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A novel P450-initiated biphasic process for sustainable biodegradation of benzo[a]pyrene in soil under nutrient-sufficient conditions by the white rot fungus *Phanerochaete chrysosporium*

Sukanta S. Bhattacharya^a, Khajamohiddin Syed^a, Jodi Shann^b, and Jagjit S. Yadav^{a,*}

^aDepartment of Environmental Health, University of Cincinnati, Cincinnati, OH, USA

^bDepartment of Biological Sciences, University of Cincinnati, Cincinnati, OH, USA

Abstract

High molecular weight polycyclic aromatic hydrocarbons (HMW-PAHs) such as benzo[a]pyrene (BaP) are resistant to biodegradation in soil. Conventionally, white rot fungus *Phanerochaete chrysosporium* has been investigated for HMW-PAH degradation in soil primarily using nutrient-deficient (ligninolytic) conditions, albeit with limited and non-sustainable biodegradation outcomes. In this study, we report development of an alternative novel biphasic process initiated under nutrient-sufficient (non-ligninolytic) culture conditions, by employing an advanced experimental design strategy. During the initial nutrient-sufficient non-ligninolytic phase (16 days), the process showed upregulation (3.6- and 22.3-fold, respectively) of two key PAH-oxidizing P450 monooxygenases *pc2* (CYP63A2) and *pah4* (CYP5136A3) and formation of typical P450-hydroxylated metabolite. This along with abrogation (84.9%) of BaP degradation activity in response to a P450-specific inhibitor implied key role of these monooxygenases. The subsequent phase triggered on continued incubation (to 25 days) switched the process from non-ligninolytic to ligninolytic resulting in a significantly higher net degradation (91.6% as against 67.4% in the control nutrient-limited set) of BaP with concomitant de novo ligninolytic enzyme expression making it a biphasic process yielding improved sustainable bioremediation of PAH-contaminated soil. To our knowledge this is the first report on development of such biphasic process for bioremediation application of a white rot fungus.

Keywords

P450; Response surface methodology; Genetic algorithm; PAH biodegradation; Transcript analysis in soil; Non-ligninolytic

1. Introduction

Soil contaminated with persistent high molecular weight polycyclic aromatic hydrocarbons (HMW-PAHs), including benzo[a]pyrene (BaP), poses human health hazard and ecological risks owing to the toxicity, mutagenicity and/or carcinogenicity of these chemicals [1–4]. Among the few microorganisms that can degrade PAHs [5–8] white rot fungi including

Phanerochaete chrysosporium, have shown an extraordinary ability to degrade even higher (4 ring) PAHs including BaP [9–12]. In conventional studies using white rot fungi, PAH biodegradation has been attempted by inducing ligninolytic conditions in nutrient-limited media. However, practical application of this nutrient starvation strategy to soil has yielded variable and less-than-efficient removal of PAHs [13–15], probably because of inconsistent colonization and non-sustainable nature of the process due to short-lived fungal viability and enzyme activity as a result of exhaustion of nutrients. To circumvent this sustainability problem and to achieve higher biodegradation, we employed a different strategy for developing a soil bioremediation process for PAHs, using BaP as the test PAH.

Whole genome sequencing of *P. chrysosporium* has revealed a large repertoire (an estimated 149 P450s) of Cytochrome P450 monooxygenases [9,16,17], seven of which have been shown to have the capacity to oxidize a range of PAH compounds including low molecular weight (LMW)- and HMW-PAHs in our recent genome-to-function studies [18–20]. An upregulated expression of majority of the P450 monooxygenases including these specific PAH-oxidizing P450s under nutrient-sufficient culture conditions [21–23] and their induction by varying ring size PAHs has been demonstrated in liquid cultures [20]. In light of the above facts, we hypothesized that a greater and more sustained biodegradation of PAHs could be achieved if the biodegradation process was initiated under P450-expressing nutrient-sufficient conditions and allowed to transition into nutrient-limited (ligninolytic) process to trigger the expression of ligninolytic enzymes.

To develop a process based on this biphasic strategy, understanding of the interaction of various physicochemical and nutritional parameters, their modeling and optimization for biodegradation was critical for scale up and deployment of the process in field conditions. For this, we employed response surface methodology (RSM) to investigate the interaction between parameters over a wide range of conditions encompassing both nutrient-limited (ligninolytic) and nutrient-sufficient (non-ligninolytic) conditions. RSM has been frequently used for such optimizations in biological systems [24]. However owing to its limitation of prediction beyond user-defined boundary parameters and often restricting to local optima, genetic algorithm (GA), an advanced computational tool capable of arriving to global optima [25] has been used. GA uses the biological principles of mutation, selection, and recombination (using the physicochemical factors as genes embedded in a chromosome) to find the best viable solution to the problem. As a part of evaluating the process optimization, the two oxidative enzyme types (P450 monooxygenases and peroxidases) known to be involved in the PAH degradation process (albeit under differing nutrient conditions) were also measured to get an insight into the relationship between their expression and the associated BaP biodegradation changes during the process. The optimized biphasic process yielded sustainable and high biodegradation of BaP in soil. To the best of our knowledge, this is the first attempt at exploiting the nutrient-sufficient soil conditions and the P450 monooxygenases expressed therein for advanced *in silico* based optimization of a sustainable biphasic PAH biodegradation process for white rot fungus.

