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Enhanced chrysene degradation by halotolerant *Achromobacter xylosoxidans* using Response Surface Methodology

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ABSTRACT

Degradation of chrysene, a four ring High Molecular Weight (HMW) Polycyclic Aromatic Hydrocarbon (PAH) is of intense environmental interest, being carcinogenic, teratogenic and mutagenic. Multiple PAH degrading halotolerant *Achromobacter xylosoxidans* was isolated from crude oil polluted saline site. Response Surface Methodology (RSM) using Central Composite Design (CCD) of Bushnell–Haas medium components was successfully employed for optimization resulting 40.79% chrysene degradation on 4th day. The interactions between variables as chrysene and glucose concentrations, pH and inoculum size on degradation were examined by RSM. Under optimum conditions, *A. xylosoxidans* exhibited 85.96% chrysene degradation on 5th day. The optimum values predicted by RSM were confirmed through confirmatory experiments. It was also noted that pH and glucose as co-substrate play a dynamic role in enhancement of chrysene degradation. Hence, *A. xylosoxidans* can be further used for subsequent microcosm and *in situ* experiments for its potential to remediate PAH contaminated saline and non-saline soils.

1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are environmentally ubiquitous and recalcitrant organic contaminants produced naturally or as a result of incomplete combustion of organic materials. The persistence of PAHs is of environmental concern as they have been reported to be carcinogenic, teratogenic and mutagenic. Many approaches have been proposed to destroy or render these contaminants, such as landfilling, solvent extraction, high-temperature incineration, and various types of chemical decomposition (Abdelhay et al., 2008). However, much less information is available on the metabolism of High Molecular Weight-PAHs (HMW-PAHs), albeit on the bacterial degradation of four ring PAHs such as fluoranthene, pyrene, benz[a]anthracene, etc. A number of strains such as *Stenotrophomonas maltophilia* strain VUN 10003, *Mycobacterium* sp., *Gardona* sp., *Rhodococcus* sp., *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Flavobacterium* sp., and *Cycloclasticus* sp., etc. have been found to utilize benz[a]anthracene, fluoranthene and pyrene as a source of carbon and energy. Most of these bacteria are Gram-positive, suggesting that these organisms play a more important role than Gram-negative bacteria in the environmental degradation of HMW PAHs (Chauhan et al., 2008). Among HMW-PAHs, chrysene, a typical four ring carcinogenic PAH, has very low water solubility, which strongly reduces its bioavailability thus, making it resistant to microbial attack. It is the first PAH detected in non-industrial environment. It is also found in various environmental matrices as drinking water (19.25–998.46 ng/L), ground water (0.249–2.7 mg/L), surface water (0.0372–0.459 ng/L), rain water (0.025–86 ng/L) and surface snow (0–25.50 pg/kg) (http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?./temp/~rz9Y-wr:1:estan). Bioremediation has been considered as a promising potential option for PAH elimination in comparison to conventional practices. As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on-site (Vidali, 2001). In this context, many review articles have described the ability of numerous soil microorganisms to biotransform, cometabolize and/or mineralize PAHs (Prabhu and Phale, 2003; Kim et al., 2005).

To accelerate the process of biodegradation of PAHs, various conditions and components of the media used for the degradation study can be manipulated, which has a direct influence on degradation. Bushnell–Haas (BH) medium is the most cited mineral salt medium and is well recognized for the stimulation of a wide range of bacteria to study biodegradation of various recalcitrant and toxic compounds (Pathak et al., 2009; Xu and Lu, 2010). Traditional technique of "one-factor at a time" used for optimizing a multivariable system is not only time-consuming but also often easy to miss the interactive effects between the components (Bandaru et al., 2006). Response Surface Methodology (RSM) is a suitable method for seeking the optimal conditions for multivariable system efficiency. This approach reduces the number of experiments, improves statistical interpretation possibilities, and indicates the interaction between two variables. However, RSM using Central Composite Design (CCD) is useful for small number of variables (up to five)

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but is impractical for large number of variables, due to high number of experimental runs required (Mohana et al., 2008).

Looking to much less work done on degradation of HMW-PAH chrysene, the present work is an attempt to optimize certain BH medium components, cultural parameters and a co-substrate glucose for enhanced chrysene degradation by a multiple PAH degrading halotolerant *Achromobacter xylosoxidans* isolated from a crude oil polluted saline site, using a statistical approach RSM. The application of RSM can assist in both modelling and enhancement of chrysene degradation. This is perhaps the first report on the degradation of chrysene, a HMW four ring recalcitrant PAH by a halotolerant *A. xylosoxidans*. Based on these statistical models, the data will aid the development of soil amendments for optimizing degradation of PAHs contaminated saline soils using *A. xylosoxidans*.

2. Methods

2.1. Reagents, materials and culture maintenance media

Chrysene (Neat) [CAS No. 218-01-9] was purchased from Supelco, Bellefonte, USA. Phenanthrene and anthracene were purchased from HiMedia, India. The solvents, chemicals and reagents used for the degradation study were procured from Thermo-Fisher Scientific (India) Pvt. Ltd. Borosilicated glasswares used were amber to prevent photo-oxidation of PAHs. The bacterial culture was routinely grown on the Bushnell–Haas (BH) medium (HiMedia, India) with phenanthrene as a sole source of carbon and energy.

