Isolation, characterization and optimization of chrysene degradation using bacteria isolated from oil-contaminated water

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ABSTRACT

Polyaromatic hydrocarbons (PAHs) are uncharged, non-polar molecules generated from natural and anthropogenic activities, where the emissions from anthropogenic activities predominate. Chrysene is a high molecular weight PAH, which is found to be highly recalcitrant and mutagenic in nature. The aim of this study was to isolate chrysene-degrading microorganisms from oil-contaminated water and to enhance their degradative conditions using design expert. From the various samples collected, 19 bacterial strains were obtained through enrichment culture and the one which showed highest activity was identified by 16S rRNA sequencing as Bacillus halotolerans. Under optimum conditions of 100 mg/l chrysene concentration, 1,000 mg/l nitrogen source, pH 6, Bacillus halotolerans exhibited 90% chrysene degradation on sixth day. Positive results for the enzymes lactase and catechol 1,2 dioxygenase confirmed the ability for chrysene degradation by the isolated strain. Major metabolic intermediate determined in GCMS analysis was dioisooctyl phthalate. Hence it can be concluded that Bacillus halotolerans can be a promising candidate for the removal of HMW hydrocarbons from contaminated environments.

Key words: Bacillus halotolerans, biodegradation, chrysene, design expert, poly aromatic hydrocarbon, 16S rRNA sequencing

INTRODUCTION

Polyaromatic hydrocarbons (PAHs) are non-polar lipophilic organic compounds present in the atmosphere as a result of incomplete combustion of automotive waste, organic matter, refineries, power plants, tobacco smoke, forest fires and smoked foods (Samanta et al. 2002). Due to their cytotoxic, mutagenic, and carcinogenic effects, PAHs are classified as dangerous compounds. In course of storage and refining of crude oil, oil refineries contain a vast amount of oily sludge in tanks. The oily sludge comprises of saturated and aromatic hydrocarbons, asphaltene, resins and heavy metals. The extraction studies of petroleum sludge revealed the presence of different PAHs that are toxic in nature (Siebielska 2014). According to the USEPA, 16 PAHs are priority pollutants out of which 8 are typically considered probable cancer-causing agents. This includes benzo[α]-fluoranthene, benzo[h]fluoranthene, benzo[a]anthracene, dibenz[a,h]anthracene, benzo[a]pyrene, benzo[g,h,i]-perylene, indenol[1,2,3,c-d]pyrene and chrysene (Tam et al. 2003).

Chrysene is an example of high molecular weight Polyaromatic hydrocarbon. It is less water-soluble (0.006 mg/l) and carcinogenic. Four fused benzene rings are used to structure chrysene, with the molecular formula C₁₈H₁₂. Chrysene fluoresces blue but appears sometimes yellow because of its impurities. Chrysene is formed when wood, fossil fuels, creosote, coal tar, and cigarette smoke are partially burned; each cigarette contains 60 ng of chrysene (Hecht et al. 1974).
Due to its cost-effectiveness, capacity to treat a variety of contaminants, non-toxic secondary by-merchandise technology, and green technology, biodegradation appears to be a promising method for removing PAH from contaminated environments (Sundararaju et al. 2020). Hydrocarbons with two or three aromatic rings (low molecular weight) are readily biodegradable under the right conditions. PAHs with four or more rings, on the other hand, are resistant to bioremediation and can survive in nature for long periods of time (Juhasz et al. 1997). Very few researches have reported the utilization and complete mineralization of chrysene by microorganisms (Nayak et al. 2011). This includes Alcaligenes faealis (John et al. 2012), Achromobacter xylosoxidans (Ghevariya et al. 2011), Pseudoxanthomonas sp. (Nayak et al. 2011), Polyporus sp. S153 (Hadibarata et al. 2009), Bacillus sp. (Dhote et al. 2010), Pseudomonas sp. (Zang et al. 2021), and Paracoccus (Zhang et al. 2004). The majority of the studies use either a fungal culture or bacterial consortiums for the biodegradation of chrysene by using it as the sole carbon substrate (Nwinyi et al. 2017). However, there is restrained statistics on metabolic intermediates of chrysene degradation. Therefore, the objective of the study was to segregate a bacterial strain that had the ability to develop in pure culture with chrysene as a sole carbon source and to characterize the intermediates by chromatographic method. In this study, chrysene-degrading microorganisms were isolated from oil-contaminated sites and characterized, medium optimization using RSM was performed and metabolic intermediates were detected.

