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RESEARCH ARTICLE

Enhanced enzymatic removal of anthracene by the mangrove soil-derived fungus, *Aspergillus sydowii* BPOI

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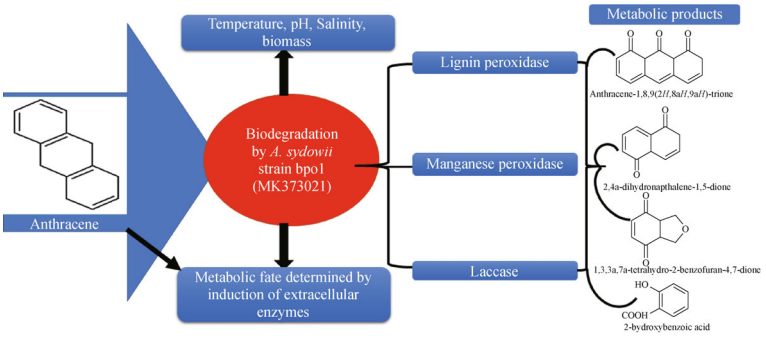
HIGHLIGHTS

- *A. sydowii* strain bpo1 exhibited 99.8% anthracene degradation efficiency.
- Four unique metabolic products were obtained after anthracene degradation.
- Ligninolytic enzymes induction played vital roles in the removal of anthracene.
- Laccase played a crucial role in comparison with other enzymes induced.

GRAPHIC ABSTRACT



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ABSTRACT

The present study investigated the efficiency of *Aspergillus sydowii* strain bpo1 (GenBank Accession Number: MK373021) in the removal of anthracene (100 mg/L). Optimal degradation efficiency (98.7%) was observed at neutral pH, temperature (30°C), biomass weight (2 g) and salinity (0.2% w/v) within 72 h. The enzyme analyses revealed 131%, 107%, and 89% induction in laccase, lignin peroxidase, and manganese peroxidase respectively during anthracene degradation. Furthermore, the degradation efficiency (99.8%) and enzyme induction were significantly enhanced with the addition of 100 mg/L of citric acid and glucose to the culture. At varying anthracene concentrations (100–500 mg/L), the degradation rate constants (k_1) peaked with increasing concentration of anthracene while the half-life ($t_{1/2}$) decreases with increase in anthracene concentration. Goodness of fit (R^2 = 0.976 and 0.982) was observed when the experimental data were subjected to Langmuir and Temkin models respectively which affirmed the monolayer and heterogeneous nature exhibited by *A. sydwoii* cells during degradation. Four distinct metabolites; anthracene-1,8,9 (2*H*,8a*H*,9a*H*)-trione, 2,4a-dihydronaphthalene-1,5-dione, 1,3,3a,7a-tetrahydro-2-benzofuran-4,7-dione and 2-hydroxybenzoic acid was obtained through Gas Chromatography-Mass spectrometry (GC-MS). *A. sydowii* exhibited promising potentials in the removal of PAHs.

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous, persistent organic pollutants commonly found in air, water, and soil. They consist of carbon and hydrogen atoms fused with the aid of two or more aromatic pentacyclic or benzene rings (Baltrons et al., 2018, Wu et al., 2020). Pipeline vandalism, sabotage due to civil unrest, militancy and war, oil spillage during exploration, over-exploitation, spillage from tankers during loading and transfers and petrochemical industry wastes are part of the anthropogenic factors contributing to the large scale and unprecedented presence of PAHs in the soil and aquatic biosphere (Jacques et al., 2008). PAHs are sometimes cofounded with heavy metals due to pollution from refinery and manufacturing plants although heavy metals do distort their complete degradation (Su et al., 2018). The acute mutagenic, carcinogenic and teratogenic toxicity of PAHs to humans and living organisms have further heightened their removal from the environment (Samanta et al., 2002).

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Naturally, organic compounds in PAHs released into the environment undergo photolysis, adsorption and volatilization in the soil although it takes several decades for recovery. A formidable alternative to the energy-sapping, expensive, physical PAH's removal methods (combustion, photolysis and landfill) is microbial degradation which is unarguably an efficient, cost-effective and eco-friendly treatment as it gives no sludge (Gong et al., 2018). Ambrosoli et al. (2005) and Balachandran et al. (2012) reported that the use of indigenous microorganisms isolated from PAH polluted environment in the degradation of PAHs is a promising technique. Extensive studies have been carried out on the capacity of fungi in the degradation of a broad range of environmental and persistent organic pollutants. The pivotal role played by enzymes during biodegradation of PAHs is attributed to the induction of non-specific, non-selective extracellular enzymes such as laccases (Lac), manganese peroxidases (MnP), and lignin peroxidases (LiP) (Wang et al., 2009). Degradation efficiency and enzyme induction are mostly influenced by optimizing salinity, pH, temperature, mediators, co-factors and stimulators such as glucose, fructose, malic acid, gallic acid and salicylic acid (Wen et al., 2009). There are diverse types of PAHs having compounds with low and high molecular weights. The degradation rate of high molecular weight PAHs is slow due to low solubility and adsorption. Researchers are however taking up the new challenge of discovering new fungi strains that can remove different PAHs in extreme conditions and low nutrients (Rashid et al., 2016). Biodegradation potency of new fungal strains isolated from PAH impacted environment is most times greater than the strains isolated from non-impacted sediments because of their acclimatization and adaptation to the pollution impacted environment (Zhang et al., 2006). The sampling location for the isolation of fungus in Nigeria is characterized by mangrove ecosystems, tidal swamps and wetlands along the coastlines of a tropical delta (Niger) with different tree species which are constantly exposed to PAH contamination due to pipeline leakages, vandalization and high oil exploration activities in the region.

Recently, Ye et al. (2011), Zhang et al. (2016) and Al-Hawash et al. (2019) reported renewed interest of researchers in the deployment of filamentous fungi in the degradation of PAHs. Anthracene has three benzene rings atomically connected by carbon and hydrogen elements. Human exposure to anthracene causes skin allergy, irritates the nose and lungs leading to coughs and sneezing. Studies have revealed that it distorts the proper functioning of the digestive and lymphatic systems in humans (Santos et al., 2008). To date, there are few reports on PAH biodegradation by *A. sydoowii.*

In this present study, the potency of *Aspergillus sydowii* strain bpo1 in the removal of anthracene was reported. The influence of organic acids and glucose in the induction of ligninolytic enzymes and enhanced anthracene degradation was investigated. Metabolites obtained after anthracene degradation were characterized through GC-MS and a metabolic pathway of degradation was proposed. Overall, the new fungal strain showed remarkable and promising potentials in the eco-friendly removal of PAHs.