2. Materials and methods

2.1. Fungal strain, chemicals, and treatment matrices

Phanerochaete chrysosporium strain BKM-F-1767 (ATCC 24725) was used. The fungus was subcultured and maintained on malt extract (ME) agar. BaP, analytical grade was obtained from Sigma chemicals, Inc., St. Louis, MO. All organic solvents were HPLC grade and obtained from Spectrum Chemical Mfg. Corp., NJ. A sandy loam type soil obtained from a field site was used. This soil type, containing low total organic carbon (TOC) of 0.93% and a cation exchange capacity (CEC) of 6.3 meq/100g, was used in the present study to minimize the effect of variables such as organic carbon and other contributing ionic

factors. The soil composition was 56% sand, 16% clay and 28% silt and the pH was 8.3. Initial pH of soil matrix in the treatment process was adjusted per the experimental conditions using 0.1 M acetate buffer of an appropriate pH. Wood sawdust was used as a source of lignocellulose substrate for soil supplementation to support colonization by the fungus. The sawdust material was procured from the carpentry of the University of Cincinnati located in the Department of Biological Sciences; it was a mixture derived from hard and soft woods, mainly from pine, and had the following composition: 41.81% cellulose, 23.57% lignin, 400:1 C:N ratio.

2.2. Fungal inoculum preparation

P. chrysosporium inoculum was grown on sawdust to minimize the lag period in colonizing the main substrate (soil amended with sawdust) in the process optimization experiments. Dried and sieved (15 mesh size) sawdust (5 g) was supplemented with basal III salts solution of the defined low-nitrogen medium [26] buffered with 0.1 M sodium acetate buffer pH 5, 2% glucose, and 20 mM ammonium tartrate to achieve a liquid:solid ratio of 2.5:1. The resulting substrate matrix was inoculated with 9 agar plugs (0.5 mm diameter) cut from a 5-day old malt extract (ME) agar culture plate of *P. chrysosporium* and incubated at 30 °C for 4 days.

2.3. Design of experiments

The design of experiments (DOE) was based on four key physiological factors (variables), namely carbon (C) (in the form of dextrose), nitrogen (N) (in the form of ammonium tartrate), pH, and sawdust (as a source of lignocellulose). These factors varied over a wide range, with respective boundary parameters as follows: C (2%, w/v and 6%, w/v), N (12.5 mM and 37.5 mM), initial pH (3.5 and 5.5) and sawdust (2.5–7.5%, w/w). RSM was employed for modeling the process and studying the interaction between the parameters for the biodegradation of BaP. The regression equation generated was subsequently used as objective function for process optimization in GA (Fig. 1).

2.3.1. Modeling and parameter interaction analysis using RSM—Central composite design (CCD) was selected, owing to its better prediction capability in the entire design space, for the optimization of biodegradation of BaP under different nutritional conditions. This design was applied using Minitab® program with four variables at five levels. The four variables used were approximated by the quadratic model equation as follows:

$$Y = k_0 + k_a A + k_b B + k_c C + k_d D + k_{aa} A^2 + k_{bb} B^2 + k_{cc} C^2 + k_{dd} D^2 + k_{ab} AB + k_{ac} AC + k_{ad} AD + k_{bc} BC + k_{bd} BD + k_{cd} CD \quad (1)$$

where Y is the predicted response; k_0 is a constant; k_a, k_b, k_c, k_d are the linear coefficients; $k_{ab}, k_{ac}, k_{ad}, k_{bc}, k_{bd}, k_{cd}$ are the cross-coefficients; $k_{aa}, k_{bb}, k_{cc}, k_{dd}$ are the quadratic coefficients. A total of 31 experiments were carried out to estimate the 15 coefficients for the BaP biodegradation as well as BaP metabolite (3OH-BaP) formation (Table 1). Data were analyzed using Minitab® to find out the interaction between the variables and the response. The quality of the fit of this model was expressed by the coefficient of determination (R^2) using the same program.

2.3.2. In silico optimization of treatment conditions using GA—GA, a stochastic computational optimization tool where the input variables (C, N, pH, and sawdust) were treated like the genes “embedded” in a chromosome, was used. The fitness function (Eq. (1)) obtained from the RSM was used as input in GA to create the initial population of the solutions (% degradation outputs), evaluation of these candidate solutions in light of

feasibility, creation of the genetic variants (of the parameters) in the offspring using genetic operators (crossover and mutation), and evaluation of the altered offspring (parameter combination variants).

“Mutation, recombination, and selection” were carried out for this “chromosome” as in the biological systems to find out the best fit solution (maximum biodegradation) subject to a constraint (biodegradation < 100%). GA was initiated with a randomly generated initial population size of 100 individuals (initial genetic variants of the parameter set). Random mutation was carried out with tournament selection (based on fitness). Here each parent was selected by randomly choosing individuals, the number of which was specified by the tournament size that is 4, and then choosing the best individual out of the set to be a parent. A uniform mutation function was used with scattered crossover which created a random binary vector. These randomizations in the GA were more likely to overcome the constraints of local optima thereby attaining the global optima for the biodegradation of BaP.