**MATERIALS AND METHODS**

**Chemicals**

Chrysene was bought from Sigma, and all the other chemicals used in this study were of laboratory quality with an analytical standard exceeding 98.0 percent.

**Isolation and characterization of chrysene-degrading bacteria**

Samples of water were collected at Ennore Beach, Chennai that was polluted in 2017 as a result of the oil spill. These samples have been transferred to sterile bottles for further analysis and stored in a refrigerator. Standard serial dilution protocol was followed and dilutions of $10^{-4}$ and $10^{-5}$ were used for isolating chrysene degrading bacteria. On serial dilution of each sample; the discrete colonies obtained were sub-cultivated on nutrient agar plate. An enriching cultivation was carried out with a minimum salt media (MSM) and a chrysene supplement as sole carbon source in order to isolate chrysene-degrading bacteria. The minimal salt media contained 1 g of NH$_4$NO$_3$; 1 g of KH$_2$PO$_4$; 1 g of K$_2$HPO$_4$; 0.1 g of MgSO$_4$.7H$_2$O; 0.1 g of FeCl$_3$.6H$_2$O and 0.01 g of CaCl$_2$.2H$_2$O per litre (Nwinyi et al. 2017). Pure chrysene solubility is very strong in acetone in comparison with other solvents. Each conical flask containing 50 ml media was transferred with 5 mg of dissolved chrysene in acetone (Nwanna et al. 2006). Before inoculation, acetone was permitted to evaporate and 2 ml of inoculum have been transferred to each flask. At room temperature in an orbital shaker at 180 rpm, the cultures were incubated. Microbial growth was detected by taking 600 nm of absorption because, unlike other wavelengths, it is not harmful to microbes. UV Visible Spectrophotometer was used to note the absorbance every day, until the absorption rate continuously declined (Tian et al. 2018). The growth was compared with a glucose-containing and abiotic control medium. The bacterial strain showing best results was chosen for further studies.

**Identification of chrysene-degrading bacteria**

DNA was extracted (phenol-chloroform) from the culture, electrophoresed in 1% Agarose, and observed under UV light. Specific primers (27F 5’-AGAGTTTGATCCTGGCTCAG-3’; 1492R 5’-GGTTACCTTGTTACGACTT-3’) have been used to amplify the 16S region and amplicon is tested for suitable size by visualisation of agarose gel. Amplicon gel has been purified by a commercial column cleaning kit (Invitrogen, USA); sequencing was carried out using an ABI 3730 XL cycle sequencer with forward and reverse primers (Hadibarata et al. 2009). After trimming the low-quality bases, the forward and reverse sequences were combined to form a contig. The maximum identity score was used in the sequence analysis, which was done using BLAST from the NCBI database. Multiple sequence alignment (Clustal W2) and a phylogenetic tree were constructed using the E value of the topmost sequences.

**Optimization of media components for enhanced biodegradation**

The response surface method, a statistical approach, was used to investigate the interaction of multiple parameters (Kaushik et al. 2006). Five ranges of each parameter were provided for MSM and OD values were
noted for continuous days before the design expert software was used for. Minimal salt media was prepared with different concentrations of chrysene (50, 100, 150, 200 and 250 mg/l), varying concentrations of nitrogen source (500, 1,000, 1,500, 2,000 and 2,500 mg/l) and different pH (3, 5, 7, 9 and 11). It was used to inoculate bacterial cultures. They were incubated separately and absorbance values were observed for 15 days. Higher and lower levels were achieved after initial optimisation and values were placed in design expert software version 11. In the software Design of Experiments, the experiment was planned and carried out using a factorial model. Four different arithmetical factors were tested in triplicate at two widely spaced levels, +1 (high level) and –1 (low level) (Kaushik et al. 2006).

