Journal of Microbiology (2014) Vol. 52, No. 11, pp. 908–917
DOI 10.1007/s12275-014-4137-6

Application of Response Surface Methodology for Rapid Chrysene Biodegradation by Newly Isolated Marine-derived Fungus *Cochliobolus lunatus* Strain CHR4D

Jwalant K. Bhatt, Chirag M. Ghevariya, Dushyant R. Dudhagara, Rahul K. Rajpara, and Bharti P. Dave*

Department of Life Sciences, Maharaja Krishnakumarsinhji Bhavnagar University, Sardar Vallabhbhai Patel Campus, Gaurishankar Lake Road, Bhavnagar, Gujarat – 364002, India

(Received Mar 3, 2014 / Revised Aug 8, 2014 / Accepted Aug 16, 2014)

For the first time, *Cochliobolus lunatus* strain CHR4D, a marine-derived ascomycete fungus isolated from historically contaminated crude oil polluted shoreline of Alang-Sosiya ship-breaking yard, at Bhavnagar coast, Gujarat has been reported showing the rapid and enhanced biodegradation of chrysene, a four ringed high molecular weight (HMW) polycyclic aromatic hydrocarbon (PAH). Mineral Salt Broth (MSB) components such as ammonium tartrate and glucose along with chrysene, pH and trace metal solution have been successfully optimized by Response Surface Methodology (RSM) using central composite design (CCD). A validated, two-step optimization protocol has yielded a substantial 93.10% chrysene degradation on the 4th day, against un-optimized 56.37% degradation on the 14th day. The results depict 1.65 fold increase in chrysene degradation and 1.40 fold increase in biomass with a considerable decrement in time. Based on the successful laboratory experiments, *C. lunatus* strain CHR4D can thus be predicted as a potential candidate for mycoremediation of HMW PAHs impacted environments.

***Keywords*:** biodegradation, chrysene, *Cochliobolus lunatus* strain CHR4D, response surface methodology, central composite design

Introduction

The marine environment suffers from unceasing oil pollution globally. Out of an array of environmentally damaging pollutants, polycyclic aromatic hydrocarbons (PAHs) are pervasive priority pollutants commonly found in soil, surface waters and sediments as a result of various anthropogenic activities. Because of their greater stability, persistence and catastrophic nature to biota, they are of great ecological concern. Apart from its chemical and physical fates in nature as photolysis, volatilization, adsorption, bioaccumulation, and chemical oxidation, biodegradation has undeniably gained wider acceptance, being economic and effective alternative for the remediation of historically contaminated marine environments (Hadibarata and Tachibana, 2009; Damare *et al*., 2012).

Hydrocarbon biodegradation using marine fungi has been first reported by Ahearn and Meyer (1972). The use of these organisms in bioremediation of polluted marine environments may be efficiently exercised because of their salt tolerance. Moreover, they possess powerful extracellular lignin degrading enzyme system that can degrade not only lignin but several aromatics such as crude oil wastes, textile effluents, organochlorides and aromatic hydrocarbons (Kiiskinen *et al*., 2004). Fungal oxidation of aromatic hydrocarbons results in the production of metabolites with higher aqueous solubility and less biological reactivity than the parent compound. The extracellular fungal enzymes can degrade bulky hazardous compounds which otherwise cannot be taken up by the cells (Hadibarata and Tachibana, 2009).

Many studies have been reported on microorganisms that are capable of degrading low molecular weight (LMW) PAHs consisting 2–4 benzene rings, for example naphthalene, acenaphthene, fluorene, anthracene, phenanthrene and pyrene, as sole carbon and energy sources; whereas degradation of high molecular weight (HMW) PAHs such as benzo[a]pyrene (BaP) and chrysene have been less reported. Solubility of these pollutants decreases with an increase in hydrophobicity and number of fused benzene rings. Another reason is the lack of induction of enzymes necessary for HMW PAH catabolism. Therefore, the appropriate co-substrates (i.e., glucose) may be useful for the bioremediation of sites polluted with PAHs because they induce the secretion of catabolic enzymes and thus promote the degradation of HMW PAHs (Hadibarata and Kristanti, 2012; Dave *et al*., 2014).

Response surface methodology (RSM) is based on experimental design with the objective of the assessment of optimal functioning of all variables with the least experimental effort. The design is based on the polynomial equation describing the behaviour of data sets with the objective of making statistical predictions. Application of central composite design (CCD) is the symmetric second order design most utilized as it has the obvious advantage over traditional one-factor-a-time approach. It has superior accuracy, high efficacy and predictions over the traditional RSM (Bezerra *et al*., 2008; Khuri and Mukhopadhyay, 2010).

The present work aims to exploit an indigenous marine-

*For correspondence. E-mail: bpd8256@gmail.com; Tel.: +91-278-2519824; Fax: +91-278-2521545

derived fungus *Cochliobolus lunatus* strain CHR4D for enhanced chrysene degradation by optimizing the media components along with substrate (chrysene) and co-substrate (glucose) by CCD. RSM was also applied for the determination of significance of interaction of variables influencing chrysene biodegradation with an experimental validation of the model. This will truly lay the foundation in marine mycotechnology for the effective remediation of shorelines heavily contaminated with PAHs.

Materials and Methods

Reagents, materials, and culture media

Chrysene (CAS 218-01-9) was purchased from Supelco, USA. The solvents and reagents used for the study were procured from Thermo-Fisher Scientific (India) Pvt. Ltd. Borosilicated amber glassware was used to prevent photo-oxidation of chrysene. The fungus was routinely grown on mineral salt medium (MSM) (Arora and Gill, 2001; Hadibarata and Tachibana, 2009) containing nutrients as (g/L): 10.0 glucose, 2.0 KH_2PO_4, 0.5 $MgSO_4{\cdot}7H_2O$, 0.1 $CaCl_2{\cdot}2H_2O$, 0.2 $(NH_4)_2C_4H_4O_6$, and 10.0 ml trace metal solution. Trace metal solution contains nutrients as (g/L) 1.5 $N(CH_2COOH)_3$, 0.48 $MnSO_4{\cdot}H_2O$, 1 NaCl, 0.01 $CoSO_4{\cdot}7H_2O$, 0.01 $FeSO_4{\cdot}7H_2O$, 0.008 $CuSO_4{\cdot}7H_2O$, 0.008 H_3BO_3, 0.008 $Na_2MoO_4{\cdot}2H_2O$, and stored at 4°C until further investigation.