2.4. Experimental conditions

All biodegradation experiments were set up using a preselected basic set of conditions (60% moisture level, 5% inoculum level (w/w), and 31 °C incubation temperature). Levels of added nutrients (C, N), sawdust, and initial pH were varied over an entire range in the soil-sawdust matrix using the Tien and Kirk’s defined medium base (without C, N, trace elements solution, veratryl alcohol, and Tween-80) prepared as described previously [27,28]. The numerical values for C, N, and pH parameter levels were those achieved in the supplementation medium whereas the sawdust level values were those relative to the soil amount used. These test parameters (C, N, sawdust, initial pH) were varied based on 31 different parameter sets generated by the DOE as described above.

2.4.1. Soil treatment conditions—The soil was prepared into a uniform material by grinding and sieving (20 mesh size) before use. For all experiments, the soil was spiked with BaP at a final concentration of 100 mg/Kg, using a working stock (1 mg/ml) in acetonitrile. Degradation studies were carried out in 150 ml Erlenmeyer flasks, each flask containing 10 g soil supplemented with media and subjected to aeration on alternate days to maintain the aerobic environment. The culture conditions (levels of C, N, pH, sawdust) were varied using the statistical design of experiments (central composite design). The modeling was based on 16 days incubation period from which the optimal degradation conditions were selected (Table 1). The optimized treatment sets (nutrient-limited set and nutrient-sufficient set) were then further incubated for 25 days to understand the biphasic nature of the process.

2.4.2. P450 inhibitor studies—To ascertain the role of P450(s) in the degradation of BaP in nutrient-sufficient conditions, the known P450 inhibitor piperonyl butoxide, was added to the optimal treatment process by incorporation (0.5 mM) in the aqueous base medium used for soil supplementation.

2.4.3. Extraction conditions—Simultaneous extraction of BaP and its P450 metabolite 3-hydroxybenzo[a]pyrene (3-OH BaP) and ergosterol (used as an index of fungal growth) was carried out using a microwave-assisted extraction method by Montgomery et al. [29] with some modifications. Briefly, 0.5 g soil was treated with 0.5 ml 2 M NaOH followed by the addition of 2 ml methanol in a 15 ml extraction vial. The contents were heated in a microwave oven (highest power setting) using three pulses of 30 s each with intermittent cooling for 30 s each. In the resulting mixture, phenol red was added as an indicator and the contents were neutralized with 1 M HCl. Methanol (2 ml) was added to the mixture followed by extraction (3× 2 ml) with pentane. The extract was dried under a stream of nitrogen gas and resolubilized in a small volume of methanol (500 µl).

2.4.4. Analyses—The treated and untreated soil-sawdust samples were analyzed for total biodegradation, enzyme activities, and fungal biomass yield.

2.4.5. HPLC analysis—Organic extracts of the soil-sawdust samples were simultaneously analyzed for BaP, 3-OH-BaP, and ergosterol using a Varian Prostar 210/215 HPLC unit (Varian Analytical Instruments, Walnut Creek, CA) equipped with a C18 column (250 mm × 4.6 mm, 5 μ m) run with a solvent mobile phase containing methanol and acetonitrile (50:50). Sample elution was carried out in the gradient mode at a flow rate of 1.5 ml/min using a gradient of methanol (50–100% methanol) in 8 min. Detection was based on a UV–vis detector set at 282 nm. BaP and 3-OH-BaP standards (Sigma) were used for identification and quantification of the parent compound and its P450 metabolite 3-OH-BaP as described in our recent studies [18,20]. Dried mycelial mass obtained from liquid cultures of *P. chrysosporium* was used to prepare a standard curve [mycelial dry weight versus ergosterol content] for quantification of fungal biomass in the samples (expressed as μ g mycelial biomass/g matrix substrate).

2.4.5.1. Enzyme assays: An aqueous extract of treated or untreated soil-sawdust matrix was prepared by suspending 1 g of the matrix substrate in 3 ml distilled water by vortexing and centrifugation (10,000 rpm for 10 min.). The resulting extract was subjected to enzyme activity assays for lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and cellobiose dehydrogenase (CBD) using standard spectrophotometric methods as described by Tien and Kirk [26], Paszczynski et al. [30] and Baminger et al. [31] respectively. One unit (expressed as International Unit) of enzyme activity was defined as 1 μ M of substrate oxidized per min. The final activity was calculated with respect to the dry weight of the matrix substrate.

2.4.5.2. Estimation of carbon: Total carbon in the matrices (soil, sawdust, soil + sawdust mixture) was estimated by the Anthrone method as described elsewhere [32].