2 Materials and methods

2.1 Chemicals

PAH (Anthracene) of 99.2% purity, potato dextrose agar and HPLC grade methanol were procured from Sigma–Aldrich, UK. Other chemicals of high analytical grade and purity used were purchased from BDH Chemicals, Mumbai, India.

2.2 Sampling, microorganism and culture media

A. sydowii strain bpo1 was isolated from a PAH contaminated mangrove soil near Gbaramatu (5°41′54″N; 5°50′56″E), Delta State, Nigeria. Enrichment of the isolate was done using potato dextrose media (dextrose-12 g, microbiological agar-2 g, sterile distilled water-250 mL, pH-7.2) in a 250 mL Erlenmeyer flask and incubated at 30°C for 48 h. The pellets obtained thereafter and centrifuged at 10000 × *g* for 15 min at 4°C and then collected for use in anthracene biodegradation (Ye at al. 2011). Screening and experiments for degradation efficiency by the fungus was done using mineral salt medium (MSM) ($CaCl_2 \cdot 2H_2O$-0.003 g, $Na_2HPO_4 \cdot 12H_2O$-13.0 g, KH_2PO_4-1.80 g, $MnSO_4 \cdot H_2O$-0.002 g, $(NH_4)_2SO_4$-0.8 g, $FeSO_4 \cdot 7H_2O$-0.002 g and $MgSO_4$-0.1 g and 1000 mL of sterile distilled water at pH 7.0) (Ramadass et al., 2016).

2.3 Molecular identification of the anthracene degrading filamentous fungus strain

Doyle (1990) method was followed in the extraction of the strain's genome was done using Cetyl Trimethyl Ammonium Bromide protocol. Amplification of the Internal Transcribed Spacer (ITS) was achieved with 2 primers: forward primer-ITS1F and reverse primer-ITS4R. Initial denaturation step was performed at 98°C for 2 min to kick-start the Polymerase Chain Reaction (PCR) process. This was strictly followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 45°C for 15 s, polymerization at 72°C for 15 s, and a final extension at 72°C for 5 min. PCR products were analyzed using 1.5% agarose gel electrophoresis. The actual sequencing of purified PCR products obtained was achieved at International Institute of Tropical Agriculture (IITA), Nigeria. Basic Local Alignment Search Tool (BLAST) query of the ITS4 sequence data was conducted using the BLASTN facility available on the National Center for Biotechnology Information (NCBI). The nucleotide sequence data were thereafter deposited on

the NCBI and DNA DataBank of Japan databases. Accession number (MK373021) was assigned after documentation in the NCBI gene library. Twenty-two (22) other sequences of high similarity and homologous identity to the strain were retrieved from NCBI portal and sequence alignment was performed using ClustalW facility on MEGA X Software (Kumar et al., 2018). Phylogenetic typing analyses were conducted using the maximum-like hood method and Tamura-Nei model (Tamura and Nei, 1993).

2.4 Biodegradation of anthracene by *A. sydowii* strain bpo1

A 0.005 g of anthracene was dissolved in methanol (50 mL) to make 100 mg/L anthracene standard solution. The procedure was repeated for concentrations (200–500 mg/L) to obtain full calibration of anthracene curve. The different anthracene solutions were added to 30 mL of mineral salt media (MSM) in 250 mL containing *A. sydowii* mycelia. The mycelia were collected after full growth in MSM as explained in 2.2 with the aid of Whatman filter paper. The mycelial pellets were of biomass weight (2 g) (pH 6). The setup was prepared in triplicates and kept for 72 h with flasks containing no fungus used as biocontrol (Ting et al., 2011).

The influence of PAH concentration, temperature, contact time, salinity (NaCl concentration) and pH the biodegradation of anthracene biodegradation was determined. The influence of anthracene concentrations and biomass of *A. sydowii* was determined in 250 mL Erlenmeyer flasks containing 50 mL MSM supplemented with the addition of anthracene (100–500 mg/L) as sole carbon source. Likewise, the incubation temperature was set at 15°C, 20°C, 25°C, 30°C, 35°C, and 40°C to determine its influence on biodegradation. The degradation efficiency was equally determined at different contact times, 12, 24, 38, 48, 60 and 72 h. Effect of salinity was determined by varying NaCl concentration in the set up at 0.2%, 0.5%, 1.0%, 1.5%, 2.0% and 2.5% while the initial pH of the experimental set up was adjusted to 5.0, 6.0, 7.0, 7.5, 8.0, 9.0 and 10.0 by adding NaOH and HCl (Al-Hawash et al., 2019). Other physicochemical parameters were kept constant while determining the effect of another parameter on degradation efficiency. Controls were prepared and monitored simultaneously in triplicates under the same experimental conditions although with no mycelial pellets of the *A. sydowii* strain bpo1. After every experimental cycle, samples were withdrawn from the flasks to determine residual biomass and anthracene at wavelength (λ = 460 nm) (Villemain et al., 2006).

$$\textit{Anthracene removal percentage (deg radation efficiency)} = \frac{C_i - C_f}{C_f} \times 100\%, \quad (1)$$

where C_i = initial anthracene (mg/L) and C_f = final anthracene concentration (mg/L).

2.5 Enzyme induction by *A. sydowii* during anthracene degradation

Induction of enzymes during degradation experiment was done using UV/Vis spectrophotometer (Shimadzu, Japan). The quantity of laccase induced was evaluated at increasing absorbance (λ = 405 nm) by measuring after serial oxidation of ABTS (0.5 mmol/L) in sodium acetate (0.1 mol/L) buffer with the pH adjusted to 5.0. Manganese peroxidase (MnP) was estimated and measured at absorbance (λ = 610 nm) in H_2O_2 and $MnSO_4$ (0.1 mmol/L) with a substrate made of phenol red (0.01%) as a substrate. MnP activity was measured through the oxidation of Mn(III) formation from Mn(II), Sodium hydroxide of 5 mol/L concentration was used in stopping the reaction. One unit of enzyme activity is equivalent to the enzyme needed to oxidize 1 μmol of substrate/min. Lignin peroxidase induction was determined from the reaction between culture filtrate (0.5 mL), sodium tartrate (1 mL), azure B (0.5 mL) and hydrogen peroxide (0.5 mL). Absorbance was read at 650 nm and expressed as 1 unit per liter (U/L) of the culture filtrate. Enzyme activity was expressed as U/L of culture filtrate. The results were presented as means of triplicates and standard error of means ($P \leqslant 0.05$) (Bankole et al., 2018; Cao et al., 2020).