Isolation of chrysene degrading fungi

A modified method (Wu *et al*., 2010) has been applied for the isolation, in which crude oil contaminated sediment samples have been collected from Alang-Sosiya ship breaking yard (latitude 21.12° N, longitude 72.30° E) at Bhavnagar coast, Gujarat, India. Sediments (0.5 g) were added into 100 ml conical flask containing 50 ml sterile Ringer's solution comprising (g/L) 7.2 NaCl, 0.17 $CaCl_2{\cdot}2H_2O$, 0.37 KCl (pH 7.3). The flasks were shaken for 30 min on an environmental shaker (New Brunswick, USA) at 150 rpm and kept at room temperature in dark. The enriched samples were established by inoculating 5 ml of sediment supernatant from each flask into 50 ml of MSM containing 60 ppm chrysene. Penicillin G and streptomycin sulfate were filter-sterilized through a 0.2 µm cellulose acetate membrane syringe filter (Millipore, USA) and added to give the final concentration of 500 mg/L of each to inhibit the growth of bacteria. The flasks were incubated at 30°C on a rotary shaker at 150 rpm in the dark. After one week of incubation, 5 ml from each flask was transferred into another freshly prepared MSM. This step was repeated four times to obtain fungal cultures with a stable chrysene degrading ability. The cultures, then, were serially diluted to the concentration of 10^{-1} to 10^{-3}, and finally plated on to MSM agar plates coated with 10% w/v ethereal solution of chrysene and incubated at 30°C in dark. Chrysene was used as a carbon source to create a constant stress in order to maintain the degradative ability of the isolates.

Identification of fungus by rRNA sequence analysis

Genomic DNA was isolated from the 5-day old culture (grown in MSB) by the cetyl-trimethylammonium bromide (CTAB) method as suggested by Griffith *et al.* (2009). Freeze dried mycelium (100 mg) was resuspended in 700 µl CTAB buffer (0.1 M Tris-HCl, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB, pH 8.0) and incubated at 65°C for 60 min. Chilled chloroform (500 µl) was added to it, vortexed and centrifuged at 13,000×g for 20 min at 4°C. The upper aqueous layer (500 µl) was carefully removed and mixed with 300 µl isopropanol. After centrifugation, the resulting DNA pellet was washed with 1 ml of hot 70% ethanol (70°C), and centrifuged again at 13,000×g for 20 min. The pellet was resuspended in 50 ml TE buffer (1 M Tris-Cl, 0.5 M EDTA, pH 8.0) and stored at -20°C until further use. PCR amplification of the D1/D2 domains of the 28S large subunit (LSU) of rRNA was performed with the set of primers DF (5′-ACCCGCTGAACTTAAGC-3′) and DR (5′-GGTCCGTGT TTCAAGACGG-3′). The amplified PCR product had been visualized on 1% agarose gel and purified using the MinElute PCR purification kit (Qiagen, India). The purified product has been sent for sequencing (Xcelris Genomic Centre, India). The sequences obtained were analyzed for sequence similarity using GenBank BLAST and phylogenetic tree was generated using the neighbour joining algorithm of MEGA5 (Tamura *et al*., 2011).

Growth linked chrysene degradation pattern

Isolate JB4 was examined for growth linked chrysene degradation ability in liquid culture experiment using 50 ml mineral salt broth (MSB). Experimental conditions such as pH, chrysene concentration, temperature, and inoculum size were kept as 5.6, 60 ppm, 30°C, and an actively growing mycelial disc / 50 ml MSB, respectively. Flasks were placed on an environmental shaker (New Brunswick, USA) at 30°C at 150 rpm in dark for 14 days. Chrysene degradation assay was performed at every alternate day upto 14 days.

Chrysene degradation assay

For the extraction of residual chrysene, a modified method as suggested by Ghevariya *et al.* (2011) had been applied in which 15 ml of dichloromethane (DCM) was added to the flasks and rigorously mixed. The flasks were then placed on a rotary shaker at 150 rpm for 30 min at an ambient temperature. To ensure complete recovery of residual adsorbed particles of chrysene onto the fungal bodies, a modified method of Jie *et al.* (2012) was employed using ultrasonication for 5 cycles, each of 3 min with a rest of 1 min with an addition of another 15 ml of DCM. Solvent phase was collected and the same procedure was repeated twice. Aqueous phase was removed by Na_2SO_4 and the collected solvent phases were pooled and evaporated with gentle stream of N_2 gas. Solid white crystals of chrysene were then dissolved in dichloromethane and subjected to GC-MS (QP2010+, Shimadzu, Japan) analysis. The conditions kept were as suggested by Mohajeri *et al.* (2010) in which Rtx-5 capillary column (Restek, USA) (60 m × 0.25 mm ID, film thickness 0.25 µm) had been used. The operating conditions kept were as follows: injector temperature 300°C, detector temperature 300°C, carrier gas helium (99.999%), make-up gas nitrogen at 30 ml/sec, oven temperature program 1 min at 60°C, then in-

creased by 10°C/min up to 160°C then 10 min in this temperature followed by 4°C/min up to 300°C, and finally 10 min at 300°C. Splitless mode injections were carried out with splitless time at 0.8 min. Mass spectrometer conditions kept were electron impact, electron energy 70 eV, filament current 100 A, multiplier voltage, 1200 V, full scan as suggested by Xu and Lu (2010).

Laboratory experimental setup

For all statistical analyses, 50 ml of MSB was taken in 250 ml Erlenmeyer flasks. Throughout the optimization approaches, unless otherwise mentioned, process variables namely temperature and inoculum size were kept constant i.e. 30°C and one actively growing mycelial disc/ 50 ml of MSB. The proportion of seawater amendment was kept constant i.e. 30% for all optimization protocols. For each statistical approach, a flask with uninoculated medium, served as a reference for determination of residual chrysene. Flasks in triplicates having heat killed inoculum were also kept to monitor the extent of abiotic removal of chrysene.

Laboratory experimental design

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).

The media engineering and process optimization had been carried out in two steps: (i) The previously optimized trace metal solution (data not shown) was combined with the components of MSB such as KH_2PO_4, $MgSO_4{\cdot}7H_2O$ and $CaCl_2{\cdot}2H_2O$ in a CCD matrix, and (ii) The chief constituents of MSB such as glucose (as a co-substrate) and ammonium tartrate (as a nitrogen source) were optimized along with the concentrations of chrysene (as a substrate) and pH.

