*. *V. radiata* (seed variety: Leela seeds, Ahamdabad) and *C. arietinum* (seed variety: Research Bengal Gram Daftari 21) seeds were collected from Krishi Raksha Kendra, Bilaspur, Chhattisgarh. Prior to performing the experiment, the seeds surface were sterilized with 70% (v/v) ethanol and 2% (v/v) solution of sodium

hypochlorite. Furthermore, repeated washing of seeds were performed with distilled water to remove the remaining sodium hypochlorite. Sterilized seeds of *V. radiata* and *C. arietinum* were sowed in control and treatment sets (In control sets, 50 mg/L of PHE was incorporated in 1 kg soil. In treatment sets, 50 mg/L of PHE incorporated in 1 kg soil was treated by fungal inoculum and after 30 days of incubation, bioremediated soil was used as treatment sets.). *V. radiata* seeds were sowed in wheat bran inoculated soil and *C. arietinum* seeds were sowed in talc powder inoculated soil. The control and treatment sets were kept in outer environmental condition for 30 days. After that plants were harvested from the soil. The length and weight (both dry and fresh) of the root and shoot, percentage of seed germination, root elongation and germination index (GI) were examined with the help of formulae as described earlier by Agrawal and Shahi (2017).

2.10 Statistical analysis

To avoid any type of experimental errors, each test of experiment was conducted in triplicate. The standard deviation was investigated with the help of Microsoft Excel (ver. 2019, Microsoft, United Kingdom) and results of experiments were presented in mean $\pm$ SD value. The mean value of various test of control and experiment samples was investigated by one way ANOVA (analysis of variance) test by applying Dunnett's multiple comparison through Graph Pad Prism 8.0, (USA) statistical software.

3 Results

3.1 Analysis of PHE degradation by plate assay

C. byrsina culture was grown in BHA media (BHA media containing PHE used as control, (Fig. 1a), after incubation period, 7 mm clearing zone formation was observed around the mycelium because of the degradation of PHE (Fig. 1b).

3.2 Measurement of PHE degradation efficiency

PHE degradation by *C. byrsina* strain APC5 was examined after 2 days of regular time intervals by HPLC chromatogram. The HPLC chromatogram of control sample showed (Fig. 1c) a single major peak of PHE, while in fungi treated sample chromatogram (Fig. 1d) showed small peak of PHE and many extra peaks of other newly formed metabolites which clearly indicated the PHE degradation efficiency of strain APC5 by their ligninolytic enzyme activity. It was observed that as the incubation period increased, PHE degradation and biomass (Fig. 1e) of *C. byrsina* also increased, after 8th day 83.40% PHE (20 mg/L) degradation was examined at pH 6.0 and 27°C temperature while at 14th day of incubation 99.90% PHE degradation was observed (Fig. 1e).

3.3 Identification of PHE degradation metabolites

FTIR analysis of *C. byrsina* treated PHE sample supported the degradation of PHE as compared with control because of the change in the functional groups of compounds (Fig. 2). The vibrational band frequencies of PHE was found in FTIR spectroscopy between 2800 and 3200 cm^{-1} (3057 cm^{-1}) (Wu et al. 2010) (Fig. 2a, b). IR spectra (Fig. 2a, b) of compounds demonstrated different skeleton, stretching vibration of functional groups. A peak at 1716–1740 cm^{-1} was observed because of the existence of stretching vibration of CO bond in carboxyl, carboxylic acids and ketones. The absorption band at 1450–1625 cm^{-1} might appear as a result of skeleton vibration of benzene ring. At 1463 cm^{-1} , 610–700 cm^{-1} , skeleton vibration of aromatic ring, $-\text{C}\equiv\text{C}-\text{H}$: C–H bend in alkynes were investigated, because of ring cleavage reaction.

In this experiment, the GC-MS analysis of ethyl extracted untreated PHE (control), the peak of PHE was detected at the retention time (RT) 13.28 min (m/z 178) (Fig. 3a). However, after the 4th day treated sample, at the RT value 17.58

min (m/z 210) 9, 10-DihydroxyPHE was investigated, (Fig. 3b). At RT value 16.23 min (m/z 166) phthalic acid was identified, after 8th day of incubation, (Fig. 4a). Acetic acid anhydride (RT 4.05 min, m/z 102), Benzene octyl (RT 11.03 min, m/z 190), 4-heptyloxy phenol (RT 15.73 min, m/z 208), 2, 2-Diphenic acid (RT 18.36 min, m/z 242), 9, 10-Dihydroxyphenanthrene (RT 21.10 min, m/z 210) were observed (Fig. 4b) after 12th day of incubation. These metabolites were degraded products of PHE after fungi treatment.