2.6 Influence of metabolic substrates on anthracene degradation rate and enzyme induction

The effects of metabolic substrates such as citric acid, tartaric acid, glucose, gallic acid and salicylic acid on anthracene degradation and activities of Lac, LiP and MnP were investigated for 3 days. In a mixture of anthracene (200 mg/L) and MSM (150 mL), citric acid, tartaric acid, glucose, gallic acid and salicylic acid doses added were of 100, 100, 100, 100 and 100 mg/L respectively (Cao et al., 2020). The control experiments were flasks with no metabolic substrates.

2.7 High-Performance Liquid Chromatography (HPLC) and Gas Chromatography Mass Spectrometry (GC-MS) analyses

Anthracene was extracted with dichloromethane in ratio 2:2 in a test tube to determine the residual anthracene (Yuan and Chang, 2007). The pH of the mixture was adjusted to 4.2 and thereafter homogenized for 5 min with a vortex mixer. The mixture was later incubated for 30 min and centrifuged at 10000 × *g* for 10 min. The organic phase was then transferred to another test tube upon cooling. The water in the supernatant was extracted with sodium tetraoxosulphate VI. The resultant solution was

dissolved in dichloromethane, diluted further in methanol, and filtered (with membrane filter of 0.22 mm pore size).

2.7.1 HPLC analysis

The resultant solution in 2.7 above was analyzed with Waters™ 2690 (HPLC machine, Milford, Massachusetts, USA). The diameter of the hypersil (C18 column) is 4.6 mm × 250 mm. The column was made of 80% methanol mobile phase and 20% water (deionized). The system was kept for 10 min at a flow rate of 1 mL/min. A 2 μL aliquot was injected and analyzed on the UV-Vis detector (λ_{max} = 460 nm).

2.7.2 GC-MS analysis

Shimadzu QP 2010 GC-MS Machine (Shimadzu, Japan) was deployed for this analysis. The analysis was performed in programming mode (temperature) with a GC is equipped with a Resket column (60 m long, 0.25 mm id, nonpolar; XTI-5). While the GC-MS interface temperature was maintained at 290°C, the injection port temperature was set at 280°C. The initial temperature in the column was set at 80°C for 2 min. For a splitless period of 7 min, the temperature was thereafter increased steadily by 10°C/min to 280°C. The system's carrier gas was made of Helium which was set at a flow rate of 1.0 mL/min. Spectra mass was observed and measured at 1 scan/s with ionization energy of 70 eV. Retention times and fragmentation patterns were used for the identification of metabolites in line with spectra available GC-MS QP 2010 software library.

2.8 Isotherm modeling and kinetics studies

This analysis was conducted to determine the interaction between *A. sydowii* mycelia pellets (adsorbate) and anthracene (adsorbent) concentration. The concentrations were varied from 100 to 500 mg/L at temperature (28°C), pH 5 and 72 h.

2.8.1 Langmuir model

The monolayer adsorption nature of *A. sydowii* during anthracene was determined mathematically with Langmuir isotherm model using the linearized equation:

$$\frac{1}{q_e} = \frac{1}{q_0} + \frac{1}{q_0 K_L C_e}, \tag{2}$$

where K_L = Adsorption constant (Langmuir equilibrium) (L/mg),

q_e = Adsorption capacity (equilibrium) of *A. sydowii* mycelia pellets (mg/g),

C_e = Anthracene (equilibrium concentration) (mg/L),

q_0 is the Langmuir maximum of the anthracene adsorbed by *A. sydowii* cells (mg/g),

The slope $\frac{1}{q_0 K_L}$ is obtained from the plot of $1/q_e$ against $1/C_e$ (Bankole et al., 2018).

2.8.2 Freundlich model

The potency of the *A. sydowii* pellets was mathematically evaluated using the Freundlich model through a linearized equation:

$$\log\ q_e = \frac{n\ \log\ K_F + \log\ C_e}{n}, \tag{3}$$

where C_e = quantity of residual anthracene after degradation experiment (mg/L),

q_e = equilibrium of anthracene adsorption by *A. sydowii* mycelia pellets (mg/g),

K_F = Freundlich constant (L/mg),

n = Freundlich constant,

The slope ($1/n$) is determined in a plot of $\log\ q_e$ against $\log\ C_e$ (Bankole et al., 2018).

2.8.3 The Temkin model

Temkin model was conducted to evaluate the role played by temperature in the degradation set up. The modeling was conducted to further determine the heterogeneous behavior of the *A. sydowii* mycelia pellets during adsorption of anthracene. The model was mathematically determined through a linearized equation:

$$q_e = B\ \ln\ K_T + B\ \ln\ C_e, \tag{4}$$

where K_T = Equilibrium isotherm (binding constant) (L/g),

B = heat sorption constant (J/mol),

C_e = anthracene concentration (mg/L),

Isotherm constants B and K_T representing the slope and the intercept respectively is extrapolated from plot q_e against $\ln\ C_e$ (Bankole et al., 2018).

2.8.4 First-order kinetic model

This was performed to evaluate effect of anthracene concentration on degradation rate (K_1) and half-life ($t_{1/2}$). The anthracene degradation experimental data were subjected to pseudo-first-order kinetics:

$$S = S_{0\ \exp}\ -k_1 t;\ t_{1/2} = \frac{\mathrm{In2}}{k_1}, \tag{5}$$

where S_0 initial anthracene concentration,

S = substrate concentration at time t,

t = experimental time (Day),

While K_1 = anthracene removal rate constant. Anthracene (%) is estimated by dividing anthracene (residual) with anthracene (initial concentration) (Ting et al., 2011).

2.9 Statistical analysis

Experiments were conducted in triplicates. Values were presented and plotted as Mean±Standard Error of Means (95% C.I). Data on anthracene removal and enzyme induction were subjected to Levene statistics to test for normality and homogeneity. Data on anthracene degradation efficiency was subjected to One-way Analyses of Variance (ANOVA) to test for homogeneity with Statistical Package for Social Sciences v25.0 software (SPSS Inc. IL, USA) ($P \leqslant 0.05$). GraphPad Prism software version 8.4.2 (2020) was used in plotting the graph and regression plots of kinetics studies.