For both the experiments, the plan of CCD in coded levels of the four independent variables was coded as:

$X_i = (X_i - X_0)/\delta X_i$

Where, X_i is experimental value of variable, X_0 is the mid-

Table 1. **Experimental set up for the optimization of chrysene degradation with MSB components by RSM using full factorial CCD by *C. lunatus* strain CHR4D**

Run No.	x_1 (KH_2PO_4)		x_2 ($MgSO_4{\cdot}7H_2O$)		x_3 ($CaCl_2{\cdot}2H_2O$)		x_4 (trace metal sol)		D (%)	Predicted D (%)
	Coded	Actual	Coded	Actual	Coded	Actual	Coded	Actual		
1	0	0.75	2	0.7	0	0.075	0	3	18.90	18.8896
2	-1	0.50	1	0.5	-1	0.050	-1	1	22.23	22.5825
3	0	0.75	0	0.3	0	0.075	-2	-1	45.99	46.1579
4	0	0.75	-2	-0.1	0	0.075	0	3	13.89	14.2179
5	-1	0.50	1	0.5	1	0.100	-1	1	22.90	22.5229
6	-1	0.50	1	0.5	-1	0.050	1	5	25.02	25.1446
7	-1	0.50	-1	0.1	-1	0.050	-1	1	37.47	37.6429
8	-2	0.25	0	0.3	0	0.075	0	3	33.89	33.7279
9	-1	0.50	1	0.5	1	0.100	1	5	41.11	41.1825
10	-1	0.50	-1	0.1	-1	0.050	1	5	35.99	35.4175
11	2	1.25	0	0.3	0	0.075	0	3	26.97	27.4496
12	0	0.75	0	0.3	0	0.075	0	3	41.81	41.3871
13	0	0.75	0	0.3	2	0.125	0	3	39.44	39.6729
14	-1	0.50	-1	0.1	1	0.100	-1	1	33.34	33.1858
15	0	0.75	0	0.3	0	0.075	0	3	41.02	41.3871
16	1	1.00	1	0.5	-1	0.050	-1	1	45.99	45.3796
17	0	0.75	0	0.3	0	0.075	0	3	41.09	41.3871
18	0	0.75	0	0.3	0	0.075	2	7	40.00	40.1496
19	0	0.75	0	0.3	0	0.075	0	3	41.00	41.3871
20	0	0.75	0	0.3	0	0.075	0	3	41.75	41.3871
21	1	1.00	-1	0.1	-1	0.050	-1	1	35.00	34.8325
22	1	1.00	1	0.5	1	0.100	-1	1	41.02	41.4975
23	0	0.75	0	0.3	0	0.075	0	3	41.04	41.3871
24	1	1.00	-1	0.1	1	0.100	1	5	18.43	17.9825
25	1	1.00	-1	0.1	1	0.100	-1	1	26.90	26.5529
26	-1	0.50	-1	0.1	1	0.100	1	5	46.67	47.0579
27	1	1.00	1	0.5	1	0.100	1	5	38.11	37.7146
28	1	1.00	-1	0.1	-1	0.050	1	5	10.01	10.1646
29	0	0.75	0	0.3	-2	0.025	0	3	31.83	31.9146
30	0	0.75	0	0.3	0	0.075	0	3	42.00	41.3871
31	1	1.00	1	0.5	-1	0.050	1	5	25.44	25.4992

D, Degradation

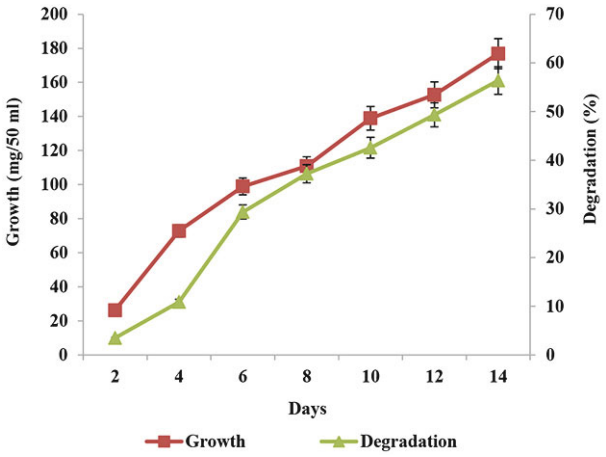

Fig. 1. Growth linked chrysene degradation pattern by isolate JB4.

point of X_i and δX_i is the step change in X_i. Coded value for X_i; i=1,2,3,4. Chrysene degradation (dependent response Y) was explained as a second order response surface model in four independent variables:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j$$

Where β_0, β_i, β_{ii} and β_{ij} represent the constant process effect between X_i and X_j on chrysene degradation process, respectively. 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 coefficient of determination, and different *R* squared (R^2) values.

Optimization of chrysene degradation with MSB components: A CCD with five coded levels was conducted to evaluate individual as well as combined effect of significant variables like KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, so as to optimize the entire medium components. Chrysene degradation corresponding to combined effect of the above four parameters were in ranges as KH_2PO_4: 0.5–1 g/L, $MgSO_4{\cdot}7H_2O$: 0.1–0.5 g/L, $CaCl_2{\cdot}2H_2O$: 0.05–0.1 g/L and trace metal solution: 1–5 ml/L. All four variables were studied at five α levels (-2, -1, 0, 1, and 2) with a set of 31 experiments having 8 axial points and 7 replicates of a centre point (coded values are given in Table 1 for each variable) using CCD according to the design combinations based on 2^k+2k+n_0, where *k* is the number of independent variables and n_0 is the number of repetitions of experiments at the central point.

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 coefficient of determination, R squared (R^2) value.

Optimization of chief components of MSB by RSM using CCD: The second optimization step involved a CCD of four chief constituents of MSB as concentrations of glucose, ammonium tartrate, chrysene and pH as independent variables at five different α levels (-2, -1, 0, 1, and 2). A set of 31 experiments with 8 axial points and 7 replicates of a centre point (coded values are given in Table 4 for each variable) had been obtained based on 2^k+2k+n_0, where *k* is the number of independent variables and n_0 is the number of repetitions of experiments at the central point. Chrysene degradation corresponding to combined effect of the above four parameters were in ranges as glucose: 0.1–0.4 g/L, ammonium tartrate: 0.05–0.25 g/L, chrysene: 10–70 ppm and pH 2–8. The other process variables were kept constant.

Experimental validation of chrysene degradation using RSM model data

Chrysene degradation was experimentally validated with all optimized variables as obtained by RSM based on the data from the above sections.

Results and Discussion

Isolation of chrysene degrading fungi

Based on zone of clearance of chrysene around the mycelial growth, twenty different chrysene degrading isolates (JB1–JB20) had been obtained (data not shown). Amongst these, isolate JB4 exhibited highest chrysene biodegradability and hence was considered for further studies.