2.4.5.3. Estimation of total nitrogen: Total nitrogen content in the matrices was estimated using the Kjeldahl method [33] with slight modifications. Briefly, 1 g of sample was digested in concentrated H₂SO₄ supplemented with 2 Kjeldahl buffer tablets followed by mixing with 25 ml of 35% (w/w) of NaOH and heating in the Kjeldahl flask for ~15 min till a clear solution was obtained. The evolved ammonia was distilled over and trapped in 0.05 M boric Acid. The total nitrogen content was determined in terms of the amount of ammonia produced by titrating the excess boric acid against 0.1 M NaOH.

2.4.5.4. Quantitative RT-PCR analysis for expression of P450 monooxygenases:

Transcriptional quantification for the P450 monooxygenases *pc2* and *pah4*, recently identified for their role in PAH oxidation in *P. chrysosporium* [18,20], was carried out in soil-sawdust samples using real-time qRT-PCR. Total fungal RNA was extracted from soil-sawdust matrix using a modified version of the method for soil nucleic acid isolation by Griffiths et al. [34] coupled with further purification using RNeasy kit per manufacturer's instructions (Qiagen, Inc., Valencia, CA). Purified RNA prep showing a A₂₆₀/A₂₈₀ value over 2 was considered acceptable. The purified total RNA (25 ng) was subjected to qRT-PCR quantification of the target P450 transcripts using gene-specific primers (Table 2) and the Brilliant SYBR green QRT-PCR master mix kit (Stratagene, La Jolla, CA) per manufacturer's specifications using 7900 HT ABI Prism unit (Applied Biosystems, Forest city, CA). A fold-change in P450 transcript level for *pc2* or *pah4* was determined by using the formula $2^{\Delta C_t}$ in comparison to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*gpd*) an internal control, which is known to have a constitutive expression in the fungus. The qRT-PCR reaction conditions involved the reverse transcription step carried

out at 50 °C for 30 min followed by the PCR reaction performed using denaturation at 95 °C for 10 min and target amplification for 40 cycles. Each amplification cycle involved denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. [22]. A melting curve analysis was also carried out to rule out the possibility of a crosslinking between the primers and a non-specific amplification.

3. Results and discussion

Soil treatment process for biodegradation of BaP was first optimized for both nutrient-limited and nutrient-sufficient conditions in a 16-day treatment using RSM coupled with GA. The latter set of conditions eventually led to development of a 25-day biphasic process with improved and sustained BaP biodegradation activity. These optimizations encompassed the entire non-ligninolytic to ligninolytic range of conditions. The biphasic process was based on a metabolic switch from non-ligninolytic to ligninolytic state as interpreted in terms of the associated enzyme activities and P450 metabolite formation and disappearance.

3.1. Understanding the biodegradation parameters and their interactions using RSM-assisted model

The RSM-assisted experimental design (Table 1), meant to investigate the effect of varying the process parameters on BaP biodegradation, led to the following regression equation (expressed in terms of uncoded variables) as a part of the modeling process:

$$\begin{aligned} \text{Biodegradation} = & 95.631 - 4.83 \cdot x_1 - 1.94 \cdot x_2 - 4.95 \cdot x_3 \\ & + 0.17 \cdot x_4 - 0.45 \cdot x_1 \cdot x_1 + 0.01 \cdot x_2 \cdot x_2 \\ & - 1.51 \cdot x_3 \cdot x_3 - 0.67 \cdot x_4 \cdot x_4 - 0.12 \cdot x_1 \cdot x_2 \\ & + 2.38 \cdot x_1 \cdot x_3 + 0.34 \cdot x_1 \cdot x_4 + 0.31 \cdot x_2 \cdot x_3 \\ & + 0.16 \cdot x_2 \cdot x_4 - 0.05 \cdot x_3 \cdot x_4 \end{aligned} \quad (2)$$

where x_1 is the added carbon level, x_2 is the added nitrogen level, x_3 is the initial pH and, x_4 is the sawdust level. The RSM model showed significance based on ANOVA (Table A.1) and its experimental validation showed the R^2 and R^2_{adj} to be 84.2% and 70.3%, respectively; these values are considered acceptable for the biological experiments involving soil owing to the problem of uniform mass transfer and extraction of PAHs for such complex matrix.

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhazmat.2013.07.055>.

In order to gain further insights on the effect of interaction of the parameters on BaP biodegradation (%), the individual response surface plots were critically analyzed. From the C and N response surface plot (Fig. 2a), an elevated degradation was observed in the high N condition over a moderate range of C (2–5%, w/v). A similar increase in the degradation was observed in the low nitrogen condition at elevated carbon levels. However, at very low levels of both the nutrients (C and N), a decrease in BaP degradation was observed which may be attributed to the poor colonization/growth of the fungus. Also at higher levels of C (7%, w/v), when N was increased beyond 40 mM, a decrease in the BaP degradation was observed which may be attributed to the physiological capacity of the fungus to perform optimally in a certain range of nutrient conditions. This response plot therefore represents one of the most important interactions as this is indicative of the two systems of degradation (ligninolytic and non-ligninolytic) coming into play. Low N conditions typically induced the ligninolytic enzyme system [26] which is known to catalyze oxidation of PAHs [11,33], in this case BaP. On the other hand, an increased degradation in high C and high N condition

during the initial phase of incubation, which is essentially a non-ligninolytic condition, highlights the presence of an alternative oxidizing enzyme system responsible for the degradation of the PAH compound. Studies by us and others have highlighted the importance of cytochrome P450 enzyme system in the oxidation of PAHs under nutrient-sufficient culture conditions [20,34,35]. Our gene-specific qRT-PCR analysis indeed confirmed the induction of two PAH degradation-relevant P450 monooxygenases in the nutrient-sufficient conditions (discussed in later sections). Enhanced initial growth of the fungi in high C and low N may be responsible for the increased depletion of nutrients which in turn causes induction of the ligninolytic conditions in extended incubation.