3 Results

3.1 Molecular identification and characterization of *A. sydowii* strain bpo1

The fungus strain was identified based on molecular characteristics. The Basic Local Alignment Search Tool (BLAST) analysis of the molecular sequence data revealed the actual identity of the fungus. The strain has between 97% and 99% homologous identities with 10 other *A. sydowii* sequence data previously documented in the NCBI database. Thereafter, an Accession Number-MK373021 was assigned upon proper documentation. The evolutionary relationship with 22 nucleotide sequences inferred through maximum likelihood method revealed that *A. sydowii* strain bpo1 belong to the genus *Aspergillus* with close affinities to the species *sydowii* (Fig. 1).

3.2 Physicochemical parameters optimization for enhanced anthracene degradation

3.2.1 Influence of varying concentrations of anthracene on degradation efficiency

The concentration of PAH during degradation experiments plays a significant role. The degradation efficiency was determined at concentrations (100–500 mg/L). Maximum degradation efficiency (98.4%) of *A. sydowii* on anthracene was observed at 100 mg/L concentration while the lowest degradation efficiency (81%) was recorded at anthracene concentration (500 mg/L). However, the biomass yield of *A. sydowii* during degradation was high (1.11 g) at 500 mg/L concentration while the lowest biomass yield was observed at 100 mg/L (Fig. 2(a)).

3.2.2 Influence of varying temperatures on anthracene removal

Temperature plays a pivotal role in the growth of organisms with respect to degradation of persistent organic pollutants. The experiments were done at varying temperatures of 15°C, 20°C, 25°C, 30°C, 35°C and 40°C. The maximum anthracene degradation efficiency (98.7%) was achieved at 30°C (Fig. 2(b)) while the lowest anthracene degradation efficiency (88.3%) was observed at

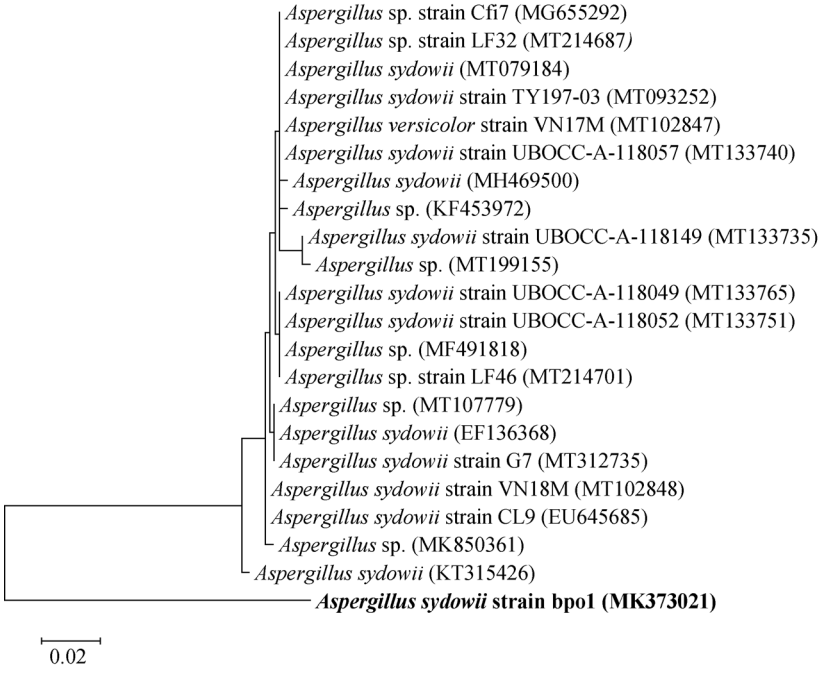

Fig. 1 Phylogenetic typing through maximum-likelihood method of *A. sydowii* strain bpo1. The tree was inferred from the analysis of sequence data (ITS genes) with a scale bar of 0.020 indicating the genetic distance.

temperature 15°C. This is not unconnected to the fact that the low temperature rendered the mycelia cells seemingly inactive for effective induction of enzymes needed for anthracene degradation. Also, decrease in anthracene degradation efficiencies (96.2% and 94%) was observed at temperatures 35°C and 40°C respectively (Fig. 2(b)). One-way ANOVA analysis conducted revealed that the degradation efficiencies recorded at 35°C and 40°C were statistically significant while the efficiencies obtained at 15°C and 20°C were not different statistically. This is due to partial denaturation of fungal enzymes at high temperatures which in turn affected anthracene degradation rate. However, at temperature 30°C, the resulting biomass generated was 1.3 g which represented the highest recorded during anthracene degradation while the lowest biomass of *A. sydowii* (0.7 g) was recorded when the