Identification of isolate JB4

The isolate JB4 (= CHR4D) exhibited shiny velvety texture and pigmentation ranging from light brown to intense dark brown colour. It possessed septate and dematiaceous hyphae producing brown geniculate spores. The conidia were curved slightly to distinctly septate with a bulging third compartment from the pore and conidium. Based on phylogenetic analysis with close relatives (Fig. 4), it has been identified as *C. lunatus*. The closest neighbour of the isolate was *C. lunatus* strain NBRC 5997 (99% similarity). The sequence has been deposited in the GenBank with accession number KC616350.

Table 2. ANOVA for the CCD of MSB media components

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*p*
Regression	14	2996.00	2996.00	214.00	949.08	0.0000
Linear	4	236.30	236.30	59.08	262.00	0.0000
Square	4	1284.29	1284.29	321.07	1423.95	0.0000
Interaction	6	1475.41	1475.41	245.90	1090.56	0.0000
Residual Error	16	3.61	3.61	0.23		0.0000
Lack of Fit	10	2.43	2.43	0.24	1.24	0.415
Pure Error	6	1.18	1.18	0.20		
Total	30	2999.61				

Table 3. Estimated regression coefficient of degradation of chrysene by CCD

Term	Coefficient	SE Coefficient	*T*	*p*
Constant	41.3871	0.17948	230.600	0.0000
x_1	-1.5696	0.09693	-16.193	0.0000
x_2	1.1679	0.09693	12.049	0.0000
x_3	1.9396	0.09693	20.011	0.0000
x_4	-1.5021	0.09693	-15.497	0.0000
x_1* x_1	-2.6996	0.08880	-30.401	0.0000
x_2* x_2	-6.2083	0.08880	-69.915	0.0000
x_3* x_3	-1.3983	0.08880	-15.747	0.0000
x_4* x_4	0.4417	0.08880	4.974	0.0000
x_1* x_2	6.4019	0.11871	53.928	0.0000
x_1* x_3	-0.9556	0.11871	-8.050	0.0000
x_1* x_4	-5.6106	0.11871	-47.262	0.0000
x_2* x_3	1.0994	0.11871	9.261	0.0000
x_2* x_4	1.1969	0.11871	10.082	0.0000
x_3* x_4	4.0244	0.11871	33.900	0.0000
R^2 = 0.9988	R^2 (Adjusted) = 0.9977		R^2 (Predicted) = 0.9948	

Growth linked chrysene degradation

Fig. 1 indicates maximum chrysene degradation (56.37± 0.07%) by *C. lunatus* strain CHR4D. In the uninoculated control, almost no abiotic loss of chrysene had been observed (0.012%) which is indicative of its high structural stability, non-polarity and high molecular weight of chrysene. The figure also indicates that maximum growth and degradation had been achieved on the 14th day.

Optimization of chrysene degradation with MSB components

Table 1 shows CCD along with experimental and model predicted values of chrysene degradation on the 5th day for all 31 combinations. As a result of optimizing media components of MSB by CCD using RSM, a regression model equation was developed which predicted maximum chrysene degradation (47.05%) that closely matched the experimentally measured value (46.67%) as obtained on the 5th day. These equations depicted a quadratic relationship between chrysene degradation activity, *f(X)*, the media constituents i.e. KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$ and amount of trace metal solution. ANOVA for chrysene degradation by CCD is as shown in Table 2. The Fisher's *F* values of both models owing to regression were found to be high.

The large *F* value indicates that most of the variation in the response can be explained by the regression model equation. The associated *p* value is used to judge whether *F* is significance or not. A *p* value <0.05 suggests that the model is considered to be statistically significant at >95% confidence level. The linear and square terms of the regression model for chrysene degradation on the 5th day were found highly significant (p=0.000). The overall interaction between variables and its regression model for chrysene degradation was found to be highly significant (p=0.0000), indicating the correlation between response and independent variables in a polynomial model.

Table 3 shows that the regression coefficients for the linear terms KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, $FeSO_4{\cdot}7H_2O$ and trace metal solution were highly significant (p = 0.0000 for each variable). The regression coefficient R^2 (0.9988), predicted R^2 (0.9948), and adjusted R^2 (0.9977) indicated the reliability of the experiment on repetition. The model also revealed a lack of fit (p = 0.415) (Table 2).

Fig. 2 represents contour plots for degradation of chrysene by *C. lunatus* strain CHR4D on the 5th day as observed after a number of repeated experiments. Each contour plot represents a number of combinations of two test variables and its effect on chrysene degradation.

Maximum degradation (%) of chrysene is indicated by the surface confirmed in the smallest curve (circular) of contour plot. Studies of the contour plots also revealed the optimum value of the process conditions as 0.5 g/L KH_2PO_4, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}H_2O$ and 5 ml/L trace metal solution. The actual proportions of these constituents in MSB are 2 g/L, 0.5 mg/L, 0.1 g/L, and 10 ml/L, respectively.

The fitted second order response surface model specified by the equation for chrysene degradation in coded process variables can be summarized as follows:

$$\eta = (41.3871) + (-1.5696)\, x_1 + (1.1679)\, x_2 + (1.9396)\, x_3 + (-1.5021)\, x_4 + (-2.6996)\, x_1^2 + (-6.2083)\, x_2^2 + (-1.3983)\, x_3^2 + (0.4417)\, x_4^2 + (6.4019)\, x_1 x_2 + (-0.9556)\, x_1 x_3 + (-5.6106)\, x_1 x_4 + (1.0994)\, x_2 x_3 + (1.1969)\, x_2 x_4 + (4.0244)\, x_3 x_4.$$

The role and concentration of trace metals are significant when linked to hydrocarbon biodegradation, as metals are present in the environment in a variety of physiological and

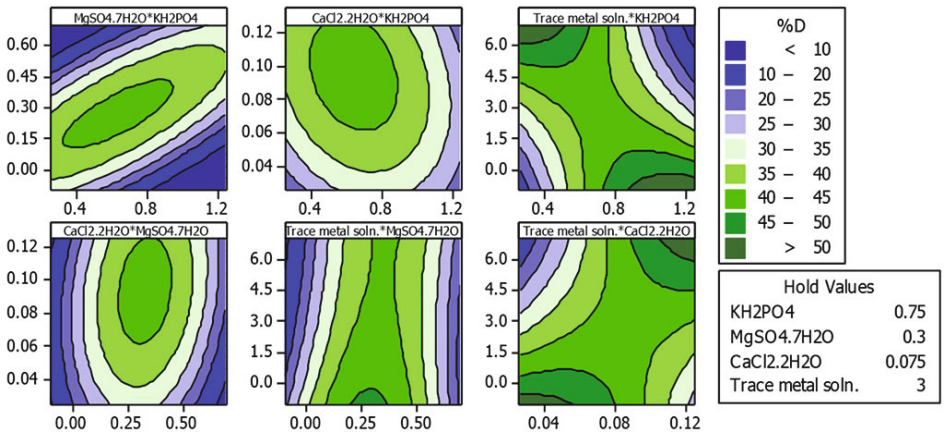

Fig. 2. Contour plots showing the response surface function effects of the interactions between KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$ and the amount of trace metal solution on chrysene degradation (%D).