The presence of ligninolytic and non-ligninolytic systems in the degradation of BaP was further indicated in the response plot between C and pH (Fig. 2b). Low C (<2%, w/v) and low pH (<3) showed enhanced BaP degradation, indicating the role of a typical ligninolytic system known to be triggered under carbon-limited conditions in acidic medium such as the Tien and Kirk's defined low N (LN) medium. In contrast, another scenario supporting enhanced BaP degradation was observed at elevated levels of C (>4.5%, w/v) and relatively higher pH (>5.5). This is a condition that may be indicative of the expression and role of PAH-oxidizing P450 systems in the degradation, such as that observed in nutrient-sufficient media [20,36].

The response surface plot between C and sawdust (Fig. 2c) revealed an optimum BaP degradation with added C at ~5% (w/v) and sawdust at ~5% (w/w). Critical analysis of the plot showed that, at higher levels of sawdust, an increase in the added C (glucose) leads to enhanced BaP degradation. This observation may be explained by the fact that increase in the added C level increases the biomass of the fungus thereby enabling a greater access of the fungus to the matrix-adsorbed BaP, as observed earlier [36,37] where sawdust was used as a source of supplemental carbon and observed here in the optimal nutrient-sufficient degradation sets compared to the nutrient-limited sets (Table 3). This is despite the fact that excess sawdust may result in a lower bioavailability of the BaP (possibly due to increased sequestration) to the fungus [38] which is also evident from the plot between pH and sawdust (Fig. 2d).

Analysis of the response surface plot between N and pH (Fig. 2e) depicted two distinct sets of conditions supporting enhanced BaP degradation viz. low N-low pH set and high N-high pH set. This is similar to the plot between sawdust and N (Fig. 2f) which also depicts two optimal sets one at high N and the other at low N. These two sets of conditions are known to represent typical ligninolytic conditions triggering exo-peroxidases [26] and typical non-ligninolytic conditions upregulating the P450 monooxygenase systems [22] respectively. Hence, the observed degradation under these two sets of physiological conditions may be attributed to the two respective enzyme systems, as discussed later in this section with the enzyme data.

3.2. Understanding the parameter interactions using RSM-assisted model for the formation of P450-generated BaP metabolite

The degradation experiments based on DOE (described above) also allowed us to understand the parameter interactions for the nature of biodegradation when interpreted in terms of formation of P450-generated metabolite 3OH-BaP; this can be represented by the following regression equation:

$$\begin{aligned}
 3\text{OH-BaP} = & -9061 + 21xa_1 - 1.94xa_2 - 4.95xa_3 + 0.17 \cdot x_4 \\
 & - 0.45 \cdot x_1 \cdot x_1 + 0.01 \cdot x_2 \cdot x_2 - 1.51 \cdot x_3 \cdot x_3 \\
 & - 0.67 \cdot x_4 \cdot x_4 - 0.12 \cdot x_1 \cdot x_2 + 2.38 \cdot x_1 \cdot x_3 \\
 & + 0.34 \cdot x_1 \cdot x_4 + 0.31 \cdot x_2 \cdot x_3 + 0.16 \cdot x_2 \cdot x_4 \\
 & - 0.05 \cdot x_3 \cdot x_4
 \end{aligned} \quad (3)$$