On the basis of above identified metabolites, the PHE degradation pathway was postulated as shown in Fig. 5. PHE was first oxidized in K-region and transformed into 9, 10-dihydroxyphenanthrene by the action of LAC, LiP and MnP enzyme. Further 2, 2-diphenic acid and phthalic acid were produced due to the oxidation and ortho-ring cleavage reaction occurred by LAC, LiP and MnP enzyme. Due to side group removal phthalic acid converted into 4-heptyloxy phenol, benzene octyl, then ring cleavage reaction occurred by the ligninolytic enzyme, finally the conversion of acetic acid anhydride was observed after degradation.

3.4 Ligninolytic enzyme activity

To observe the ligninolytic activity during the PHE degradation, LAC, LiP and MnP activity of *C. byrsina* was investigated at regular 2 days time intervals. MnP activity was found maximum at the initial stage of PHE degradation whereas LAC activity was noted maximum 2629.00 U/L after 8th day incubated sample. LiP and MnP activity was found to be noted maximum 1727.00 U/L, 20147.00 U/L after 6 and 8 days of incubation respectively (Table 2). During the experiment it was observed that the degradation of PHE was significantly increased in the 6th day treated sample.

Table 2
Ligninolytic enzyme production by *C. byrsina* in PHE containing MSB.

Incubation time (days)	LAC activity (U/L)		LiP activity (U/L)		MnP activity (U/L)	
	Without PHE (Agrawal and Shahi, 2017)	With PHE	Without PHE (Agrawal and Shahi, 2017)	With PHE	Without PHE (Agrawal and Shahi, 2017)	With PHE
2	95.60 ± 8.62	250.50 ± 35.88 ^{***}	272.30 ± 35.61	646.40 ± 84.17 ^{***}	367.00 ± 47.75	882.30 ± 31.89 ^{****}
4	368.10 ± 15.12	460.40 ± 65.46 ^{ns}	553.60 ± 85.74	911.10 ± 47.24 ^{***}	2183.00 ± 40.86	5490.00 ± 17.74 ^{****}
6	729.50 ± 21.73	814.70 ± 83.26 ^{ns}	1170.00 ± 306.80	1727.00 ± 210.60 [*]	2932.00 ± 234.60	6586.00 ± 415.30 ^{***}
8	1523.00 ± 54.46	2629.00 ± 124.30 ^{****}	938.80 ± 135.20	1266.00 ± 58.10 ^{ns}	11020.00 ± 31.34	20147.00 ± 669.40 ^{****}
10	893.70 ± 67.88	1434.00 ± 106.80 ^{***}	305.30 ± 20.96	541.00 ± 111.60 ^{ns}	15987.00 ± 130.60	18969.00 ± 469.60 ^{***}
12	935.20 ± 64.11	128.30 ± 17.90 ^{****}	318.20 ± 22.22	687.20 ± 66.72 [*]	1847.00 ± 259.20	4301.00 ± 458.60 ^{***}
14	307.00 ± 21.78	381.50 ± 8.66 ^{ns}	480.00 ± 78.11	768.20 ± 97.06 ^{ns}	2110.00 ± 22.03	3391.00 ± 196.70 ^{**}
Footnote - Values represent of three independent replicate (n = 3) with mean ± standard deviation. According to one way Anova, values represent in column are significant different, ns-non significant p > 0.05, ****p < 0.0001, ***p < 0.001, ** p < 0.01 and *p < 0.05.						

3.5 Optimization of culture conditions for the ligninolytic enzyme production and degradation

3.5.1 Effect of physical parameters

The effect of abiotic environmental parameters like pH, temperature, and saline condition on the degradation of PHE showed that at pH 6.0 the maximum ligninolytic activity (1213.00 U/L LAC, 1028.00 U/L LiP, 7300.00 U/L MnP) and PHE degradation (85.60%) was observed (Fig. 6 (a)). It showed that when the production of enzyme increased, the degradation of PHE was simultaneously increased. The highest biomass (25.00 mg) and protein concentration (261.50 mg/L) were observed at pH 7.0 and 8.0 respectively. Further more increase in pH, inhibited the enzyme production, degradation and growth of fungi. Increase the incubation temperature upto 15–35 °C enhanced the degradation and enzyme activity. The maximum activity of ligninolytic enzymes (LAC 1640.00 U/L, LiP 957.00 U/L, MnP 20800.00 U/L, and degradation of PHE (87.50%) and growth (248.50 mg biomass, 110.50 mg/L protein) was observed at optimum temperature of 25 °C (Fig. 6b). Further increase in the temperature up 55 °C adversely affected

