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RESEARCH ARTICLE

Process optimization of benzo[ghi]perylene biodegradation by yeast consortium in presence of ZnO nanoparticles and produced biosurfactant using Box-Behnken design

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BACKGROUND: Benzo[ghi]perylene (BghiP), a polycyclic aromatic hydrocarbon (PAH) containing six fused benzene rings is considered as priority pollutant because of its carcinogenicity, mutagenicity and acute toxicity.
METHODS: The synthesis of ZnO nanoparticles was done following the standard method. Biosurfactant production by yeast consortium YC04 in MSM was confirmed by various tests viz. drop collapse test, methylene blue agar plate method and emulsification test (E24) using the standard procedures. Efficiency of YC04 was tested to remediate BghiP in presence of ZnO nanoparticles and produced biosurfactant in the growth medium.
RESULTS: Response surface methodology (RSM), 3-level five variables Box-Behnken design (BBD) was employed to optimize the factors viz. pH 7.0, temperature 30°C, shaking speed 130 rpm, inoculum dosage 3% and ZnO nanoparticles concentration 2 g/L after a period of 6 days of incubation for the enhanced degradation of BghiP (63.83 ± 0.01%). It was well in close agreement with the predicated value obtained by RSM model yield (63.83 ± 0.08%). Analysis of variance (ANOVA) showed F-value of 51.70, R2 of 0.9764, probability of < 0.0001 and coefficient of variation of 1.25% confirmed the validity of the model. Degradation of BghiP was assessed using GC-MS and FTIR analysis. Kinetic study demonstrated that BghiP degradation fitted first order kinetic model.
CONCLUSIONS: To the best of our knowledge, this is the first report on process optimization toward nanobioremediation of BghiP using yeast consortium in presence of ZnO nanoparticles and produced biosurfactant in medium.

Keywords biodegradation, bioremediation, optimization, pollutants, yeasts

Introduction

Benzo[*ghi*]perylene (BghiP), a high molecular weight (HMW) polycyclic aromatic hydrocarbon (PAH) containing six fused benzene rings, is considered to be one of the 16 PAHs defined as priority pollutant by US EPA due to its toxicity, mutagenic and carcinogenic behavior (Habs et al., 1984; Cerniglia, 1992; Wilson and Jones, 1993; Wei et al., 2015). The HMW PAHs with more than four rings are highly hydrophobic, minimally bioavailable, and more recalcitrant to be degraded (Seo et al., 2007). BghiP has been reported to show enough evidence of mutagenicity/genotoxicity in animal somatic cells (EFSA, 2008). Recently, the toxicity of BghiP on human bronchial cell line has been reported by Zaragoza-Ojeda et al. (2016).

The utilization of the microorganisms has drawn a great attention toward the remediation of PAHs contaminated sites because of its biodegradative capabilities and environmental sustainability (Patowary et al., 2015; Su et al., 2018). There are few reports on degradation of BghiP using fungi (Winquist et al., 2014; Garcia-Delgado et al., 2015) and yeast (Hesham et al., 2006). Though yeasts are tolerant to changes in the adverse environmental conditions (Ooi et al., 2003), reports are scanty on yeast as biodegrader of high molecular weight PAHs (Ren et al., 2004).

Microbial compounds having evident surface activity are classified as biosurfactants and have gained considerable attention for numerous industrial, medical and environmental

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applications including remediation of number of pollutants (Cuny et al., 1999; Ibrahim, 2018).

Nanoparticles have been used as reductants or catalysts to improve various reactions due to their high surface areas and other characteristics. ZnO nanoparticles are extensively used as they are recyclable, easy to handle, inexpensive, non-volatile, nonexplosive and can serve as eco-friendly catalyst for many organic transformations (Safaei-Ghomi and Ghasemzadeh, 2017). The effect of nanoparticles on microorganisms has additionally attracted attention now-a-days because of their unique impact on microbiological responses being located on the cells to stimulate the activity of microbes (Shin and Cha, 2008; Muller et al., 2014; Zhang et al., 2017). But extremely limited studies have been reported on effect of nanoparticles on biodegradation of pollutants in presence of produced biosurfactants in the growth mediu1m using microbes (El-Sheshtawy and Ahmed, 2017).

Remediation of BghiP using microorganisms is receiving attention now-a- days. There are relatively few publications on successful microbial remediation of BghiP (Hesham et al., 2006; Winquist et al., 2014; Garcia-Delgado et al., 2015; Mandal and Das, 2018). In addition, the use of microbial consortia is considered to be more stable and effective than using single organism (Hesham et al., 2006; Mishra et al., 2014; Mandal and Das, 2018). So far, no report is available on nanobioremediation of BghiP in the presence of nanoparticles and produced biosurfactant in the medium using microbial consortium.