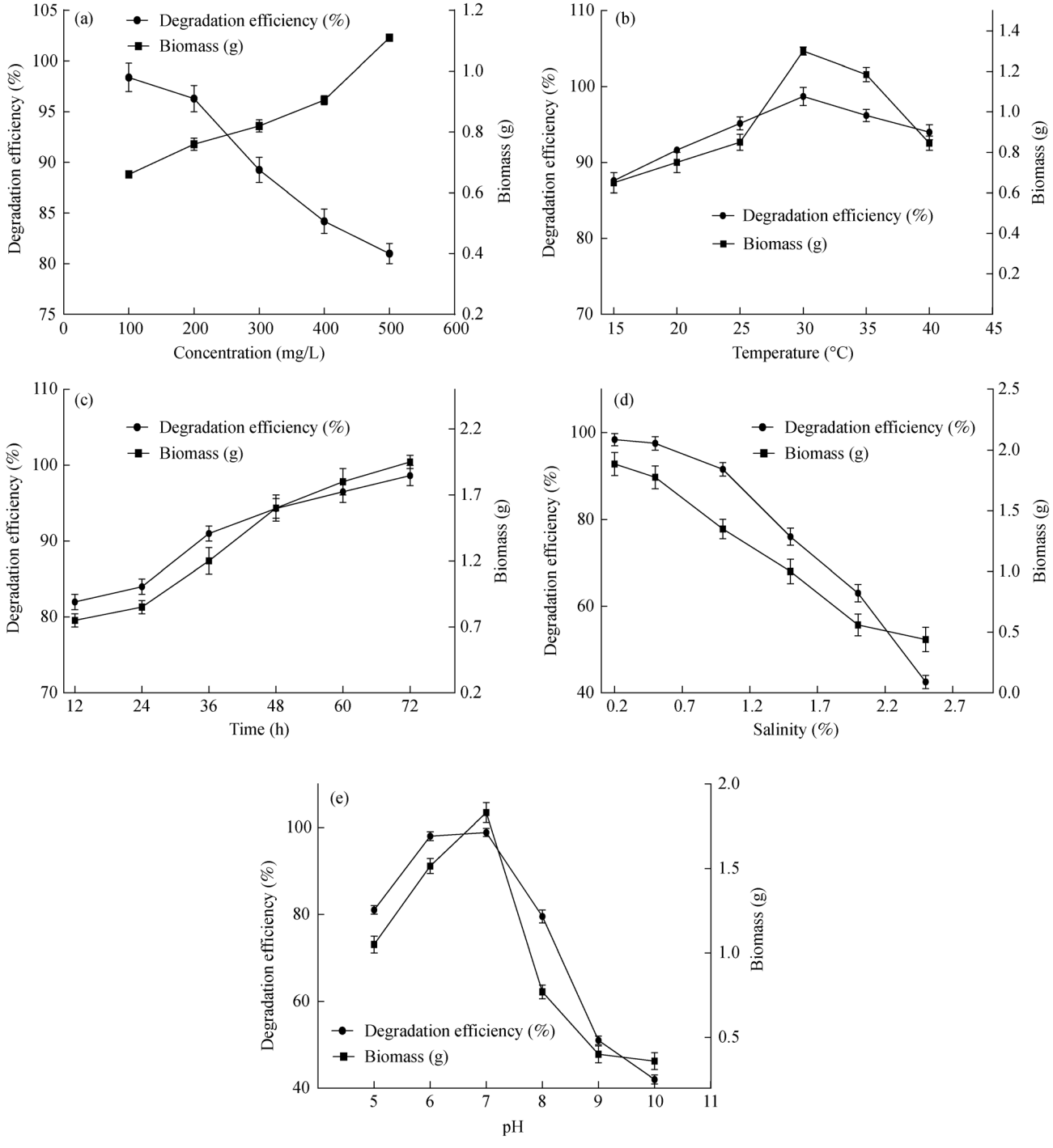

Fig. 2 Influence of (a) concentration, (b) temperature, (c) contact time, (d) salinity and (e) pH on degradation efficiencies and biomass. Graphs were plotted as mean±standard error of means.

temperature was set at 15°C (Fig. 2(b)). The results revealed that the temperature required for the highest degradation of anthracene by *A. sydowii* was 30°C.

3.2.3 Influence of incubation times on anthracene removal rate

Anthracene degradation efficiency and *A. sydowii* biomass were evaluated at contact times 12, 24, 36, 48, 60 and 72 h. The experiment revealed maximum anthracene degradation efficiency (98.6%) at 72 h while the lowest efficiency (81%) was observed at 12 h (Fig. 2(c)). A one-way ANOVA test showed that the degradation efficiency recorded at 72 h was statistically significant in comparison with the efficiencies recorded at lower contact times. Also, it was observed that the biomass of *A. sydowii* kept increasing with increase in experimental times. The highest biomass (1.95 g) was recorded at 72 h while the lowest biomass (0.75 g) was observed at 12 h (Fig. 2(c)).

3.2.4 Effect of salinity on anthracene degradation efficiency

The degradation set up was supplemented with sodium chloride of 0.2%, 0.5%, 1.0%, 1.5%, 2.0% and 2.5% (w/v). Highest degradation rate (98.3%) was observed when the set up was supplemented with 0.2% NaCl while the lowest degradation efficiency 42.5% was recorded at 2.5% NaCl concentration (Fig. 2(d)). Optimal anthracene removal and microbial growth rate were enhanced at 0.2% (w/v) NaCl. One-way ANOVA tests revealed that there was significant difference between degradation efficiency recorded at 0.2% saline condition and that recorded at high salt concentrations.

3.2.5 Influence of varying pH on anthracene degradation efficiency

A. sydowii exhibited maximum anthracene degradation efficiencies (98% and 98.9%) at pH 6 and 7 while the lowest anthracene degradation efficiencies (51% and 42%) were observed when the pH was set at 9 and 10 respectively. Similarly, the amount of *A. sydowii* biomass recorded was high (1.83 g) at neutral pH while the lowest biomass (0.36 g) was observed at pH 10 (Fig. 2(e)). The growth of fungi biomass and enzyme induction is mostly optimal at pH 5 and 6 which accounted for the slight increase in degradation efficiencies (81% and 98% respectively) but a bit lower than the efficiency recorded at neutral pH (Fig. 2(e)). The influence of pH 5 and 6 on degradation efficiency was corroborated with one-way ANOVA results which showed significant difference in comparison with efficiencies recorded at other pH levels.

3.3 Changes in degradation efficiency and enzyme induction with metabolic substrates supplements

To further optimise and enhance enzyme induction and anthracene degradation efficiency by *A. sydowii*; 100, 100, 100, 100 and 100 mg/L doses of citric acid, tartaric acid, glucose, gallic acid and salicylic acid respectively were added. Anthracene degradation efficiency by *A. sydowii* was significantly enhanced on day 6 by 98.5%, 98%, 89.5%, 86.5% and 83% when glucose, citric acid, tartaric acid, salicylic acid and gallic acid respectively was added to the mixture (Fig. 3(a)). The anthracene degradation efficiency in the control experiment was 0%. The enhancement in anthracene degradation by the metabolic substrates is as follows: glucose>citric acid>tartaric acid>salicylic acid>gallic acid.

Induction of laccase significantly peaked by 63%, 44%, 36%, 27% and 17% within 4 days when the culture was supplemented with glucose, citric acid, tartaric acid, salicylic acid and gallic acid respectively (Fig. 3(b)). The induction on Lac in the control experiment was 0%. Overall, laccase production during anthracene degradation was highly improved by the metabolic substrates in the following order: glucose>citric acid>gallic acid>tartaric acid>salicylic acid. In sharp contrast, LiP production was significantly enhanced on day 4 by 75%, 62%, 47%, 42% and 31% in the presence of citric acid, tartaric acid, glucose, salicylic acid and gallic acid respectively (Fig. 3(c)). The induction on LiP in the control experiment was 0%. Manganese peroxidase induction was improved significantly by 81%, 74%, 65%, 52% and 45% on addition of tartaric acid, citric acid, glucose, salicylic acid and gallic acid respectively to the cultures (Fig. 3(d)). The induction on MnP in the control experiment was 0%. Glucose and citric acid resulted in remarkable inductions in Lac and LiP concentrations and lower induction of MnP. The production of ligninolytic enzymes was enhanced by the addition of glucose and citric acid in the assay medium. The skewness and kurtosis for enhanced degradation of anthracene and enzyme induction were found to be less than 1 and 2 respectively which suggests that the data were normally distributed. The data were affirmed to be normal with significant values of 0.187 and 0.913 (>0.05) when subjected to Shapiro-Wilk and Kolmogorov–Smirnov[a] tests respectively. Similarly, the data on anthracene removal and enzyme induction was found to be homogenous with significant values of 0.014 and 0.017 respectively when subjected to Levene statistics. A one-way Analysis of Variance (ANOVA) revealed that the data for anthracene degradation efficiency and enzyme induction was significantly different and statistically relevant on separation of means with Tukey-b.