chemical forms. Additionally, pH and ionic strength of water phase also affect its state which in turn allows effective biodegradation of targeted organic pollutant (Sandrin and Maier, 2003). The constituents of MSB when linked to trace metal solution, 46.67% of chrysene degradation had been achieved when the concentration of KH_2PO_4 was 0.5 g/L. The concentrations above and below this had resulted in a decrease in degradation. However, $MgSO_4{\cdot}7H_2O$ and $CaCl_2{\cdot}2H_2O$ showed optimum degradation at 0.1 g/L. The concentrations of $CaCl_2{\cdot}2H_2O$ above or below 0.1 g/L exhibited marginal difference in chrysene degradation efficiency when clubbed with other process variables and hence it showed no acute contour plots (Fig. 2).

Decrement in chrysene degradation at extremes of trace metal concentrations can be explained by the relationships between trace metal concentration and degradation process. In many cases, low metal concentration stimulate biodegradation until a maxima is reached, thereafter, degradation decreases with an increase in metal concentration; whereas, in other cases, low metal concentration inhibits biodegradation until a maximum level of inhibition, followed by a decrease in toxicity (Sandrin and Maier, 2003).

Trace metal solution was found to be significantly effective when added at 5 ml/L (run No. 26, Table 1). An ambiguous observation revealed that, irrespective of the addition of trace metals, the rate of chrysene degradation obtained was almost similar (run No. 3 and 26) (Table 1). Hence we can say that, elevated or decreased proportions of trace metal solution showed no striking difference in terms of chrysene degradation. Here, CCD model has failed to demonstrate the responses generated by trace metal solution (x_4) when clubbed with KH_2PO_4 (x_1), $MgSO_4.7H_2O$ (x_2), and $CaCl_2{\cdot}2H_2O$ (x_3) (Fig. 2). By these interactions, no specific range of variables could be set, which is otherwise easy with CCD matrix. These outcomes suggest that there is no apparent role of trace metal solution in chrysene degradation, which may be the probable reason for the complete overlook or negligence of its optimization for higher degradation rates, in previous studies. The probable failure to establish the role of trace metal solution could be due to the integration of both physiological and ecological impacts of metals on chrysene biodegradation. Similar values in degradation at different concentrations of each variable indicated that the fungus was highly robust and adaptive. The results presented herein, are thus

Table 4. Experimental set up for the optimization of chief components of MSB by RSM using full factorial CCD by *C. lunatus* strain CHR4D

Run No.	x_1 (chrysene)		x_2 (glucose)		x_3 (pH)		x_4 (ammo. tartrate)		D (%)	Predicted D (%)
	Coded	Actual	Coded	Actual	Coded	Actual	Coded	Actual		
1	0	40	0	0.25	0	5	0	0.15	76.31	76.3586
2	0	40	0	0.25	0	5	0	0.15	76.40	76.3586
3	0	40	0	0.25	0	5	0	0.15	76.30	76.3586
4	0	40	-2	-0.05	0	5	0	0.15	43.42	44.1363
5	-1	10	1	0.40	-1	2	1	0.25	19.40	18.9708
6	0	40	0	0.25	2	11	0	0.15	42.38	42.3729
7	1	70	-1	0.10	1	8	1	0.25	56.84	56.6913
8	1	70	1	0.40	1	8	-1	0.05	53.00	53.0329
9	0	40	0	0.25	0	5	0	0.15	76.32	76.3586
10	0	40	0	0.25	0	5	2	0.35	47.22	46.9946
11	1	70	1	0.40	-1	2	1	0.25	40.09	40.2996
12	-2	-20	0	0.25	0	5	0	0.15	00.00	6.0163
13	-1	10	-1	0.10	1	8	1	0.25	57.76	57.2275
14	0	40	0	0.25	0	5	0	0.15	76.41	76.3586
15	1	70	-1	0.10	-1	2	-1	0.05	16.98	17.8113
16	0	40	2	0.55	0	5	0	0.15	61.77	61.3013
17	1	70	1	0.40	-1	2	-1	0.05	52.00	51.6075
18	-1	10	1	0.40	-1	2	-1	0.05	16.31	17.1363
19	-1	10	1	0.40	1	8	-1	0.05	25.20	24.7292
20	0	40	0	0.25	-2	-1	0	0.15	00.23	9.1546
21	0	40	0	0.25	0	5	-2	-0.05	17.40	17.8729
22	0	40	0	0.25	0	5	0	0.15	76.33	76.3586
23	-1	10	-1	0.10	1	8	-1	0.05	16.33	16.7979
24	1	70	1	0.40	1	8	1	0.25	54.69	55.7575
25	0	40	0	0.25	0	5	0	0.15	76.44	76.3586
26	-1	10	1	0.40	1	8	1	0.25	40.75	40.5962
27	-1	10	-1	0.10	-1	2	-1	0.05	1.03	-0.9625
28	2	100	0	0.25	0	5	0	0.15	40.40	39.9512
29	1	70	-1	0.10	1	8	-1	0.05	29.90	29.4042
30	1	70	-1	0.10	-1	2	1	0.25	31.52	31.0658
31	-1	10	-1	0.10	-1	2	1	0.25	24.79	25.4346

D, Degradation

Table 5. ANOVA for the CCD of other MSB media components

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*p*
Regression	14	17505.5	17505.5	1250.39	1945.87	0.0000
Linear	4	5096.6	5096.6	1274.16	1982.86	0.0000
Square	4	11048.1	11048.1	2762.02	4298.28	0.0000
Interaction	6	1360.8	1360.8	226.80	352.94	0.0000
Residual Error	16	10.3	10.3	0.64		
Lack of Fit	10	10.3	10.3	1.03	322.62	0.589
Pure Error	6	0.0	0.0	0.00		
Total	30	17515.8				

in partial accordance to the conditions described (Sandrin and Maier, 2003) because metals may impact physiology and ecology of the isolate used in bioremediation of organic pollutants. Nevertheless, the model was statistically fit and significant as ANOVA showed *p* values 0.0000 for all combinations and R^2 value was found to be significant (p = 0.9988) while adjusted and predicted R^2 values were found to be 0.9977 and 0.9948, respectively (Table 3). The lack of fit was insignificant (p = 0.415) as indicated in Table 4.