In the present study, an attempt has been made to optimize essential variables using 3-level Box-Behnken design that could enhance the biodegradability of BghiP using yeast consortium in presence of ZnO nanoparticles and produced biosurfactant. To the best of our knowledge, this is the first study in which process optimization has been done toward nanobioremediation of BghiP using yeast consortium in presence of ZnO nanoparticles and produced biosurfactant in medium.

Materials and methods

Microorganisms

In our previous study, the yeast consortium YC04 was already reported as potential degrader of BghiP (Mandal and Das, 2018) which consist of three yeast isolates viz. *Rhodotorula* sp. NS01, *Debaryomyces hansenii* NS03 and *Hanseniaspora valbyensis* NS04. So, YC04 was selected for the present study.

Synthesis and characterization of ZnO nanoparticles

The synthesis of ZnO nanoparticles was done following the standard method (Litt and Almquist, 2009) as discussed in supporting information (Supporting section S1) and were characterized using UV spectroscopy, X-ray diffraction (XRD), Fourier transform Infrared spectroscopy (FTIR) analysis, TEM and EDX analysis.

Production of biosurfactant in culture media

For biosurfactant production, a mineral salt medium (MSM) was prepared. Trace element solution containing (g/L): 0.116 of $FeSO_4.7H_2O$, 0.232 of H_3BO_3, 0.41 of $CoCl_2.6H_2O$, 0.008 of $CuSO_4.5H_2O$, 0.008 of $MnSO_4.H_2O$, 0.02 of $(NH_4)_6Mo_7O_{24}$ and 0.174 of $ZnSO_4$ were added to MSM (Haghighat et al., 2008). The yeast consortium YC04 was inoculated in 500 mL Erlenmeyer flasks containing 150 mL MSM of initial pH 7.0 and incubated at 30°C for 3 days under shaking condition of 130 r/min.

Biosurfactant production by YC04 in MSM was confirmed by various tests viz. drop collapsing test (Bodour and Maier, 1998), methylene blue agar plate method (Satpute et al., 2008) and emulsification test (E_{24}) (Bodour et al., 2004) following the standard procedures. Biosurfactant was simultaneously produced in the culture medium, extracted, purified and estimated using orcinol assay method (Tuleva et al., 2002). The rhamnolipid concentration was calculated from a standard curve prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mg/mL). FT-IR analysis of purified biosurfactant was done.

Biodegradation of BghiP in presence of ZnO nanoparticles and produced biosurfactant

The biodegradation experiments were conducted in sterilized 500 ml Erlenmeyer flask containing 100 ml of sterilized mineral salt medium supplemented with BghiP (40 mg/L) under different set of conditions as follows: (i) BghiP + YC04 (ii) BghiP + YC04 + produced biosurfactant (iii) BghiP + YC04 + ZnO nanoparticles (0.5 g l^{-1}) (iv) BghiP + YC04 + produced biosurfactant + ZnO nanoparticles (0.5 g/L). Flasks without inoculation were maintained as control. The residual BghiP was extracted from the different set of conditions and biodegradation percentage was calculated.

Instrumental analysis

GC-MS analysis was done to determine the residual BghiP and the degraded products in the culture broths (Arulazhagan et al., 2010). Flasks from different set of conditions were withdrawn after 6 days of incubation. The degraded products were extracted using ethyl acetate. The solvent was removed under vacuum by rotary evaporation (Superfit™ Rotary vacuum Digital bath) prior to analysis. Aliquots of 2-5 μL were injected directly for GC–MS analysis, (JEOL GC MATEII) using silica as stationary phase. The inlet temperature was 220°C; oven temperature was increased from 50 to 250°C at 10°C rev min^{-1}; the GC interface temperature was 250°C; carrier gas was nitrogen at a flow rate of 1.0 mL rev min^{-1}. Mass spectrum conditions had the

ionization energy 70 eV, ion chamber temperature was maintained at 250°C with tungsten filament which was used for the ionization of molecules. The concentration of BghiP was calculated by comparing the peak areas of each treated sample with that of the peak area of the abiotic control. For identification of the degraded products, the mass spectra of the products formed were compared with respective mass spectra of authentic compounds and also with the mass profile of the same compound available in the National Institute of Standard Technology (NIST) library, USA.

The FTIR spectra of BghiP and degraded products were used to determine the vibrational frequency changes in functional groups. The extended degraded products dissolved in ethyl acetate, were mixed with KBr and made in the form of pellets (13 mm in diameter and 1 mm thickness). IR spectroscopy was investigated with the IR affinity-1 FT-IR spectrophotometer (Shimadzu). The scanning wavenumber ranged from 4000 to 400 cm^{-1} and the spectral resolution was 4 cm^{-1}.