the degradation efficiency, enzyme activity, and growth of fungi. Similarly, at low salt concentration of 10 g/L, more enzyme activity (290.90 U/L LAC, 6799.00 U/L LiP, 2113.00 U/L MnP), degradation of PHE (34.50%) and growth (150.00 mg biomass, 85.30 mg/L protein) of fungi was observed as compared to high salt concentration (32 g/L: 10.30 U/L LAC, 2193.90 U/L LiP, 604.80 U/L MnP, 20.50% PHE degradation, 100 mg biomass, 15.70 mg/L protein) (Fig. 6 (c)).

3.5.2 Effect of different concentration of PHE

When the concentration of PHE increased in the medium, it was observed that after 10 days of incubation the ligninolytic enzyme activity of organisms significantly increased ($p < 0.0001$) (Table 3) and the degradation % of PHE decreased (Fig. 1f). At the 10 and 20 mg/L concentration of PHE maximum degradation up to 90.50% and 85.60% was observed. At 100 mg/L concentration of PHE, maximum Lac (3729.00 U/L), LiP (12911.00 U/L) and MnP (30247.00 U/L) activity was reported.

Table 3
Ligninolytic enzyme production by strain APC5 at different concentration of PHE.

Concentration of PHE (mg/L)	LAC activity (U/L)	LiP activity (U/L)	MnP activity (U/L)
0	1787.00 ± 479.80	1009.00 ± 28.90	11061.00 ± 182.20
10	2556.00 ± 347.50*	1077.00 ± 242.70ns	19887.00 ± 287.30****
20	2629.00 ± 124.30*	1266.00 ± 58.10ns	20147.00 ± 669.40****
50	2622.00 ± 206.90*	8128.00 ± 152.70****	21588.00 ± 545.60****
100	3729.00 ± 196.70****	12911.00 ± 275.30****	30247.00 ± 446.60****

3.6 Degradation of PHE in soil

Degradation of PHE was investigated in soil through the analysis of HPLC chromatogram peak area. When wheat bran inoculum was applied 77.48% of PHE degradation was investigated, whereas 68.74% of PHE degradation was observed with the talc powder inoculum.

3.7 Phytotoxicity assessment of PHE degraded metabolites

The phytotoxicity of control (PHE contaminated) and treated soil (bioremediated soil) sample was investigated by observing GI index, germination %, measuring the length and weight of root and shoot of the both *V. radiata* and *C. arietinum* plant shown in Fig. 7, and Table 4. In present study, the 100% seed germination was observed in control and treated soil sample plants of *V. radiata* and *C. arietinum*. It was observed that control sample was highly toxic, only 51.89% and 38.89% GI was found in the control plants of *V. radiata* and *C. arietinum* respectively, however 218.56% and 101.83% GI were found in the treated sample plants of *V. radiata* and *C. arietinum* respectively, which defines no toxicity of PHE degradation metabolites towards the plant.

Table 4
Phytotoxicity effect of PHEdegraded metabolites on *Vigna radiata* and *Cicer arietinum*.

	<i>Vigna radiata</i>			<i>Cicer arietinum</i>		
Parameter	Native soil	PHE contaminated soil	Bioremediated soil	Native soil (Agrawal and Shahi,2017)	PHE contaminated soil	Bioremediated soil
Root Length (cm)	9.00 ± 1.4	4.67 ± 0.58	19.67 ± 4.5*	18.00 ± 3.00	7.00 ± 2.5	18.33 ± 0.58**
Shoot Length (cm)	39.80 ± 0.24	9.33 ± 1.16	23.00 ± 2.6***	37.33 ± 0.58	15.33 ± 5.7	35.67 ± 5.5***
Fresh Root Weight (gm)	0.045 ± 0.002	0.03 ± 0.016	0.07 ± 0.03 ^{ns}	0.63 ± 0.15	0.09 ± 0.01	0.58 ± 0.13**
Fresh Shoot Weight (gm)	4.43 ± 0.33	0.18 ± 0.06	1.50 ± 0.13 ^{ns}	1.22 ± 0.16	0.23 ± 0.03	1.25 ± 0.25**
Dry Root Weight (gm)	0.028 ± 0.002	0.017 ± 0.008	0.03 ± 0.01 ^{ns}	0.08 ± 0.01	0.02 ± 0.003	0.06 ± 0.02*
Dry Shoot Weight (gm)	0.93 ± 0.007	0.052 ± 0.007	0.46 ± 0.05***	0.54 ± 0.37	0.04 ± 0.006	0.14 ± 0.04**
Germination %	100	100	100	100	100	100
Root elongation %	–	51.89	218.56	–	38.89	101.83
Germination Index (GI)	–	51.89	218.56	–	38.89	101.83
Values represent of three independent replicate (n = 3) with mean ± standard deviation. According to one way ANOVA analysis, the values represent in column are significant different, ns-non significant p > 0.05, ***p < 0.0001, ***p < 0.001, ** p < 0.01 and *p < 0.05.						