Optimization of chief components of MSB by RSM using CCD

RSM was again applied to examine the effect of chief constituents of MSB such as concentrations of glucose, ammonium tartrate, chrysene and pH on chrysene degradation by *C. lunatus* strain CHR4D. A 31 run CCD for four independent variables *viz.*, concentrations of glucose, ammonium tartrate, chrysene and pH were manipulated and optimized for chrysene degradation (Table 4).

The data obtained for degradation of chrysene (%) are representing combined effect of these four factors at various levels. Degradation varied markedly with the conditions tested in the ranges of 0–76.44% on the 4th day. Maximum chrysene degradation was at 0 level combination of each variable. This observation indicated that each variable was accurately optimized, as its lower and higher levels resulted in decrease in chrysene degradation. ANOVA for degradation showed that fitted second order response surface model is highly significant with F = 1945.87 (p = 0.000) with a lack of fit (p = 0.589) as shown in Table 5.

Table 6. Estimated regression coefficient of degradation of chrysene by CCD

Term	Coefficient	SE Coefficient	*T*	*p*
Constant	76.359	0.3030	252.024	0.0000
x_1	8.484	0.1636	51.848	0.0000
x_2	4.291	0.1636	26.226	0.0000
x_3	8.305	0.1636	50.753	0.0000
x_4	7.280	0.1636	44.493	0.0000
x_1* x_1	-13.344	0.1499	-89.015	0.0000
x_2* x_2	-5.910	0.1499	-39.425	0.0000
x_3* x_3	-12.649	0.1499	-84.378	0.0000
x_4* x_4	-10.981	0.1499	-73.255	0.0000
x_1* x_2	3.924	0.2004	19.582	0.0000
x_1* x_3	-1.542	0.2004	-7.694	0.0000
x_1* x_4	-3.286	0.2004	-16.395	0.0000
x_2* x_3	-2.542	0.2004	-12.684	0.0000
x_2* x_4	-6.141	0.2004	-30.641	0.0000
x_3* x_4	3.508	0.2004	17.505	0.0000
R^2 = 0.9994	R^2 (Adjusted) = 0.9989		R^2 (Predicted) = 0.9966	

The coefficient of determination R^2 was 0.9994 (Table 6). Therefore, this equation can be used for predicting response at any combination of four variables in the experimental range. The contour plots showcase the behaviour of response (degradation) with respect to simultaneous change in two variables. This R^2 value and contour plot patterns were observed only after a number of repetitions by trying different ranges of variables and incubation times to achieve a circular response effect on the contour plot and applicable regression coefficient value. Apart from the linear effect of the variables on the degradation process, the second order RSM also gives an insight into their quadratic and interaction effects.

From the signs and magnitude of regression coefficient for these four variables in fitted regression, chrysene degradation can be well interpreted. Substrate concentration can influence the efficiency of degradation through a combination of factors including toxicity of PAH metabolites (Kim *et al.*, 2005). Higher concentration of glucose is inhibitory for PAHs bioremediation (Mohana *et al.*, 2008). Hence, a proper choice of level combination of glucose is desirable for enhancing PAH degradation.

The choices for level combinations of the four variables; glucose, ammonium tartrate, chrysene and pH can be predicted easily from contour plots. These combinations are valid for future trials because they are based on well fitted response surface model (R^2 = 0.9994). Contour plots of degradation of chrysene are shown in Fig. 3, indicating degradation with respect to changes in chrysene (substrate) and glucose (co-substrate) concentrations. Degradation values above 75% were obtained when concentrations of glucose, ammonium tartrate, chrysene and pH were at the coded value zero (0.25 g/L, 0.15 g/L, 40 ppm, and pH 5, respectively). Concentrations above these values resulted in decreased degradation (Fig. 3).

Fig. 3 represents contour plots for degradation of chrysene by the isolate on 4th day as observed after a number of repeated experiments. Each contour plot represents a number of combinations of two test variables and its effect on chrysene degradation. The fitted second order response surface model specified by the equation for chrysene degradation in coded process variables can be summarized as follows:

$$\eta = (76.359) + (8.484)x_1 + (4.291)\,x_2 + (8.305)\,x_3 + (7.280)\,x_4 + (-13.334)\,x_1^2 + (-5.910)\,x_2^2 + (-12.649)\,x_3^2 + (-10.981)\,x_4^2 + (3.924)\,x_1\,x_2 + (-1.542)\,x_1\,x_3 + (-3.286)\,x_1x_4 + (-2.542)\,x_2\,x_3 + (-6.141)\,x_2\,x_4 + (3.508)\,x_3\,x_4.$$

The addition of readily utilizable carbon source (i.e. glu-

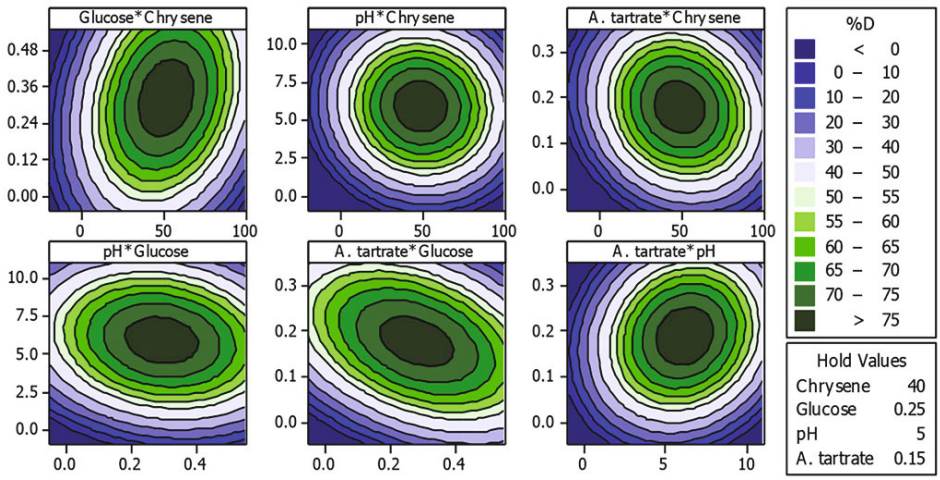

Fig. 3. **Contour plots showing the response surface function effects of the interactions between chrysene, glucose, pH and ammonium tartrate on chrysene degradation (%D).**

cose as a co-substrate) is considered as an alternative to promote biodegradation resulting in an increase in biomass thereby increasing growth linked degradative ability of the isolate (Simarro *et al*., 2012). Similar results have also been demonstrated reporting 65% of chrysene removal by a white rot fungus *Polyporus* sp. S133 in MSB when additional carbon source was added (Hadibarata and Tachibana, 2009). Similarly, 2.5 fold increase in the degradation of benzo[a]-pyrene by a white rot fungus *Armillaria* sp. F022 on the addition of glucose as a co-substrate had been demonstrated (Hadibarata and Kristanti, 2012). The results illustrated herein are in accordance to the above studies showing 76.35% chrysene degradation on the 4th day. Addition of co-substrate (glucose) also induces the secretion of extracellular lignin modifying enzymes (LMEs) such as lignin peroxidases, manganese peroxidases and laccases which can non-specifically accelerate the degradation of HMW PAHs such as chrysene via co-metabolism (Hadibarata and Kristanti, 2012).