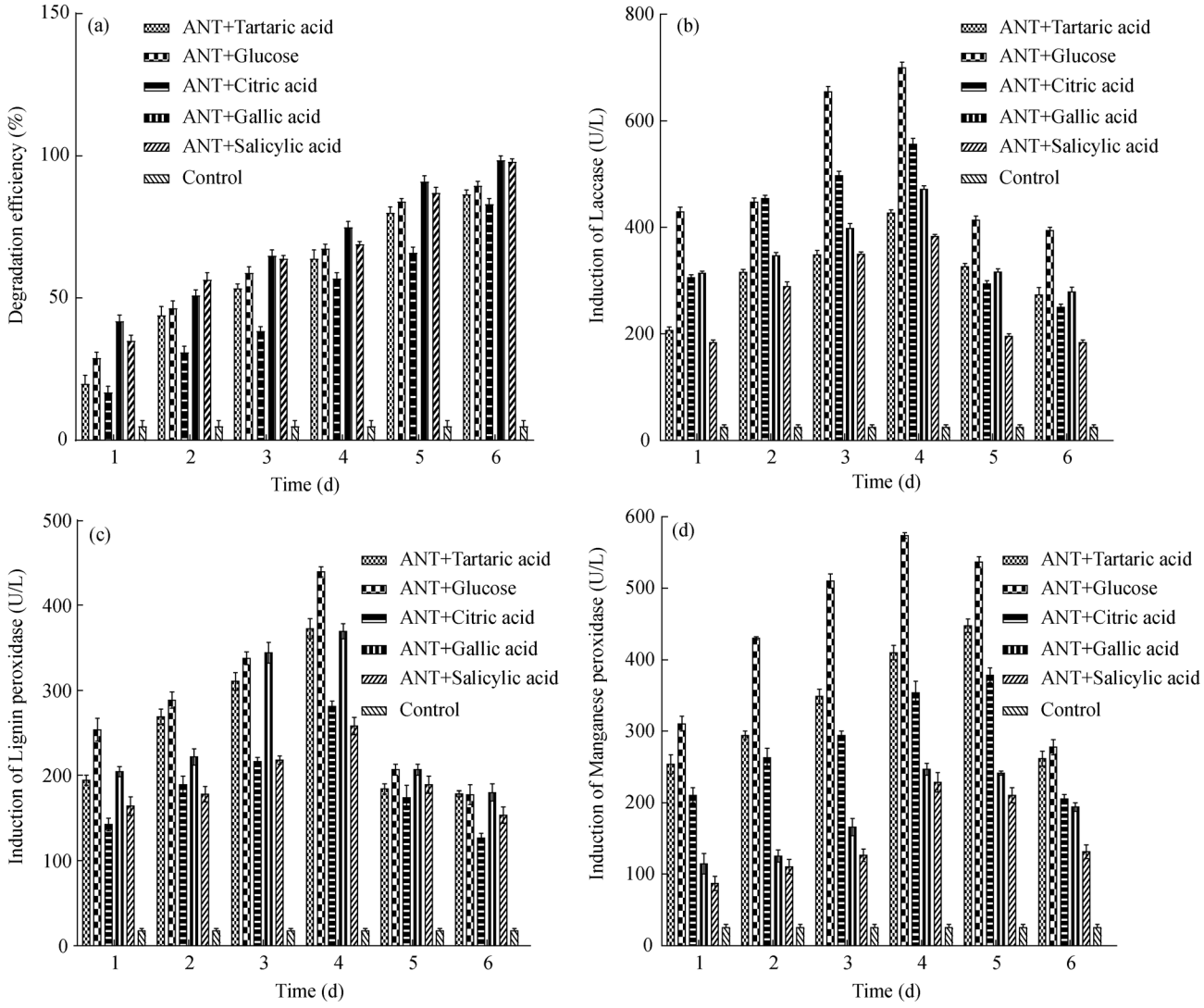

Fig. 3 Influence of organic acids and glucose on (a) anthracene degradation efficiency, (b) induction of laccase, (c) induction of lignin peroxidase and (d) induction manganese peroxidase.

3.4 Determination of metabolic fate with GC-MS and HPLC analyses

Four metabolites were obtained during the anthracene degradation by *A. sydowii* (Table SM1). The identity of four of the metabolites were confirmed with other spectra obtainable in the GC-MS library. The first metabolite; Anthracene-1,8,9 (2*H*,8a*H*,9a*H*)-trione (*m/z* = 226, *mw* = 226.2 g/mol) at R_t = 28.90 min was as a result of complete carboxylation of the three aromatic rings in the anthracene structure hence the formation of three different carboxyl groups (Table SM1). The second metabolite was formed as a result of the loss of cyclohexa-2,4-dien-1-one hence the formation of 2,4a-dihydronaphthalene-1,5-dione (*m/z* = 160, *mw* = 160.2 g/mol) at R_t = 21.73 min. The transformation of the second metabolites gave rise to two different compounds (tetrahydrofuran and cyclohex-2-ene-1,4-dione) which fused to form the third metabolite, 1,3,3a,7a-tetrahydro-2-benzofuran-4,7-dione (*m/z* = 152, *mw* = 152.1 g/mol) at R_t = 13.80 min (Table SM1). The fourth metabolite, 2-hydroxybenzoic acid (*m/z* = 138, *mw* = 136.1 g/mol) at R_t = 11.60 min was formed as a result of complete transformation into carboxylic acid (Fig. 4).

HPLC spectra (data not shown) of the metabolites after experiment revealed the formation of new peaks with retention times 1.417, 1.523, 1.771 and 1.884 min while 2 peaks with retention times 1.225 and 1.372 min were observed in the untreated anthracene (Table SM2).