2.2. Isolation by enrichment and screening of multiple PAH degrading isolate

For the isolation of chrysene degrading bacteria, samples were collected from crude oil polluted saline site near Bhavnagar coast, Gujarat, India. This site is internationally known for its ship breaking and other anthropogenic activities as oil spillage and heavy metal contamination into the sea and coastal regions (Reddy et al., 2005). Samples were enriched in Nagel and Andreson Mineral Salt Medium (MSM) amended with chrysene (50 ppm) as a sole source of carbon and energy (Atlas, 2005). After seven days of enrichment, 100 μL of enrichment broth was spreaded onto the BH agar plates followed by coating the plate with 10% ethereal solution of chrysene (w/v) (Kiyohara et al., 1982) and incubated at ambient temperature for seven days in dark. Colonies with zone of clearance were purified and further examined for their ability to degrade other PAHs as phenanthrene and anthracene as they were the major PAHs detected at the contaminated sites (data not shown). The isolate that showed the ability to degrade these PAHs was designated as CG542 and was exploited for further degradation studies on chrysene.

2.3. Bacterial identification by biochemical reactions and rDNA sequencing analysis

The isolate CG542 was identified by biochemical tests, its ability to oxidize 95 different carbon sources using BIOLOG™ GN Microtitre plates and 16S rDNA sequencing. The obtained sequence was analysed using BLAST program from NCBI server. The sequences of neighbour strains were downloaded and aligned through Clustal W 1.6 program at http://www.ebi.ac.uk/clustalw. The isolate CG542 was also examined for its salt tolerance ability to characterize it as halophilic or halotolerant by growing it at 0 to 3.5 M NaCl concentration.

2.4. Growth linked chrysene degradation pattern

Isolate CG542 was inoculated into BH broth spiked with chrysene (50 ppm). The flasks were kept on a rotary shaker (150 rpm) at ambient temperature. Growth was measured by taking absorbance at 600 nm and percent degradation was measured by estimating residual chrysene. The flasks were harvested at regular intervals of three days up to 14th day. An uninoculated flask served as control. Unless otherwise mentioned, the experiments were conducted in triplicates.

2.5. Chrysene degradation assay

A modified method (Willison, 2004) for the estimation of chrysene degradation has been used. Equal volume of dichloromethane was added to the BH broth and sonicated for 5 min thrice with one minute of rest. Solvent phase was collected and the same procedure was repeated twice. Aqueous phase was removed by Na_2SO_4 and the collected solvent was pooled and evaporated with the gentle stream of N_2 gas. After evaporation of solvent, solid white crystals of chrysene were dissolved in dichloromethane and the extract was then scanned between 210 and 350 nm, using a UV–vis spectrophotometer (Shimadzu-1800, Japan). Residual chrysene was determined to calculate percent degradation from the height of the characteristic absorbance peak at 267 nm, using an experimentally determined extinction coefficient (ε267) of 126 $mM^{-1}cm^{-1}$.

2.6. Optimization of chrysene degradation

2.6.1. Optimization of chrysene degradation with screened media components by RSM using CCD

RSM is a sequential, exploratory approach to establish the relation between more than one variable and a given response. This methodology is useful for optimizing (or minimizing) biological processes. First, a screening phase is conducted to establish the range of each variable level to be tested. All the possible combinations of the variables (points) within these ranges comprise the design surface (Launen et al., 1999). RSM was used to evaluate the individual as well as the combined effect of screened BH media components like $FeCl_3$, $CaCl_2$ and NH_4NO_3 as determined by Plackett–Burman design (data not shown) on chrysene degradation using CCD. According to this design the total number of experimental combinations was $2^k + 2k + n_0$, where k is the number of independent variables and n_0 is number of repetition of experiments at the centre point. The trial version of Minitab 15 was used for experimental design, regression and graphical analysis of the data obtained (Mohana et al., 2008). $FeCl_3$, $CaCl_2$ and NH_4NO_3 were studied at five different levels (coded values are given in Table 1 for each variable) in a set of 20 experiments. The data obtained from RSM on chrysene degradation was subjected to the analysis of variance (ANOVA) and the results of RSM were used to fit a second order polynomial Eq. (1).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 BC + \beta_1\beta_1 A^2 + \beta_2\beta_2 B^2 + \beta_3\beta_3 C^2 \quad (1)$$

where Y is response variable (dependent variable), β is intercept (constant), β_1, β_2, β_3 are linear coefficients, $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_2\beta_3$ are interaction coefficients, $\beta_1\beta_1$, $\beta_2\beta_2$, $\beta_3\beta_3$ are squared coefficients and A, B, C, AB, AC, BC, A^2, B^2 and C^2 are level of independent variables. Statistical significance of the above model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple co-efficient of determination, R squared (R^2) value.