As compared to control the root, shoot length of treated *V. radiata* plants increased p < 0.05, p < 0.001 significantly. The root length of *V. radiata* plant was observed in bioremediated soil, 4.2 and 2.2 times longer than the seed showed in the soil of PHE contaminated and native soil respectively. In the case of shoot length of *V. radiata* 4.27 and 1.73 times reduction were found in PHE contaminated and bioremediated soil plant as compared to native plant. 51.89 germination index was found in the PHE contaminated soil, while 218.56 germination index was observed in the bioremediated soil which represents the non toxic condition of soil after the degradation of PHE by *C. byrsina* fungus.

In the case of *C. arietinum* plants, as compared to control the root, shoot length of bioremediated soil plants was increased p < 0.01, p < 0.001 significantly. The root length of *C. arietinum* plant was observed in bioremediated soil, 2.62 times longer than the seed showed in the soil of PHE contaminated. The shoot length of *C. arietinum* 2.44 times reduction were found in PHE contaminated as compared to native plant. 38.89 germination index was found in the PHE contaminated soil, while 101.83 germination index was observed in the bioremediated soil which represents the non toxic condition of soil after the degradation of PHE by *C. byrsina* fungus. On the basis of above data, it was investigated that after the degradation of PHE from soil any types of toxic metabolites were not formed, GI index and the vegetative growth parameters of *V. radiata* and *C. arietinum* plants increased significantly.

4. Discussion

PAHs transformation by wood decaying ligninolytic white rot fungi might be a universal phenomenon (Mao and Guan, 2016; Park et al., 2019). In this experiment, we demonstrated that white rot fungi *C. byrsina* strain APC5 have a high ability to degrade 99.90% of 20 mg/L PHE after 14 days of incubation in MSB. In the previous study, Hadibarata and Tachibana (2010) investigated that *Polyporus* sp. S133 degraded 92% of 1mmol/L PHE after 30 days of incubation with 120 rpm agitation in MSB and 44% degradation in non-agitated culture. Wu et al. (2016) reported that *Pleurotus eryngii* degraded 61.21% of 20 mg/L PHE after 15 days incubation in Potato dextrose liquid (PDL) medium while Pozdnyakova et al. (2018) investigated *P. ostreatus* D1 degraded $95.10 \pm 2\%$ of 50 mg/L PHE in Kirk's medium after 21 days of incubation shown in Table 1. As we known, PHE is degraded by different white rot fungi like *P. ostreatus*, *Ganoderma lucidum*, *Phanerochaete Chrysosporium*, *Trametes versicolor* (Bumpus, 1989; Dhawale et al., 1992; Han et al., 2004; Sutherland et al., 1991; Ting et al., 2011), but in this study white rot fungus *C. byrsina* strain APC5 is first time reported for the degradation of PHE.

Table 1
Comparison of PHE degradation by *Corioloopsisbyrsina* strain APC5 with some other reference fungi.