3.5 Isotherm modeling and kinetic studies during anthracene degradation

Langmuir, Freundlich and Temkin isotherm kinetic studies were done to confirm the interactions and influence of anthracene concentration, *A. sydowii* mycelia pellets and temperature. The correlation coefficient (R^2 = 0.976)

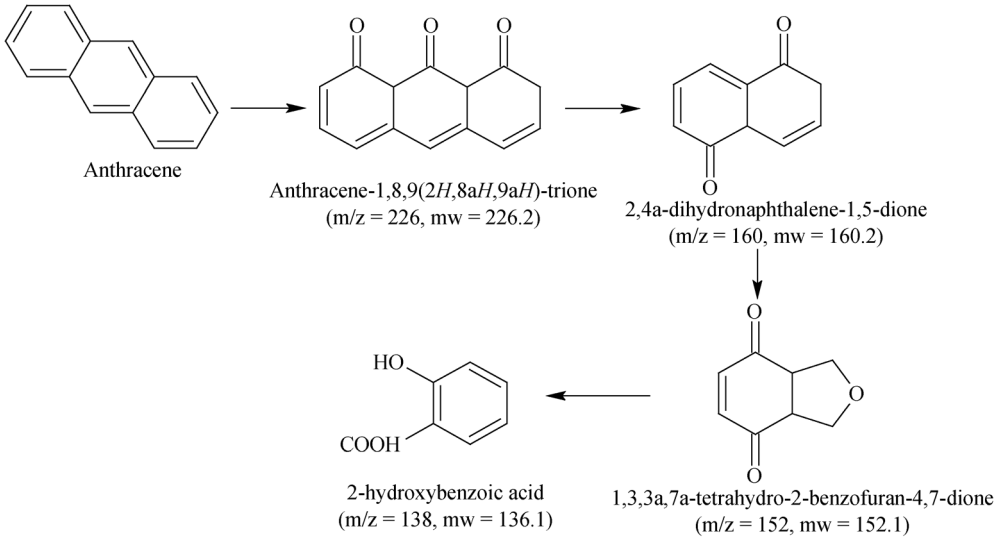

Fig. 4 Proposed metabolic pathway of anthracene degradation by *A. sydowii*

obtained when the experimental data were subjected to Langmuir model showed the best goodness of fit during linear regression analysis (Fig. SM1(a)). Likewise, isotherm correlation coefficients (R^2 = 0.948 and 0.982) obtained when the data was subjected to Freundlich and Temkin models respectively (Figs. SM1(b) and SM1(c)). Extrapolation from linear regression analysis revealed high correlation coefficients (R^2) and coefficient of determinations (AdjR^2) on fitting the experimental data into Langmuir and Temkin models. However, low correlation coefficient (R^2) was obtained on subjecting the experimental data to Freundlich isotherm model in comparison with Langmuir and Temkin model coefficients. The best goodness of fit followed this order for the three models: Temkin (0.982)>Langmuir (0.876)>Freundlich (0.948). The high coefficients of determination (R^2) obtained when the data were subjected to Temkin and Langmuir models suggest that temperature and fungi mycelia play a vital role during the removal of anthracene. The low coefficient of determination (R^2) when the experimental data was subjected to Freundlich model implies that the model is of less significance in elucidating the role played by fungal cells in biodegradation.

On the one hand, highest degradation rates (K_1 = 0.55 and 0.89 d^{-1}) was observed at 100 and 200 mg/L respectively on subjecting the experimental data to first-order kinetic model. On the other hand, highest half-lives ($t_{1/2}$ = 3.2 and 5.5 days) was observed at 400 and 500 mg/L respectively on subjecting the data to first-order kinetic model (Fig. SM2). Overall, there were decreasing degradation rates with increasing anthracene concentrations while increasing half-lives was recorded with increasing temperatures. It is noteworthy to state that at 200 mg/L anthracene concentration, the lowest half-life ($t_{1/2}$ = 1.4 days) was observed at a degradation rate constant (K_1 = 0.89 d^{-1}). The significance of this analysis is to determine the rate at which it takes each anthracene concentration to be reduced by half.

4 Discussion

The optimization of physicochemical parameters revealed that fungi exhibit potent degradation efficiency at low PAH concentrations. This result corroborated further corroborated the report of Zheng and Obbard (2002). Proliferation of fungi and extracellular enzymes induction is usually distorted at high PAH concentrations due to acute toxicity (Hadibarata and Kristanti, 2014). The temperature required for maximum degradation of anthracene by *A. sydowii* was 30°C. These results of this study corroborated the reports of Ike et al. (2019). High degradation efficiency observed at optimum temperature (30°C) is attributed to mass production of fungal mycelia, enzymes and kinetic energy in the degradation system. The low degradation efficiency recorded at high temperature is as a result of low oxygen solubility which in turn gave rise to reduced metabolic aerophilic activity by the fungus (Hwang et al., 2007). At much higher temperatures above 45°C especially for non-thermo-tolerant microbes, degradation rate might be negligible as a result of apoptosis and denaturation of fungal enzymes. Setting the PAH degradation systems at high temperature could negatively lead to the formation of highly toxic metabolic products (Müller et al., 1998). However, the relatively low PAH degradation efficiency at high temperatures is as a result of largely due to the distortion of the positively charges ions dissipated on the active sites of the fungus mycelia cells during degradation. The low efficiency recorded at high salinity is due to relatively inactive cells caused by an increase in osmotic