Where x_1 is the added carbon level, x_2 is the added nitrogen level, x_3 is the initial pH and, x_4 is the sawdust level. Greater amounts of 3OH-BaP formation for the parameter interactions that are representative of nutrient-sufficient conditions (Fig. 3) implied a higher expression of P450 activity and its role in BaP degradation under these non-ligninolytic conditions. For instance, the response surface plot on the effect of C and N on the production of 3OH-BaP (Fig. 3a) reveals that high levels of nitrogen (both at low and high levels of carbon) lead to greater amounts of 3OH-BaP. This indicates the nitrogen concentration is a critical factor for the accumulation of 3OH-BaP (or expression of P450 monooxygenases). The response surface plot between C and pH (Fig. 3b) reveals that a carbon concentration of 5–6% (w/v) and a pH over 4.5 favor an increase in the 3OH-BaP concentration; these represent non-ligninolytic conditions. An increase in 3OH-BaP formation at a lower carbon concentration (1.5–2.5%) in conjunction with a lower pH may apparently seem contrary to the trend but actually these conditions in conjunction with the hold value of 25 mM nitrogen, also represent non-ligninolytic conditions. Evaluation of the surface plot between sawdust and C (Fig. 3c) revealed that an increase in the sawdust level leads to an increase in the concentration of 3OH-BaP, with maximum amount being present at high C and high sawdust concentrations. This may be attributed to the nutrient-rich conditions and facilitated aeration. Surface plot of N and pH showed that high nitrogen - low pH (Fig. 3d) and low nitrogen- high pH combinations with a hold value of high C favored a higher accumulation of 3OH-BaP. Sawdust and N plot showed that higher sawdust levels favored the production of 3OH-BaP with the maximum accumulation being at higher levels of N and sawdust (Fig. 3e). The response surface plot between sawdust level and pH (Fig. 3f) with hold values of high C and high N revealed that an increase in the sawdust level and pH resulted in an increase in 3OH-BaP (Fig. 3f). Taken together, higher 3OH-BaP formation in various parameter combinations could in general be attributed to nutrient-sufficient conditions favoring better growth and/or metabolic status conducive for expression of P450 monooxygenase enzymes.

Collectively, the above observations on biodegradation and metabolite formation highlighted the role of interaction of various parameters in the biodegradation process and were strongly indicative of a dual degradative enzyme system operating in this process over the tested ranges of parameters. In order to obtain an enhanced efficiency of such process, optimization was carried out using GA, as it can easily overcome the user-defined boundary parameters, is less likely to be limited by local optima and thus has a greater chance to arrive to the global optima than the conventional RSM programs.

3.3. Process optimization and validation using RSM-GA combination strategy

The regression equation (Eq. (2)) obtained in the second order RSM-based model above was utilized as the objective function for optimization using GA. After running the GA for a set of parameters, two optimal points were obtained involving very different sets of parameters. One optimal point gave a predicted response of 61.88% degradation (the experimental value was 60.3%) at C 2.0% (w/v), N 2.5 mM, pH 3.5, sawdust 2% (w/w). Temperature (31 °C) and initial moisture content (60% of the water holding capacity of soil) were kept constant. This is essentially a ligninolytic condition scenario. Using the same RSM regression

equation, a very different set of conditions was observed in the other GA-predicted response. At the higher values of the parameters viz. C 5% (w/v), N 55 mM, pH 6.5, sawdust 7.83% (w/w), a 69.04% degradation was predicted (the experimental value was 66%). This set of parameters coincides with the non-ligninolytic condition, where alternative enzyme system(s) such as P450 monooxygenases may be responsible for the degradation activity. These assumptions were confirmed by the enzyme assays for the known ligninolytic enzymes (LiP and MnP) and transcript analysis for the specific PAH-oxidizing P450s *pc2* (CYP62A2) and *pah4* (CYP5136A3) as shown in Table 3. In this context, the two subject P450 enzymes were identified in our recent studies as the most promising P450 monooxygenases for role in PAH oxidations in this fungus [18–20] and *pc2* was found to be critical in oxidation of higher molecular weight PAHs including BaP [18].

On day 16, samples from the optimal set of nutrient-limited conditions showed high ligninolytic enzyme activities (LiPs, MnPs). On the other hand, the optimal set of nutrient-sufficient conditions showed no LiP activity and very low MnP activity. In contrast to peroxidases, P450 analysis relative to the housekeeping control gene revealed a much higher expression of both *pc2* (3.6-fold) and *pah4* (22.3-fold) in nutrient-sufficient conditions as against the low or constitutive level of expression under nutrient-limited ligninolytic conditions. Further validation of the role of P450 monooxygenases under nutrient-sufficient conditions was carried out using piperonyl butoxide, a known P450 inhibitor [23]. Abrogation (84.9%) of degradation activity was observed in presence of the inhibitor as compared to the control treatments without the inhibitor. This along with a higher concentration of 3OH-BaP (3036.84 $\mu\text{g kg}^{-1}$), the known P450 metabolite of BaP [18], in the nutrient-sufficient conditions confirmed the significant role of P450 monooxygenases under these conditions. CBD expression was higher and more sustained under nutrient-sufficient conditions, consistent with that in liquid cultures [39], although its role in PAH biodegradation is not clear.