**Extraction of residual chrysene and identification of chrysene metabolites**

To investigate the bacterial strain’s degradation efficiency, researchers used high-performance liquid chromatography (HPLC) to quantify chrysene. Centrifugation at 10,000 rpm for 10 minutes separated the cells from the liquid culture, and the clear liquid was used for HPLC (Agilent 1260 infinity II) analysis. Gas chromatography was used in conjunction with mass spectrometry to identify the metabolites. The supernatant was extracted with hexane (Nwinyi et al. 2013, 2017) and subjected to GC-MS Shimadzu QP 2010S fitted with a Rxi-5Sil MS column (30 m length, id: 0.25 mm).

**Enzyme assays**

Enzymatic activity in the culture medium was determined both intra- and extracellularly. The cells were separated from the liquid by centrifuging at 10,000 rpm for a duration of 10 min. under cooling conditions. The supernatant was directly taken for extra-cellular enzyme assay. The pellets were resuspended in the same buffer after being cleaned twice with 30 mL of 50 mM sodium phosphate buffer (pH 7.0). The sample was then sonicated for 5 min.at 50 amp. At 8,000 rpm at 4 °C, the sonicated sample was centrifuged for 30 min. The cell debris were removed after centrifugation, and the supernatant was collected for intracellular enzyme testing (Nadaf & Ghosh 2011). A blank was also prepared that contains distilled water instead of culture filtrate for all the assays.

**Laccase assay**

Both supernatant and pellet samples were screened for the presence of laccase enzyme. A reaction mixture of 900 μl was prepared by taking equal volumes of 1 mM guaiacol, 0.2 M sodium acetate buffer (pH 4.5) and culture filtrate. Presence of laccase can be confirmed by the formation of reddish-brown colour due to the oxidation of guaiacol by the enzyme (Muthukumarasamy & Murugan 2014).

**Catechol 1,2dioxygenase and 2,3 dioxygenase assays**

Reaction mixture contained 4 ml of 0.02 M Tris-HCl buffer (pH 8.0), 4 ml of 0.01 M catechol as substrate and culture filtrate. Appearance of bright yellow colour indicates the presence of catechol 2, 3dioxygenase enzymes. In the absence of colouration, the sample was taken for further testing.1 g of (NH4)2SO3, few drops of 1% of freshly prepared sodium nitroprusside solution and 0.5 ml of Ammonia were added to the reaction mixture. Appearance of deep violet colour indicates the presence of catechol 1, 2dioxygenase (free et al. 1958).

**Lipase assay**

The plate was streaked with a Tributyrin agar base containing olive oil and inoculum. For 48 hours, the plates were incubated. The organism’s lipase breaks down the olive oil, leaving a clear halo around the areas where lipase was produced, indicating the organism’s ability to do so (Ertuğrul et al. 2007).

**Manganese peroxidase assay**

Bacterial culture for the assay was prepared by inoculating into MSM with chrysene and incubated for 24 h. Hydrogen peroxide was added drop by drop to one millilitre of screening media and an equal volume of broth until the pink colour appeared. Appearance of pink colour shows the presence of the enzyme (Raja Rao & Kavya 2014).
RESULTS AND DISCUSSION

Isolation and screening of culture

Samples of different dilutions were placed on an agar plate following serial dilution. Single colonies were then picked and moved to a minimum salt-medium broth containing 100 mg/l of chrysene. Growth of the organism was continuously monitored for all the samples until the bacterial growth declined. Based on the OD values, the growth curve was plotted. Though an initial lag phase was observed for 2 days, the cells later turned to log-arithmetic phase. The growth of bacteria, with chrysene as sole carbon source was compared with both biotic and abiotic controls. The organism grown on glucose-containing medium serves as the biotic control. This was done for all the 19 samples isolated. Out of these, the one which showed a good growth profile was selected as the best chrysene degrader and named as S10 (Figure 1).

The selected bacterial strain (S10) was further taken for morphological and biochemical studies and the properties of the isolate are given in Table 1. The strain S10 was an aerobic gram-positive bacterium and the optimum growth was in the range of 30–35 °C. Methyl red (MR) and Indole tests were negative whereas Voges Proskauer test was positive. It could assimilate citrate and catalase positively. No urease activity was detected. Positive results are shown in Figure 2.