S. No.	White rot fungi	PHE concentration	Degradation (%)	Enzymes	Incubation medium	Incubation period (days)	References
1.	<i>Corioloopsis byrsina</i>	20 mg/L	99.90	Lac, LiP, MnP	Mineral salt broth	14	Present work
2.	<i>Pleurotus ostreatus</i> D1	50 mg/L	95.10 ± 20	Lac, MnP, Versatile peroxidase	Kirk's medium	21	Pozdnyakova et al. (2018)
3.	<i>Pleurotus eryngii</i>	20 mg/L	61.21	Lac, MnP, Superoxide dismutase	Potato dextrose liquid medium	15	Wu et al. (2016)
4.	<i>Ganoderma lucidum</i> strain BCRC36021	10 mg/L	98.70	Lac	Basal medium	10	Ting et al. (2011)
5.	<i>Polyporus</i> sp. S133	1 mmol/L	92	Lac, LiP, MnP, 1,2-Dioxygenase, 2,3-Dioxygenase	Mineral salt broth	30	Hadibarata and Tachibana (2010)

Ligninolytic enzyme of white rot fungi was described as the key for degradation of PAHs (Ghosal et al., 2016; Kunjadia et al., 2016; Qian and Chen, 2012). Our data represented that *C. byrsina* fungi produced significant amount ($p < 0.0001$) of ligninolytic enzyme therefore degrade PHE more efficiently. *C. byrsina* strain APC5 produced 2629 U/L LAC, 1727 U/L LiP, 20147 U/L MnP enzyme in the PHE containing media, while previously maximum 2000 U/L LAC activity was observed in *C. byrsina* SXS16 in the wheat bran substrate, but the activity of LiP and MnP was very low as compared to LAC reported by Gomes et al. (2009), although in the presence of PHE in MSB *C. byrsina* produced much higher amount of these three types of enzymes. While other PHE degrading fungi *P. eryngii* showed (996.25 U/L) LAC activity (Wu et al., 2016) and *Polyporus* sp. S133 showed 77.60 U/L LAC activity, 54.30 U/L LiP activity and 69.70 U/L MnP activity in MSB (with PHE) after 15 days of incubation (Hadibarata and Tachibana, 2010).

The metabolites of PHE degradation identified by GC-MS were 1, 2 dihydroxy phenanthrene, 4-heptyloxy phenol, benzene octyl and acetic acid anhydride. During degradation, toxic metabolites like quinones, which is formed after the degradation of PAHs by many other fungi (Singh, 2006) were not detected in GC-MS analysis. PHE was oxidized at K-region by the ligninolytic enzyme and formed 1, 2 dihydroxy PHE. Kadri et al. (2017) and Pozdnyakova et al. (2018) also supported that ligninolytic enzymes are responsible for the initial oxidation of PAHs. After that the formation of 2, 2-Diphenic acid was investigated by the oxidation and ring cleavage reaction occurred by enzymes. 2, 2-Diphenic acid produced in *P. chrysosporium* by the action of MnP and LiP enzyme investigated by Hammel et al. (1992), Hammel, (1995). Further produced phthalic acid entered into basal metabolism (also suggested by Pozdnyakova et al. (2010, 2018)). Our data support the degradation pathway described by Bezalel et al. (1997); Hadibarata and Tachibana, (2010). On the basis of above results, the detected metabolites of PHE was transformed and mineralized by *C. byrsina* strain APC5 through their enzyme activity.

PHE is considered as an indicator or model compound for the PAHs degradation study, because their presence in high concentration in the PAHs polluted sites and contain K-region and bay-region which are present in the structure of higher molecular weight PAHs (Bezalel et al., 1996; Sack et al., 1997). The efficiency of PAHs degradation depends on the production of ligninolytic enzyme (Kim et al., 1998; Field et al., 1992). Therefore to check the PAHs removal efficiency by strain APC5 in the contaminated area, different physical parameters and the concentration of PHE were optimized for the production of ligninolytic enzyme and PHE degradation. In this study *Corioloropsis* fungi showed ligninolytic enzyme activity in the temperature range 15–55 °C at 3 to 8 pH, therefore able to degrade PHE in pH and temperature stress condition, while most fungi have shown maximum growth and ligninolytic activity at pH 3 to 6 (Yamanaka et al., 2008). Tekere et al. (2001) investigated that the optimum temperature for the growth of *T. versicolor* is 30 °C. Han et al. (2004) investigated that PHE degradation by *T. versicolor* was maximum at pH 6.0 and 30 °C temperature. In the present investigation the best temperature for the ligninolytic enzyme production, PHE degradation and growth of *C. byrsina* is 25 °C at pH 6.0.

Salinity stress also affects the PHE degradation, fungal growth and enzyme production. Dixon et al. (1993) reported that every single fungus has revealed their different salt tolerance capacity due to their changed cell characteristics. In the presence of 10 g/L of salt *Corioloropsis* fungi showed high growth, PHE degradation and enzymatic activity. As the salt concentration increased fungal growth, PHE degradation and enzyme production decreased. Kamei et al. (2008) also reported that the growth of *P. chrysosporium* and *T. versicolor* was suppressed in 32 g/L sea salt concentration. But at the salt concentration of 32 g/L *Corioloropsis* fungi showed their growth, enzyme production and degradation.