Importantly, further incubation of the optimal sets of both nutrient-sufficient and nutrient-limited sets until day 25 resulted in a much higher degradation in nutrient-sufficient set (91.6%) but only slightly higher degradation in nutrient-limited set (67%). The presence of 3OH-BaP was not detected in either of the sets in the 25-day process, implying its disappearance due to amenability to further degradation in the system. Enzyme analysis revealed no LiP and CBD activities and low residual MnP activity (10.52 IU/gds) for the nutrient-limited set which may be attributed to the loss of fungal viability due to lack of nutrients. Enzyme analysis for nutrient-sufficient set revealed much higher activity for LiPs (302.39 IU/gds), MnPs (48.96 IU/gds) and CBD (131.6 IU/gds) and a 2.4-fold expression of *pah4*. Also, this set showed sustained fungal growth as depicted in Table 3. These observations collectively indicate that the 25-day nutrient-sufficient process could support an enhanced initial degradation of PAHs due to expression of PAH-oxidizing P450s in the first 16 days and could provide a sustained and more extensive degradation in the extended incubation (25 days) by switching to ligninolytic conditions (wherein ligninolytic enzymes are expressed). The low carbon and nitrogen levels in the nutrient-limited conditions may be the major limiting factors for a sustainable growth of the fungus in extended incubation as compared to the nutrient sufficient set of conditions that result in more sustainable growth. Comparison of the data on fungal biomass, total C and total N (Table 3) in 16-day versus 25-day bioprocess supports this phenomenon. Taken together, the above data demonstrate that the optimized 25-day nutrient-sufficient treatment set is a biphasic process involving initial P450-initiated (non-ligninolytic) oxidation (phase I) followed by a ligninolytic degradation (phase II) and is an alternative, more sustained approach for bioremediation.

4. Conclusions

This study, based on an advanced experimental design using RSM coupled with GA, led to optimization of two separate sets of conditions for biodegradation of BaP in soil in a 16-day process, one for a nutrient-limited (ligninolytic) process and the other for a nutrient-sufficient (non-ligninolytic) process. A notable overexpression of PAH-specific genes *pc2* and *pah4* in the nutrient-sufficient process as compared to the nutrient-limited process confirmed the role of P450 monooxygenases in the degradation of BaP under the nutrient-sufficient conditions, which was also confirmed by analysis of the known P450 metabolite 3OH-BaP. More importantly, the study led to the development of a 25-day biphasic process initiated under nutrient-sufficient (non-ligninolytic) conditions and transitioned into nutrient-limited (ligninolytic) conditions. This biphasic process sustained better fungal colonization/growth and led to a much greater PAH biodegradation efficiency due to a concerted activity of the non-ligninolytic (P450) and ligninolytic (peroxidase) enzyme systems expressed in tandem during the process. The developed biphasic process overcomes the bottlenecks in the conventional strategies currently adopted for soil bioremediation by white rot fungi and is a significant step in the direction of designing field applications of these promising bioremediation agents. Further studies on scale up of this process, extrapolation of the process to other soil conditions, as well as elucidation of the degradation pathways during the process are being pursued.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- A novel biphasic process developed for soil bioremediation using white rot fungus.
- Bioprocess optimization by experimental design based on RSM coupled with GA.
- Report on biodegradation of a normally recalcitrant higher PAH (5-rings) in soil.
- First report on soil bioremediation by a ligninolytic fungus in a nutrient-rich process.
- First demonstration of expression of PAH-oxidizing P450s of white rot fungus in soil.

Fig. 1. Overall design of the optimization strategy for the developed Biphasic process for BaP degradation in soil.

Fig. 2.

Response surface plots depicting influence of parameter interactions on the biodegradation of BaP by *P. chrysosporium* in the soil treatment process. (a) Carbon and nitrogen interaction; (b) carbon and pH interaction; (c) carbon and sawdust interaction; (d) pH and sawdust interaction; (e) nitrogen and pH interaction; (f) nitrogen and sawdust interaction. In addition to the variables considered in the individual plots, hold values were 4% (w/v) carbon, 25 mM nitrogen, 4.5 initial pH, and 5% (w/w) sawdust.

Fig. 3.

Response surface plots depicting influence of parameter interactions on the concentration of the P450 monooxygenase metabolite 3OH-BaP generated by *P. chrysosporium* in the soil treatment. (a) Carbon and nitrogen interaction; (b) carbon and pH interaction; (c) carbon and sawdust interaction; (d) nitrogen and pH interaction; (e) nitrogen and sawdust interaction; (f) sawdust and pH interaction. In addition to the variables considered in the individual plots, hold values were 4% (w/v) carbon, 25 mM nitrogen, 4.5 initial pH, and 5% (w/w) sawdust.

Optimization of biodegradation of BaP in soil by *P. chrysosporium* using various sets of treatment conditions designed using the central composite design of experiments*.