PCR amplification and sequence alignment of 16S rRNA

Genomic DNA was isolated from the selected strain (S10) and the 16S region was amplified for characterization. Amplification and sequencing of the gel purified product was done using the respective primers. The 16S region was sequenced very well and was excellent for predicting the identity of the organism (Dhote et al. 2010). 1114 base pair contig was obtained for 16S region after trimming off the low-quality bases and good consensus was obtained. The amplified sequence was further analysed and compared with other sequences available in GenBank database using BLAST. BLAST annotation indicates an identity of 96% of Bacillus halotolerans (Bacillus axarquiensis) with a value of 0-E and 98% of the nucleotide homology queries. Hence the isolate was most similar to Bacillus halotolerans.

Table 1 | Biochemical characterization (S10)

| Test                  | Result |
|-----------------------|--------|
| Gram staining         | +      |
| Indole Test           | –      |
| Methyl Red Test       | –      |
| Voges Proskauer Test  | +      |
| Citrate test          | +      |
| Catalase Test         | +      |
| Urease Test           | –      |
Phylogenetic analysis

Phylogenetic analyses showed that the isolated strain was very closely related to *Bacillus halotolerans* in the genetic relationship. Therefore, S10 was identified as *Bacillus halotolerans* based on the 16S rRNA sequence analysis along with the results of physio-biochemical tests and morphological observations. The sequence data were submitted to GenBank with the accession number **MK439524** (https://www.ncbi.nlm.nih.gov/nuccore/1562068489). Phylogenetic tree was constructed comparing the closely related species and is shown in Figure 3.

**Figure 2** | morphological and biochemical test (only positive tests included).

**Figure 3** | Phylogenetic tree of S10 based on 16S rRNA sequencing.
Optimization of media components

Response surface methodology is an excellent tool for optimizing various parameters in bioprocess (Kaushik et al. 2006). Percentage of biodegradation can be enhanced through the optimization of media components. Four parameters were selected for the present study and these include pH, growth, chrysene concentration and time period. Five ranges of each parameter were chosen for the study as part of the initial screening. The growth curve was plotted, and two parameters’ limits were chosen and assigned to the design expert limits.

Figure 4(a) shows the role of pH on the growth of organism and chrysene degradation. The different pH conditions affected the degrading activity significantly over the whole incubation period. The bacterial growth was determined to be maximum at pH 5 and hence found favourable for the degradation of chrysene. From Figure 4(b), it was clear that the nitrogen concentration has an impact on the growth of the organism. The bacterial growth was comparatively less at higher and lower concentrations of nitrogen. Even though the growth was good at 2,000 mg/l, a better growth curve could be observed at 1,000 mg/l.

The growth of an organism with chrysene at different time intervals is shown in Figure 4(c). Since we are using MSM with chrysene as the sole carbon source, there was a lag in the growth of the organism. After two days, we could observe an exponential increase in the growth of the organism. The growth was at its best on the sixth day and then slowly declined.

Response of the bacterial growth towards varying concentration of chrysene was shown in Figure 4(d). From the results it is clear that high levels of chrysene were found to be inhibitory, decreasing the growth of the organism. Lower concentrations of chrysene also adversely affected the bacterial growth. Optimum growth of bacteria was observed when cells were supplemented with 100 mg/l of chrysene.

Optimization using design expert

The parameters taken for optimization were chrysene concentration, pH, number of days, and concentration of nitrogen sources. The upper and lower limits of each variables were selected based on early optimization and the values obtained were put into the design expert (Kaushik et al. 2006). The limits for various factors were shown in the Table 2.

Based on the initial screening, a total of twenty-four experimental runs were provided by the software. Optical density (OD) was measured at 600 nm for all the combinations. All the trials were done in triplicates and average value reported as Response R1.

Figure 4 | (a) Bacterial growth at different pH values; (b) Effect of varying concentration of nitrogen on growth; (c) Bacterial growth (OD₆₀₀) v/s time (hour); (d) Effect of chrysene concentration on growth.
ANOVA for optimization study

The model F-value was found to be 43.90 which implies that the model is significant (Table 3). The model terms are significant when the P-values are less than 0.05. In the present study, factors A, B, AB, AC and AD are significant model terms. Model terms C and D were found to be insignificant as the P-values are higher than 0.1000.