Concentrations below and above 0.25 g/L can significantly affect the rate of chrysene degradation. The isolate has shown 43.42% degradation of chrysene in absence of glucose which is an interesting finding. This indicates the purported presence of the specific enzyme systems which can degrade chrysene as sole source of carbon and energy, in absence of glucose. This indicates the ability of the isolate to secrete number of non-specific and specific enzymes functional both in presence and absence of glucose (Table 4).

pH plays a crucial role in physiology and integrity of organisms. The optimum pH was found to be 5, at coded value 0. However, the organism could withstand the wide range of pH (pH 2–11) demonstrating its degradative ability even at the extremes of pH i.e. 40.09 and 52.00% at pH 2 (run Nos. 11 and 17, respectively), 56.84, 53, and 54.69% at pH 8 (run Nos. 7, 8, and 24) and 42.38% at pH 11 (run No. 6) (Table 4).

Ammonium tartrate is the prime source of nitrogen in MSB and the optimized value was found to be 0.15 g/L in combination with other process variables. It is very important for the growth of microorganisms and hence holds very crucial role in growth linked chrysene degradation. As observed, in run No. 21 (Table 4), absence of ammonium tartrate has resulted in a steep decrease in chrysene degradation (17.40%). The lower levels of ammonium tartrate as 0.05 g/L showed significant decrease in chrysene degradation and higher values such as 0.25 g/L showed significant decrease when combined with other process variables (Table 4). The ANOVA for the model showed highly significant output (Table 5) as p values for all interactions were found to be highly significant (p = 0.0000). The significant R^2 value (0.9994) indicated the repeatability and reproducibility of the CCD model. The adjusted and predicted R^2 values were found to be 0.9989 and 0.9966, respectively (Table 6). Similar effect has been shown reported 72% degradation of phenanthrene by *Trichoderma* sp. S019 when ammonium tartrate was added as the source of inorganic nitrogen (Hadibarata *et al*., 2009).

The flasks having heat killed fungus (an abiotic negative control) have shown negligible amount of chrysene degradation with the maximum value 0.09±0.02% on the 14th day. The lipophilic and hydrophobic nature of HMW PAH chrysene, its strong adsorbing nature onto the mycelia and photooxidation can be considered as reasons behind this meagre extent of chrysene degradation. Hence, the absolute

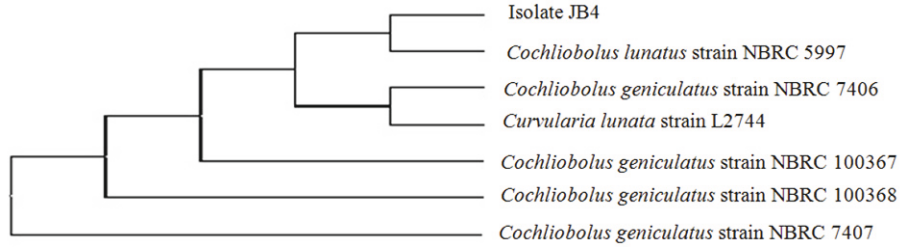

Fig. 4. **Phylogenetic relationship of *C. lunatus* strain CHR4D with its closest neighbours.**

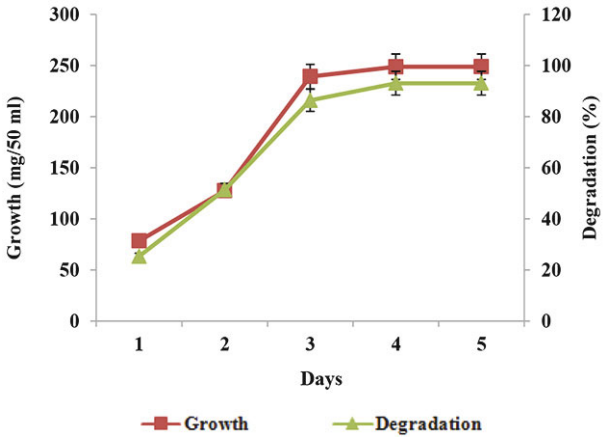

Fig. 5. Experimental validation of chrysene degradation as obtained by RSM model data.

recovery of adsorbed and untransformed / undegraded chrysene was confirmed by using rigorous and repetitive extraction procedures.

The data presented herein thus extrapolate the significance of trace metals and media components on organism's vital physiology and on effective chrysene degradation. The critically discussed role of trace metals and media components will provide an expedient insight on the behaviour of fungi and biodegradation of high molecular weight refractory PAH chrysene in marine environments.

Experimental validation of chrysene degradation as obtained by RSM model data

A growth linked experimental set having all optimized values as determined by RSM was performed to ascertain and corroborate the cumulative effect of various optimized independent variables on chrysene degradation. Fig. 5 shows the cumulative effect of all optimized process variables on chrysene degradation. Maximum chrysene degradation obtained was 93.10% with maximum biomass in dry cell mass - 248.88 mg/50 ml on the 4th day. These findings demonstrate 1.65 fold increase in chrysene degradation and 1.40 fold increase in biomass on the 4th day as compared to unoptimized condition (56.37%, 176.86 mg/50 ml) on the 14th day. The time as a process variable for chrysene degradation has been drastically reduced from the 14th day to the 4th day. This has epitomized the efficacy of all optimization models, selection of design matrices, media engineering and process optimization for enhanced and rapid chrysene biodegradation by *C. lunatus* strain CHR4D.

C. lunatus strain CHR4D as a future candidate for *in situ* bioremediation

Statistically validated laboratory experiments for microbial exploitation for eukaryotic biotransformation of HMW PAHs are one of the initial steps for evaluation of microorganisms and their successful future applications. Positive outcomes always lead researchers for the scale up of the laboratory model for *in situ* remediation approaches using comprehensive data available for characterization of isolates.