Table 1

Run Order (Treatment set)	C (%w/v)	N (mM)	Initial pH	Sawdust (%w/w)	X1	X2	Y1	Y2
1	2	12.5	3.5	2.50	59.89	54.74	1701.56	1599.07
2	6	12.5	3.5	2.50	46.47	51.61	2021.59	1782.25
3	2	37.5	3.5	2.50	44.06	43.42	2836.31	2909.25
4	6	37.5	3.5	2.50	31.81	27.84	2935.58	2859.95
5	2	12.5	5.5	2.50	36.77	34.83	3010.71	3215.83
6	6	12.5	5.5	2.50	47.07	50.76	3272.35	3002.45
7	2	37.5	5.5	2.50	37.14	39.17	2303.24	2286.72
8	6	37.5	5.5	2.50	41.25	42.65	1856.05	1840.85
9	2	12.5	3.5	7.50	32.41	34.88	3679.30	3581.94
10	6	12.5	3.5	7.50	41.68	38.49	4848.40	4474.58
11	2	37.5	3.5	7.50	48.68	43.83	5237.12	5116.69
12	6	37.5	3.5	7.50	29.19	34.99	6094.54	5776.85
13	2	12.5	5.5	7.50	11.64	14.45	2714.35	2399.65
14	6	12.5	5.5	7.50	32.61	37.12	3081.24	2895.73
15	2	37.5	5.5	7.50	40.33	39.05	1568.33	1695.11
16	6	37.5	5.5	7.50	45.28	49.27	2246.55	1958.71
17	0	25.0	4.5	5.00	31.22	35.85	2927.63	2799.51
18	8	25.0	4.5	5.00	50.27	42.93	2615.27	3246.29
19	4	00.0	4.5	5.00	51.85	49.03	2576.38	3013.93
20	4	50.0	4.5	5.00	49.76	49.87	3321.74	3387.09
21	4	25.0	2.5	5.00	39.79	43.34	2937.80	3313.26
22	4	25.0	6.5	5.00	43.97	37.72	984.44	1111.88
23	4	25.0	4.5	0.00	35.44	36.52	2393.50	2362.56
24	4	25.0	4.5	10.00	27.06	23.28	3929.45	4463.29
25	4	25.0	4.5	5.00	43.04	46.55	2688.56	2745.20
26	4	25.0	4.5	5.00	47.73	46.55	2813.21	2745.20
27	4	25.0	4.5	5.00	49.54	46.55	2707.98	2745.20
28	4	25.0	4.5	5.00	41.20	46.55	2813.25	2745.20

Run Order (Treatment set)	C (%w/v)	N (mM)	Initial pH	Sawdust (%w/w)	X1	X2	Y1	Y2
29	4	25.0	4.5	5.00	48.21	46.55	2704.21	2745.20
30	4	25.0	4.5	5.00	48.03	46.55	2770.84	2745.20
31	4	25.0	4.5	5.00	48.11	46.55	2718.35	2745.20

* Experimental variables included dextrose as carbon source [C], ammonium tartrate as nitrogen source [N], pH, and sawdust. Boundary parameters were C (2%, w/v and 6%, w/v), N (12.5 mM and 37.5 mM), pH (3.5 and 5.5) and sawdust (2.5–7.5%, w/w). X1 = experimental degradation, X2 = predicted degradation, Y1 = experimental 3OH-BaP level, Y2 = predicted 3OH-BaP level.

Table 2

Primers used in the study.

Target gene*	Forward primer (5' 3')	Reverse primer (5'.3')	Amplicon (bp)
<i>pah4</i>	CAAGCAAGTGCAGCACATCC	CCGCGTGCAACGATTTCG	354
<i>pc2</i>	CGACTTCCAGGACCTCATCTCG	CACTCTCCCACCGTTCTCAC	346
<i>gpd</i>	GGCATTGTGCAGGGTCTCATG	GAGTAGCCCCACTCGTTGTC	458

pc2, *pah4*: cytochrome P45 genes; *gpd*: glyceraldehyde-3-phosphate dehydrogenase gene.

Table 3

Relative BaP biodegradation activity and associated fungal enzymatic activities and growth in the optimized nutrient-limited and nutrient-sufficient processes for soil treatment with *P. chrysosporium*.

Test parameter	Nutrient-limited process ^c		Nutrient-sufficient process ^d	
	Day16	Day25	Day16	Day25 (biphasic process)
BaP degradation (%)	60.27	67.39	66.14	91.60
LiPs (IU gds ⁻¹) ^a	106.43	–	–	302.39
MnPs (IU gds ⁻¹)	40.83	10.52	12.13	48.96
CBD (IU gds ⁻¹)	16.48	0	124.5	131.6
P450 <i>pc2</i> (fold-change)	0.44	–	3.58	–
P450 <i>pah4</i> (fold-change)	2.39	–	22.3	2.4
Fungal biomass (µg g ⁻¹) ^b	331.59	387.37	513.28	753.84
Total C (mg gds ⁻¹) ^e	13.94	6.39	45.37	30.43
Total N (µg gds ⁻¹) ^f	34.54	29.47	1320.62	1162.21

^a IU gds⁻¹ means International units per gram dry substrate.

^b Fungal growth was estimated in terms of biomass yield, measured based on ergosterol analysis (µg biomass per gram dry substrate). The initial levels of added C (as glucose), added N (as ammonium tartarate), and pH indicated for the two processes are as follows; these levels were those achieved in the liquid base medium for supplementation.

^c Nutrient-limited process: 2.0% (w/v) C, 2.0 mM N, 2.0% (w/w) sawdust, 3.5 pH

^d Nutrient-sufficient process: 5.0% (w/v) C, 55.0 mM N, 7.8% (w/w) sawdust, 6.5 pH.

^e Final total carbon was measured in terms of total reducing sugar. The amounts represent their added form plus the native form present in the matrices (soil and sawdust). Initial total carbon levels were as follows: nutrient deficient set 21.2 mg/gds; nutrient sufficient set 56.9 mg/gds.

^f Total N measured using Kjeldahl method represents the added nitrogen plus the native nitrogen present in the matrices.