Fit statistics

The Predicted $R^2$ value and adjusted $R^2$ were found to be 0.8886 and 0.9289 respectively. Since the difference between predicted and adjusted value is less than 0.2, both are in good correlation (Table 4). The signal to noise ratio was expected to be more than 4 for an adequate signal. Here the ratio is 18.4629 which indicates that the model is significant.

Coefficient estimates for the design

The coefficient estimate corresponding to each factor is presented in the Table 5. The average of responses from all the runs were represented as intercept. 95% CI low and high values were significant. Variance Inflation Factor (VIF) is significant for the model as it is less than 10.

Different combinations of selected variables were predicted from surface plots. The optimal conditions of the selected parameters were obtained as graphs based on the response inserted to each run. From Figure 5(a), it is clear that the nitrogen concentration has an impact on the microbial growth and thereby affects the removal of chrysene. The optimum growth for the organism was obtained at 1,000 mg/l. Microbes are highly sensitive to the

Table 2 | Optimization factors

| Factor | Name         | Low level | High level |
|--------|--------------|-----------|------------|
| A      | Chrysene     | 50 mg/l   | 100 mg/l   |
| B      | Nitrogen     | 1,000 mg/l| 2,000 mg/l |
| C      | pH           | 7         | 5          |
| D      | Incubation period | 144 hours | 120 hours |

Table 3 | Anova analysis

| Source                  | Sum of squares | df | Mean square | F-value | p-value   |
|-------------------------|----------------|----|-------------|---------|-----------|
| Model                   | 0.1923         | 7  | 0.0275      | 43.90   | <0.0001   |
| Chrysene concentration (A) | 0.1655      | 1  | 0.1655      | 264.45  | <0.0001   |
| Nitrogen concentration (B) | 0.0067      | 1  | 0.0067      | 10.71   | 0.0048    |
| pH (C)                  | 0.0001         | 1  | 0.0001      | 0.0911  | 0.7666    |
| Incubation period (D)   | 0.0000         | 1  | 0.0000      | 0.0560  | 0.8159    |
| Chrysene -Nitrogen (AB) | 0.0061         | 1  | 0.0061      | 9.77    | 0.0065    |
| Chrysene -pH (AC)       | 0.0047         | 1  | 0.0047      | 7.56    | 0.0142    |
| Chrysene -incubation period (AD) | 0.0092     | 1  | 0.0092      | 14.64   | 0.0015    |
| Pure error              | 0.0100         | 16 | 0.0006      |         |           |
| Cor total               | 0.023          | 23 |             |         |           |

Table 4 | Statistics

| Standard Deviation | 0.0250 | R2 | 0.9505 |
|--------------------|--------|----|--------|
| Mean               | 0.4161 | Adjusted R2 | 0.9289 |
| C.V.%              | 6.01   | Predicted $R^2$ | 0.8886 |
|                    |        | Adequate Precision | 18.4629 |
hydrogen ion concentration and the response to changing pH is shown in Figure 5(b). The organism shows optimum growth at pH 5. Figure 5(c) represents the growth response of the organism at different time intervals and it can be seen that the growth rate of the bacteria increased until day 6, after which a decline in growth was observed. The optimum medium conditions for growing microorganisms, such as ideal pH, chrysene concentration, nitrogen concentration and incubation time, are 5, 100 mg/l, 1,000 mg/l, and sixth day, respectively, according to the graph.

Table 5 | Coded factors

| Factor         | Coefficient estimate | Degrees of freedom | Standard error | 95% CI low | 95% CI high | VIF |
|----------------|----------------------|--------------------|----------------|------------|-------------|-----|
| Intercept      | 0.4161               | 1                  | 0.0051         | 0.4053     | 0.4270      |     |
| A-Chrysene concentration | 0.0830               | 1                  | 0.0051         | 0.0722     | 0.0939      | 1.0000 |
| B-Nitrogen concentration | −0.0167             | 1                  | 0.0051         | −0.0275    | −0.0059     | 1.0000 |
| C-pH           | 0.0015               | 1                  | 0.0051         | −0.0093    | 0.0124      | 1.0000 |
| D-incubation period | −0.0012              | 1                  | 0.0051         | −0.0120    | 0.0096      | 1.0000 |
| AB             | −0.0160              | 1                  | 0.0051         | −0.0268    | −0.0051     | 1.0000 |
| AC             | −0.0140              | 1                  | 0.0051         | −0.0249    | −0.0032     | 1.0000 |
| AD             | 0.0195               | 1                  | 0.0051         | 0.0087     | 0.0304      | 1.0000 |