Based on the experimental data obtained and discussed, it can be extrapolated that the studied isolate is a native microorganism and thus have intrinsic capability to withstand high concentrations of pollutants and extremes of environmental factors (such as temperature and pH shifts, low nutrients, elevated salinity, low water activity etc.) and can be used effectively for degradation of chrysene even in the fields as the isolate can tolerate high concentrations of chrysene and other HMW PAHs (data not shown). Moreover, the isolate can function even at extremes of pH (i.e., pH 2–11) and can degrade chrysene considerably well. The isolate had exhibited 51.60% and 42.38% chrysene degradation at pH 2 and 11, respectively. This shows the tremendous adaptability of the isolate to degrade chrysene even in such hostile extremes, making it suitable for on-site remediation approaches.

Thus, in present study, it is strongly believed that *C. lunatus* strain CHR4D can be applied for future *in situ* remediation techniques for PAHs impacted environments.

Conclusion

Selection of CCD using RSM has been successfully employed for the complete optimization of MSB for enhanced chrysene biodegradation by marine-derived fungus *C. lunatus* strain CHR4D. The present study demonstrates a critically strategized and evaluated two step optimization protocol yielding a colossal 93.10% chrysene degradation on the 4th day against 56.37% on the 14th day in unoptimized conditions. These outcomes ensure 1.65 fold increase in chrysene degradation and 1.40 fold increase in cell biomass. Marine-derived *C. lunatus* strain CHR4D has thus emerged as a proficient candidate to be exploited for extensive mycoremediation as it has robust physiology to withstand wider pH range and elevated concentrations of HMW PAHs.

Acknowledgements

The authors are thankful to Dr Dilip P. Barad, Professor & Head, Department of English, Maharaja Krishnakumarsinhji Bhavnagar University, Bhavnagar, Gujarat, India for his help in preparation of this manuscript.

References

Ahearn, D.G. and Meyers, S.P. 1972. The Role of Fungi in the Decomposition of Hydrocarbons in the Marine Environment, pp. 12–18. *In* Walters, A.H. and Hueck-van der Plas, E.H. (eds.), Biodeterioration of Materials, Applied Science, London, UK.

Arora, D.S. and Gill, P.K. 2001. Comparison of two assay procedures for lignin peroxidase. *Enzyme Microb. Technol.* **28**, 602–605.

Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., and Escaleira, L.A. 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta* **76**, 965–977.

Damare, S., Singh, P., and Raghukumar, S. 2012. Biotechnology of Marine Fungi, pp. 277–297. *In* Raghukumar, C. (ed.), Progress in Molecular and Subcellular Biology, Springer-Verlag Berlin, Heidelberg, Germany.

Dave, B.P., Ghevariya, C.M., Bhatt, J.K., Dudhagara, D.R., and

Rajpara, R.K. 2014. Enhanced biodegradation of total polycyclic aromatic hydrocarbons (TPAHs) by marine halotolerant *Achromobacter xylosoxidans* using Triton X-100 and β-cyclodextrin – A microcosm approach. *Mar. Pollut. Bull.* **79**, 123–129.

Ghevariya, C.G., Bhatt, J.K., and Dave, B.P. 2011. Enhanced chrysene degradation by halotolerant *Achromobacter xylosoxidans* using response surface methodology. *Bioresour. Technol.* **102**, 9668–9674.

Griffith, G.W., Ozkose, E., Theodorou, M.K., and Davies, D.R. 2009. Diversity of anaerobic fungal populations in cattle revealed by selective enrichment culture using different carbon sources. *Fungal Ecol.* **2**, 87–97.

Hadibarata, T. and Kristanti, R.A. 2012. Fate and cometabolic degradation of benzo[a]pyrene by white-rot fungus *Armillaria* sp. F022. *Bioresour. Technol.* **107**, 314–318.

Hadibarata, T. and Tachibana, S. 2009. Bioremediation of phenanthrene, chrysene, and benzo[a]pyrene by fungi screened from nature. *ITB J Sci.* **41A(2)**, 88–97.

Hadibarata, T., Tachibana, S., and Itoh, K. 2009. Biodegradation of chrysene, an aromatic hydrocarbon by *Polyporus* sp. S133 in liquid medium. *J. Hazard. Mater.* **164**, 911–917.

Jie, D., BaoLiang, C., and LiZhong, Z. 2012. Biosorption and biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium* in aqueous solution. *Environ. Chem.* **58**, 1–9.

Khuri, A.I. and Mukhopadhyay, S. 2010. Response surface methodology. *Adv. Rev.* **2**, 128–149.

Kiiskinen, L.L., Rättö, M., and Kruus, K. 2004. Screening for novel laccase producing microbes. *J. Appl. Microbiol.* **97**, 640–646.

Kim, J.D., Shim, S., and Lee, C. 2005. Degradation of phenanthrene by bacterial strains isolated from soil in oil refinery fields in Korea. *J. Microbiol. Biotechnol.* **15**, 337–345.

Launen, L.A., Pinto, L.J., and Moore, M.M. 1999. Optimization of pyrene oxidation by *Penicillium janthinellum* using response-surface methodology. *Appl. Microbiol. Biotechnol.* **51**, 510–515.

Mohajeri, L., Aziz, H.A., Isa, M.H., and Zahed, M.A. 2010. A statistical experiment design approach for optimizing biodegradation of weathered crude oil in coastal sediments. *Bioresour. Technol.* **101**, 893–900.

Mohana, S., Shrivastava, S., Divecha, J., and Madamwar, D. 2008. Response surface methodology for optimization of medium for decolorization of textile dye Direct Black 22 by a novel bacterial consortium. *Bioresour. Technol.* **99**, 562–569.

Sandrin, T.R. and Maier, R.M. 2003. Impact of metals on the biodegradation of organic pollutants. *Environ. Health Perspect.* **111**, 1093–1101.

Simarro, R., González, N., Bautista, L.F., Monila, M.C., and Schiavi, E. 2012. Evaluation of the influence of multiple environmental factors on the biodegradation of dibenzofuran, phenanthrene, and pyrene by a bacterial consortium using an orthogonal experimental design. *Water Air Soil Pollut.* **223**, 3437–3444.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739.

Wu, Y.R., Luo, Z.H., and Vrijmoed, L.L.P. 2010. Biodegradation of anthracene and benz[a]anthracene by two *Fusarium solani* strains isolated from mangrove sediments. *Bioresour. Technol.* **101**, 9666–9672.

Xu, Y. and Lu, M. 2010. Bioremediation of crude oil-contaminated soil: comparison of different biostimulation and bioaugmentation treatments. *J. Hazard. Mater.* **183**, 395–401.