The production of ligninolytic enzyme by fungi dependent on the concentration of carbon and nitrogen in the medium (D'Souza et al., 1999). It was investigated that as the concentration of PHE increased in the medium, the production of ligninolytic enzyme was increased significantly ($p < 0.0001$), which assist in the degradation of higher concentration of PHE by *Corioloropsis* fungi.

Formulation of *C. byrsina* effectively degraded PHE from soil. Degradation of PAHs in soil cannot be considered complete bioremediation of soil due to the formation of toxic metabolites after degradation or incomplete degradation of PAHs enhanced the, soil toxicity (Singh, 2006). Therefore phytotoxicity of PHE and their degraded metabolites were determined in the present study. After that we can say that *C. byrsina* strain APC5 is a potent PHE degrader from the environment and also promote the growth of plants.

5. Conclusions

This study shows that *C. byrsina* strain APC5 capable of degrading recalcitrant compound, PHE (PAHs) in *in-vitro* and *in-vivo* conditions. *C. byrsina* produced significant amounts ($p < 0.0001$) of ligninolytic enzyme at different concentrations of PHE. *C. byrsina* also degrade PHE and produced ligninolytic enzyme in worst environmental condition like pH (3.0 to 8.0), temperature (15 to 55 °C) and salinity (10 and 32 g/L). After the degradation of PHE by *C. byrsina* any types of toxic metabolites like quinone was not formed and toxicity of soil reduces therefore germination index and plant growth increases. *C. byrsina* can be used further in the process of phytoremediation as well as plant growth improvement. Furthermore, genetic engineering play a significant role in the enhancement of phytoremediation, to combine the knowledge of system biology with gene editing and gene manipulation technique efficiency of phytoremediation can be increased.

Declarations

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Authors' contributions

Nikki Agrawal: performed the experiments and completed the manuscript writing. **Vineet Kumar:** Manuscript writing and editing. **Sushil Kumar Shahi:** guided during research experiments and manuscript writing, reviewing and editing. All the authors approved the final manuscript.

Ethical approval

All the authors have read and agreed to the ethics of publishing the manuscript.

Consent for publication

Not applicable

Consent to participate

Not applicable.

Declaration of interest

The authors declare that they have no known competing interests that could have appeared to influence the research work described in this paper.

Availability of data and material

The gene sequence data of *Corioloropsis byrsina* strain APC5 were deposited in NCBI GenBank database and assigned the accession number KY418163.1, <https://www.ncbi.nlm.nih.gov/nuccore/KY418163>

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Figures

Figure 1

Qualitative analysis of PHE degradation, control (BHA media containing PHE) (a), Clear zone formation by strain APC5 after PHE degradation (b), Quantification of PHE degradation by HPLC chromatogram, chromatogram of PHE before (c) and after (d) treated by strain APC5, PHE degradation percentage and production of biomass, protein after degradation by strain APC5 (e), degradation of different concentration of PHE by strain APC5 (f).

Figure 2
 FTIR spectra of PHE before (a) and extracted metabolites (b) after PHE degradation by *C. byrsina*.

Figure 3

GC chromatogram of PHE (RT-13.28) before (a) and after 4th day (b), degradation by *C. byrsina*. Peak of 9, 10-Dihydroxyphenanthrene (RT-17.58) (b) was detected by GC-MS library search.

Figure 4

PHE degradation metabolites after 8th day (c), 12th day (d) of incubation by *C. byrsina*, Phthalic acid (RT-16.23) (c), and acetic acid anhydride (RT-4.05), benzene octyl (RT-11.03), 4-heptyloxy phenol (RT-15.73), 2, 2-Diphenic acid (RT-18.36), 9, 10-Dihydroxyphenanthrene (RT-21.10) (d) were detected by GC-MS library search.

Figure 5

Proposed pathway of PHE degradation by *C. byrsina* on the basis of GCMS analysis.

Figure 6

Degradation of PHE and ligninolytic enzyme production by *C. byrsina* under different pH (a), temperature (b) and saline condition (c).

Figure 7

The effect of PHE metabolites on the growth of *Vigna radiata* (a) and *Cicer arietinum* (b) growth cultivated in PHE degraded soil