Figure 5 | (a) Showing the interactions between chrysene and nitrogen concentration on microbial growth; (b) Showing the interactions between chrysene concentration and pH on microbial growth; (c) Showing the interactions between chrysene concentration and incubation period on microbial growth.
Extraction of residual chrysene and identification of chrysene metabolites

Following the biodegradation step, HPLC was conducted on the samples to detect chrysene residue with standard chrysene (1 mg/ml). The instrument’s detection limit is 0.1 ppm. The initial amount of chrysene in the medium was 100 ppm, and the test results revealed 9.74 ppm chrysene in the medium, indicating that Bacillus halotolerans degraded 90 percent of the chrysene by the sixth day.

During the chrysene biodegradation, major metabolic intermediates were identified by GCMS and are 1-hydroxy 2-naphthoic acid, salicylic acid, 1,2-dihydroxynaphthalene (Hadibarata et al. 2009), catechol (Hidayat & Tachibana 2013), chrysenequinone, phthalic acid, protocatechuic acid, gentisic acid, hydroxyphenanthroic acid (Samanta et al. 2002). The main metabolite detected by GCMS in this study was esters of phthalic acid, which had an Rf value of 25.107 and an M/z ratio of 149.05 (Figure 7). In the aromatic degradation pathway, phthalates are found to be common intermediates. Other intermediates detected in the spectrum include eicosane (Rf-12.066), 2, 4 di butylphenol (Rf-12.46), Isobutyl phthalate (Rf-17.403), Dibutyl Phthalate(Rf-18.63), Henecicosane(Rf-19.121), Eicosane,7-hexyl-(Rf-21.444), Heptadecane (Rf-22.531), Eicosane,10-methyl-(Rf-23.577), 2-methyloctacosane(Rf-24.658) and Tritetracontane (Rf-25.921).

Figure 6 | HPLC chromatogram.

Figure 7 | GCMS metabolites.
Analysis of enzyme activity

*Bacillus halotolerans* was grown in a chrysene-containing medium, and the enzyme activity was determined. Positive results were obtained for laccase and catechol 1,2-dioxygenase, but no lipase or manganese peroxidase were detected in the medium.

Catechol 1,2 dioxygenase assay

Formation of purple colour ring in β keto adipic acid test indicated the presence of catechol 1,2 dioxygenase enzyme (Figure 8(a)). The intracellular sample gave positive result for catechol 1,2 dioxygenase. Catechol dioxygenase is the common enzyme in the aromatic degradation pathway and can be used to metabolize chrysene via either the meta or ortho cleavage pathways. Oxidation of aromatic ring is the first step in chrysene degradation. This is catalysed by oxygenase enzymes to produce catechol via pyridine nucleotide-dependent dehydrogenation reaction (Hidayat & Tachibana 2013).

Laccase assay

Reddish-brown colour was formed within 15 min of incubation. This indicated the presence of laccase enzyme in the extracellular sample (Figure 8(b)). The crude laccase enzyme present in culture filtrate oxidized guaiacol and generates free radicals to produce the reddish-brown colour. The positive results reconfirm the role of laccase in the degradation of chrysene as already reported (Hidayat & Tachibana 2013)

As shown in Figure 8(c), There was no clear zone near the bacterial growth. This indicated the absence of lipase enzyme in the specified culture conditions. No colour change was observed in Manganese peroxidase assay which indicated the absence of the enzyme (Figure 8(d)). Even though, there were reports (Hadibarata et al. 2009) showing the presence of lipase and manganese peroxidase in the biodegradation sample, we could not get a positive result. This might be due to the extreme low concentrations of the enzyme.