pressure in the fungus mycelia cells. The degradation efficiencies were significantly impeded and kept below 80% due to reduced activity of *A. sydowii* which was in disagreement with the work of Ye et al. (2011) when *A. fumigatus* was used in the removal of anthracene. From this study, we could deduce that the most significant anthracene degradation efficiency was observed at pH 6 and 7 than the efficiencies observed at more acidic and alkaline pHs (5, 8, 9 and 10) (Jacques et al., 2005). The anthracene degradation efficiency of *A. sydowii* could be attributed to the close affinity existing between the anions present in anthracene molecules and the positively charged cations on fungal mycelia pellets. The degradation of persistent organic pollutants like PAHs could be enhanced, influenced and controlled efficiently by adjusting the pH of the MSM. This study further buttressed the need to control the pH during PAH degradation in large scale field studies where there are constant and irregular changes in physicochemical parameters due to changing soil, weather, and atmospheric conditions. In addition, changes in pH poses a significant challenge during biodegradation by microbes due to fluctuation in pollutants' organic composition (Alva and Peyton, 2003). The enzyme analyses revealed that the addition of salicylic and gallic acids resulted in low inductions of Lac, LiP, and MnP. This is presumably due to the presence of benzene (aromatic) rings in the salicylic and gallic acids structures which interfered partly with enzyme production activities by *A. sydowii* (Cao et al., 2020). On average, citric acid supported the induction of the three enzymes followed closely by glucose, tartaric acid, salicylic acid and gallic acid. This is attributable to the abundance of carbon atoms available for rapid growth by the microorganism hence increase in enzyme induction. The results of this study corroborated the earlier work done by Ting et al. (2011) who reported the positive influence of organic acids in the induction of extracellular enzymes and degradation rates. The proliferation of microbes and induction extracellular, non-selective enzymes is further enhanced on addition of metabolic substrates to persistent organic pollutants biodegradation set up (Cerniglia and Sutherland, 2010). In addition, intermolecular interactions between of ions of metabolic substrates and fungi favors enhanced induction of enzymes which in turn, increases degradation efficiency. To enhance the proliferation of microorganism in growth media and degradation cultures, glucose is mostly used as sole carbon and energy sources (Cao et al., 2020). In this study, the citric acid of a higher molecular weight improved the removal of anthracene of low molecular weight. Collins et al. (1996) reported the role played by crude laccase in the biodegradation of anthracene. Other researches have equally reported that the efficiency of Lac and MnP in the oxidation of PAH during biodegradation (Punnapayak et al., 2009). Several recent global researches have been carried out on the use of fungi in anthracene biodegradation (Capotorti et al., 2005; Villemain et al., 2006, Ye et al., 2011; Birolli et al., 2018, Ike et al., 2019) under similar culture conditions (Table 1). In similar studies conducted by Capotorti et al. (2005) and Ye et al. (2011), 60% anthracene degradation efficiency was achieved in a culture of *Aspergillus terreus* and *Aspergillus fumigatus* although within 7 and 5 days respectively. Remarkable anthracene degradation efficiency (94%) was achieved in the work done by Villemain et al. (2006) in a culture of *Absidia fusca* within 5 days although of a lower anthracene concentration (50 mg/L) in comparison with this present work. Ike et al. (2019) also reported significant anthracene degradation efficiency (71.8%) in a culture of *Leucoagaricus gongylophorus* with laccase playing a vital role however the anthracene concentration is much lower (10 mg/L) (Table 1). Furthermore, Arun et al. (2008) reported the anthracene degradation efficiency of 15.6%, 22.4%, 18%, 31.7% and 2.4% by *Pycnoporus sanguineus*, *Coriolus versicolor, Pleurotus ostreatus, Fomitopsis*

Table 1 Comparison of anthracene biodegradation by *A. sydowii* strain bpo1 with reference to other fungi in similar conditions

PAH	Fungi	Concentration (mg/L)	Temperature (°C)	Degradation efficiency (%)	Time (days)	Enzymes	Reference
Anthracene	*A. sydowii*	200	30	99.8	3	MnP, LiP, Lac	Present study
	A. fumigatus	10	*30*	60	*5*	LiP	Ye et al., 2011
	A. terreus	50	30	60	7	ND	Capotorti et al., 2005
	A. fusca	50	22	94	5	ND	Villemain et al., 2006
	L. gongylophorus	10	30	71.8	11	Lac	Ike et al., 2019
	F. palustris	1000	24	31.7	20	MnP, LiP, Lac	Arun et al., 2008
	D. elegans	1000	24	2.4	20	MnP, LiP, Lac	Arun et al., 2008
	P. ostreatus	1000	24	19	20	MnP, LiP, Lac	Arun et al., 2008
	C. versicolor	1000	24	22.4	20	MnP, LiP, Lac	Arun et al., 2008
	P. sanguineus	1000	24	15.6	20	MnP, LiP, Lac	Arun et al., 2008

Notes: ND, Not determined.

palustris and *Daedalea elegans* respectively within 20 days of incubation. However, this present study reported anthracene degradation efficiency (99.5%) by *A. sydowii* achieved within 3 days with a much higher anthracene concentration (200 mg/L) and Lac, LiP and MnP playing significant role during biodegradation (Table 1). The formation of these metabolites was propelled by the activities of enzymes produced by the fungus during degradation. Although this study was unable to ascertain the specific enzymes responsible for decarboxylation, carboxylation, ring and asymmetric cleavages of the metabolites. However, the transformation, decarboxylation and polymerization of most phenolic, non-phenolic and aromatic compounds through oxidation is attributed to the action of laccases and lignin peroxidases which are non-selective and non-specific (Majcherczyk et al., 1998). Furthermore, Bogan and Lamar (1996) reported that crude LiP, Lac and MnP has been implicated in the biotransformation of anthracene and other PAHs to less toxic metabolic products. The production of aryl cation radicals from organic pollutants to form quinones is attributed to the radical oxidation action of ligninolytic enzymes (Cerniglia, 1997). Although, anthraquinone was not part of the intermediate metabolites obtained in this study which constituted a remarkable difference and sharp deviation from the report of Hammel et al. (1991). However, the further breakdown of the intermediate quinones obtained in the study resulted in a less toxic benzoic acid in comparison with the phthalic acid obtained in the report of Ye et al. (2011).

The results showed that the experimental data is best analyzed with Langmuir and Temkin models which suggest that the active sites on the mycelia pellets of *A. sydowii* were responsible for binding the negatively charged ions of the anthracene molecules, hence efficient biodegradation. In addition, the Langmuir isotherm model elucidated and affirmed the monolayer nature exhibited by *A. sydowii* mycelia cells during anthracene degradation. Furthermore, the Temkin isotherm model affirmed the significant role played by temperature during anthracene by *A. sydowii.* The Temkin isotherm modeling confirmed the heterogeneous feature exhibited by *A. sydowii* during anthracene degradation (Juang et al., 1996).

5 Conclusions

The anthracene degradation efficiency by *A. sydowii* strain bpo1 was enhanced with the optimisation of pH, salinity, temperature, biomass, anthracene concentration and on addition of citric acid. On addition of metabolic substrates, 99.8% degradation efficiency was achieved as a result of improved inductions in Lac, LiP and MnP enzymes. This study further revealed four different metabolites of anthracene obtained after GC-MS analysis. *A. sydowii* is a promising tool and an excellent candidate for the bioremediation of PAH in the environment. Further studies are however recommended to explore large scale applicability of PAH's degradation by different fungi strains in bioreactor and in solid-state fermentation systems.

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