Process optimization

Box-Behnken design was used for optimization of parameters and to determine the significant single factors, interactions and quadratic terms using statistical analysis software [Design expert software version 11.0.5.1 Stat ease Minneapolis USA]. Each factor was varied at three different levels − 1, 0 and + 1 signifying low, medium and high values. The design consisted of 46 runs performed twice to optimize the levels of the selected variables viz., pH (A), temperature (B) shaking speed (C) inoculum dosage(D) and zinc oxide nanoparticle concentrations (E). The range of the variables was chosen based on preliminary experiments.

Kinetic studies

Degradation kinetics was performed in triplicates. The zero order (Jianlong et al., 2002), first order (Agarry et al., 2013) and second order (Capellos and Bielski, 1972) kinetic models were used to define the degradation of BghiP in mineral medium.

Results and discussion

Synthesis and characterization of ZnO nanoparticles

ZnO nanoparticles were synthesized and then characterized using UV spectroscopy, XRD, FT-IR analysis, TEM and EDX analysis as discussed in supporting information (Fig. S1). The XRD pattern of ZnO nanoparticle exhibited well-defined peaks at 2θ values of 13.13, 15.28, 26.13, 26.90, 33.31, 38.94 and 59.69 which correspond to the 010, 011, 113,104, 213, 123 and 401 planes respectively. The intensity of ZnO nanoparticle peaks at 2θ values of 15.28, 26.90, 33.31 and 59.69 reflected high degree of crystallinity in nanoparticles. Similar result was demonstrated by Kumar and Rani (2013).

Biosurfactant production

Biosurfactant produced by yeast consortium (YC04) in the MS medium was tested by methylene blue agar test, drop collapsing test and emulsification index (%). The positive results of all the tests confirmed the biosurfactant producing ability of YC04. The flat drop appearance in microtiter plate confirmed the positive results for drop collapse test. Dark blue halo zone in the methylene blue agar plate supplemented with CTAB confirmed the presence of anionic biosurfactant. The emulsification index was found to be 58.4%. Orcinol assay was done for the direct assessment of the amount of glycolipids present in the purified sample. The rhamnolipid concentration was found to be 0.72 mg/mL. Rhamnolipid type of biosurfactant produced by YC04 was confirmed by FT-IR analysis (Carbonyl stretching, C-O stretching) and shown in supporting information (Fig. S2). The findings of this study was in accordance with recent work done by Bahia et al. (2018) who reported rhamnolipid type biosurfactant production by engineered yeast *Saccharomyces cerevisiae.*

GC-MS analysis for BghiP biodegradation

The degradation of BghiP (40 mg/L) by yeast consortium YC04 in MSM was found to be 60.0% after 6 days of incubation. Improvement in degradation (60.7%) was noted when MSM was supplemented with ZnO nanoparticles (0.5 g/L). Maximum degradation of BghiP (62.0%) was observed in presence of ZnO nanoparticles (0.5 g/L) and produced biosurfactant in MSM (Fig. 1A). Hesham et al. (2006) reported the degradation of BghiP (2.17 mg/Kg) by a mixture of five yeast strains which was found to be 78% after a period of 42 days. The soil fungi viz. *Pleurotus ostreatus* was reported to be capable of degrading BghiP (40.0%) after a period of 20 days at the concentration 3.19 mg/Kg (García-Delgado et al., 2015); *Phanerochaete velutina* was reported to be capable of degrading BghiP (47.0%) after a period of 90 days at the concentration 2.7 mg/Kg (Winquist et al., 2014). Therefore, yeast consortium YC04 used in the present study was found to be more efficient in degrading BghiP at much higher concentration (40 mg/L) within a short period (6 days) compared to the earlier reports.

FT-IR analysis

FTIR spectra of control benzo[*ghi*]perylene (Fig. 1B) showed distinctive absorption peaks at 3020.25 cm^{-1} (C-H stretch in aromatic rings), 1730.15 cm^{-1} (weak overtone and combination bands in aromatic compounds), 752.24 cm^{-1} (strong out of plane CH deformations in aromatic compounds), 667.37 (strong CH out of plan deformations in cis substituted