![Figure 8](http://iwaponline.com/wst/article-pdf/doi/10.2166/wst.2021.227/898676/wst2021227.pdf)

Figure 8 | (a) Assay for catechol 1,2 dioxygenase (A- blank, B and C-culture filtrate containing enzyme sample); (b) Laccase assay (A – Blank, B- culture filtrate containing enzyme sample); (c) Lipase assay (d) Manganese peroxidase assay.
CONCLUSION

The main objective of the present study was to search for potent strains capable of degrading chrysene, a high molecular weight hydrocarbon from oil contaminated water. A Good number of organisms capable of growing in the presence of chrysene were isolated. However, the organism with best growth under the conditions of this work was selected for further studies. The strain was confirmed to be Bacillus Halotolerans by physio-chemical test and 16S rRNA sequence analysis. The optimum conditions of chrysene and nitrogen concentration were 100 mg/l and 1,000 mg/l respectively. A maximum degradation rate of 90% was obtained on the 6th day and at a pH 5. One of the prevalent intermediate detected was dioctyl phthalate with an m/z ratio of 149. Presence of catechol 1,2dioxygenase and laccase confirmed bacillus halotolerans as a potential degrader of chrysene. This is in agreement with the reports by Hadibarata et al. 2009. These enzymes degrade PAHs by forming phthalates and catechol. catechol 1,2dioxygenase catalyses the oxidation of catechol to muco-degrader of chrysene. This is in agreement with the reports by Vaidya et al. 2018. However more studies are needed to elucidate the entire degradation pathway. Bacillus halotolerans can be recommended for the use in situ bioremediation of water contaminated with PAHs. This is a great promise towards reducing the impact of high molecular weight PAHs.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

REFERENCES

Dhote, M., Juwarkar, A., Kumar, A., Kanade, G. S. & Chakrabarti, T. 2010 Biodegradation of chrysene by the bacterial strains isolated from oily sludge. World Journal of Microbiology and Biotechnology 26 (2), 329–335.

Ertugrul, S., Dünne, G. & Takač, S. 2007 Isolation of lipase producing Bacillus sp. from olive mill wastewater and improving its enzyme activity. Journal of Hazardous Materials 149 (3), 720–724.

Free, H. M., Smeby, R. R., Cook, M. H. & Free, A. H. 1958 A comparative study of qualitative tests for ketones in urine and serum. Clinical Chemistry 4 (4), 325–330.

Ghevariya, C. M., Bhatt, J. K. & Dave, B. P. 2011 Enhanced chrysene degradation by halotolerant Achromobacter xylosoxidans using Response Surface Methodology. Biosource Technology 102 (20), 9668–9674.

Hadibarata, T., Tachibana, S. & Itoh, K. 2009 Biodegradation of chrysene, an aromatic hydrocarbon by Polyporus sp. s133 in liquid medium. Journal of Hazardous Materials 164 (2–3), 911–917.

Hecht, S. S., Bondinell, W. E. & Hoffmann, D. 1974 Chrysene and methylchrysene: presence in tobacco smoke and carcinogenicity. Journal of the National Cancer Institute 55 (4), 1121–1133.

Hidayat, A. & Tachibana, S. 2013 Simple screening for potential chrysene degrading fungi. The 3rd International Conference on Biological Science. Knowledge publishing service, 2 (2015) 364–370.

John, R. C., Essien, J. P., Akpan, S. B. & Opkokwasili, G. C. 2012 Polycyclic aromatic hydrocarbon-degrading bacteria from aviation fuel spill site at Ibeno, Nigeria. Bulletin of Environmental Contamination and Toxicology 88 (6), 1014–1019.

Juhasz, A. L., Britz, M. L. & Stanley, G. A. 1997 Degradation of Benzo[a]pyrene,dibenz[a,h]anthracene and coronene by Burkholderia cepacia. Water Science and Technology 36 (10), 45–51.

Kaushik, S., Saran, S., Isar, J. & Saxena, R. K. 2006 Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by Aspergillus carneus. Journal of Molecular Catalysis B: Enzymatic 40 (3), 121–126.

Muthukumarasamy, N. P. & Murugan, S. 2014 Production, purification and application of bacterial laccase: a review. Biotechnology 15 (5), 196–205.

Nadaf, N. H. & Ghosh, J. S. 2011 Purification and characterization of catechol 1,2-dioxygenase from Rhodococcus sp NCIM 2891. Research Journal of Environmental and Earth Sciences 3 (5), 608–615.

Nayak, A. S., Sanjeev Kumar, S., Santosh Kumar, M., Anjaneya, O. & Karegoudar, T. B. 2011 A catabolic pathway for the degradation of chrysene by Pseudoxanthomonas sp. PNK-04. FEMS Microbiology Letters 320 (2), 128–134.

Nwanna, I. E. M., George, G. O. & Olusoji, I. M. 2006 Growth study on chrysene degraders isolated from polycyclic aromatic hydrocarbon polluted soils in Nigeria. African Journal of Biotechnology 5 (10), 823–828.
Nwinyi, O. C., Picardal, F. W., An, T. T. & Amund, O. O. 2013 Aerobic degradation of naphthalene, fluoranthene, pyrene and chrysene using indigenous strains of bacteria isolated from a former industrial site. *Canadian Journal of Pure and Applied Sciences* 7 (2), 2303–2314.

Nwinyi, O. C., Amund, O. O., Chinedu, S. N. & Chioma, N. M. 2017 Physiological behavior of newly isolated Bacterium CB1 and Stenotrophomonas maltophilia strain CB2 on Chrysene, Pyrene, Naphthalene and Fluoranthene. *Romanian Biotechnological Letters* 22 (2), 12409.

Raja Rao, P. & Kavya, P. 2014 Production, isolation and purification of peroxidase using Bacillus Subtilis. In *1st International Congress on Environmental, Biotechnology, and Chemistry Engineering*. IACSIT Press, Singapore, Vol. 64(5).

Samanta, S. K., Singh, O. V. & Jain, R. K. 2002 Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. *Trends in Biotechnology* 20 (6), 243–248.

Siebielska, I. 2014 Comparison of changes in selected polycyclic aromatic hydrocarbons concentrations during the composting and anaerobic digestion processes of municipal waste and sewage sludge mixtures. *Water Science and Technology* 70 (10), 1617–1624.

Sundararaju, S., Arumugam, M. & Bhuyar, P. 2020 Microbacterium sp. MRS-1, a potential bacterium for cobalt reduction and synthesis of less/non-toxic cobalt oxide nanoparticles (Co$_3$O$_4$). *Beni-Suef University Journal of Basic and Applied Science* 9 (44).

Tam, N. F. Y., Guo, C. L., Yau, C., Ke, L. & Wong, Y. S. 2003 Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by microbial consortia enriched from mangrove sediments. *Water Science and Technology* 48 (8), 177–183.

Tian, X., Wang, X., Peng, S., Wang, Z., Zhou, R. a. & Tian, H. 2018 Isolation, screening, and crude oil degradation characteristics of hydrocarbons-degrading bacteria for treatment of oily wastewater. *Water Science and Technology* 78 (12), 2626–2638.

Vaidya, S., Devpura, N., Jain, K. & Madamwar, D. 2018 Degradation of chrysene by enriched bacterial consortium. *Frontiers in Microbiology* 9 (6), 1–14.

Vasantharaj, K., Jerold, M., Deepanraj, B., Velan, M. & Sivasubramanian, V. 2017 Assessment of a sulfidogenic system utilizing microalgal biomass of Chlorella pyrenoidosa as an electron donor: Taguchi based grey relational analysis. *International Journal of Hydrogen Energy* 42, 26545–26554.

Zang, T., Wu, H., Zhang, Y. & Wei, C. 2021 The response of polycyclic aromatic hydrocarbon degradation in coking wastewater treatment after bioaugmentation with biosurfactant-producing bacteria Pseudomonas aeruginosa S5. *Water Science and Technology*, 1017–1027.

Zhang, H., Kallimanis, A., Koukkou, A. I. & Drainas, C. 2004 Isolation and characterization of novel bacteria degrading polycyclic aromatic hydrocarbons from polluted Greek soils. *Applied Microbiology and Biotechnology* 65 (1), 124–131.

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