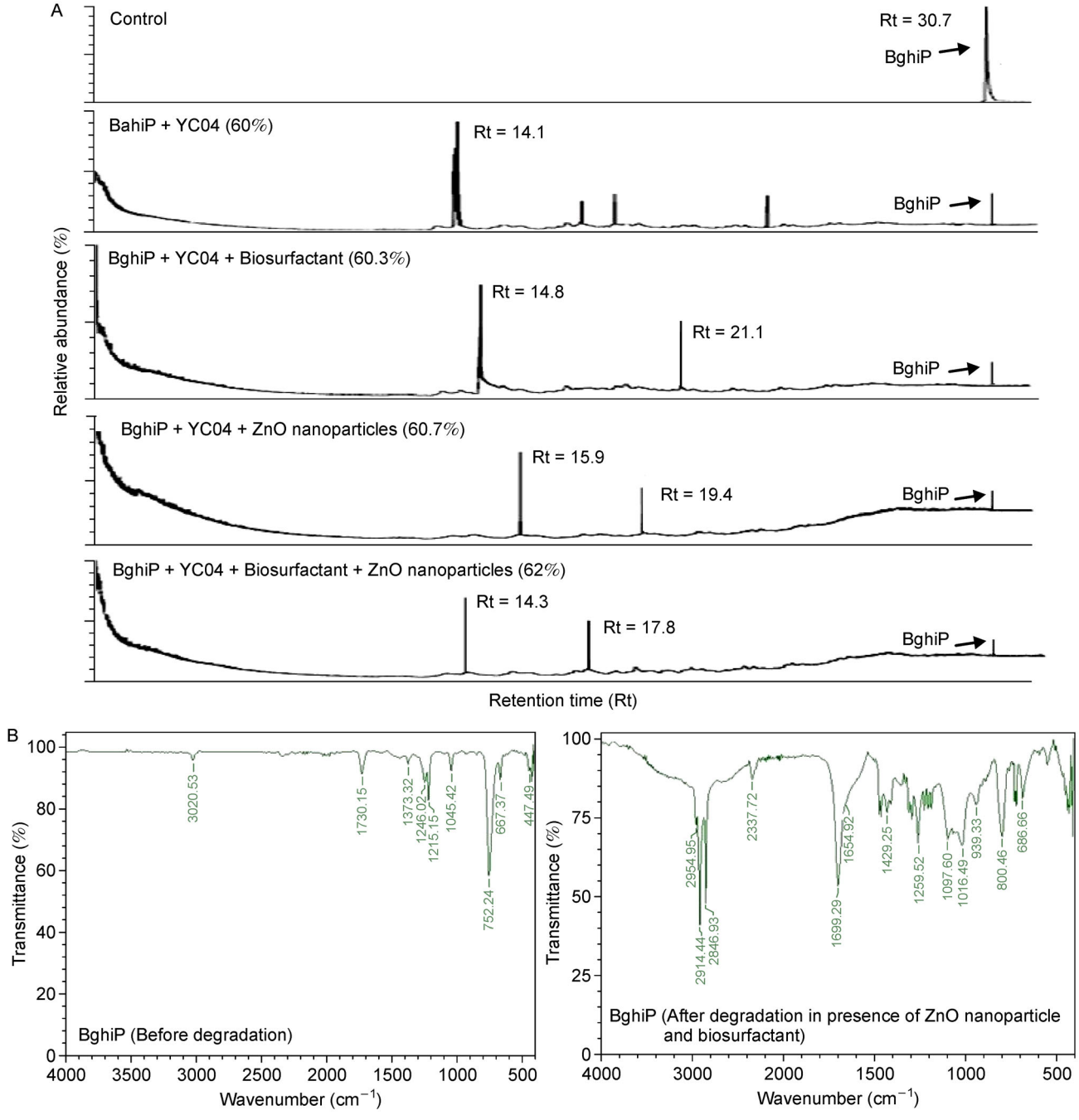

Figure 1 Biodegradation studies of benzo[*ghi*]perylene (BghiP) using yeast consortium YC04 through instrumental analysis. (A) GC-MS analysis of BghiP degradation under different sets of condition after 6 days of incubation; (B) FT-IR spectrum of BghiP before and after degradation in presence of ZnO nanoparticle and biosurfactant.

alkenes) and 447.35 cm^{-1} (medium-strong ring deformations in aromatic compounds). The second spectra illustrated, degraded benzo[*ghi*]perylene products by yeast consortium, YC04 showed a presence of 2954.95-2846.93 cm^{-1} representing H-bonded OH stretch in carboxylic acid. Sharp absorption peak of 1699.29 cm^{-1} (overtone and combination bonds in aromatic compounds), 1654.92 cm^{-1} (C = C stretch in alkenes),1654.92 cm^{-1} (OH bending in carboxylic acid), 1259.52 cm^{-1} (C-O-C antisym stretch in vinyl ethers), 1097.50-1016.49 cm^{-1} (C-O stretch in alcohols), 939.33 cm^{-1} (CH_2 out of plane wag in vinyl compounds), 800.46-686.66 cm^{-1} (out of plane CH deformations in aromatic

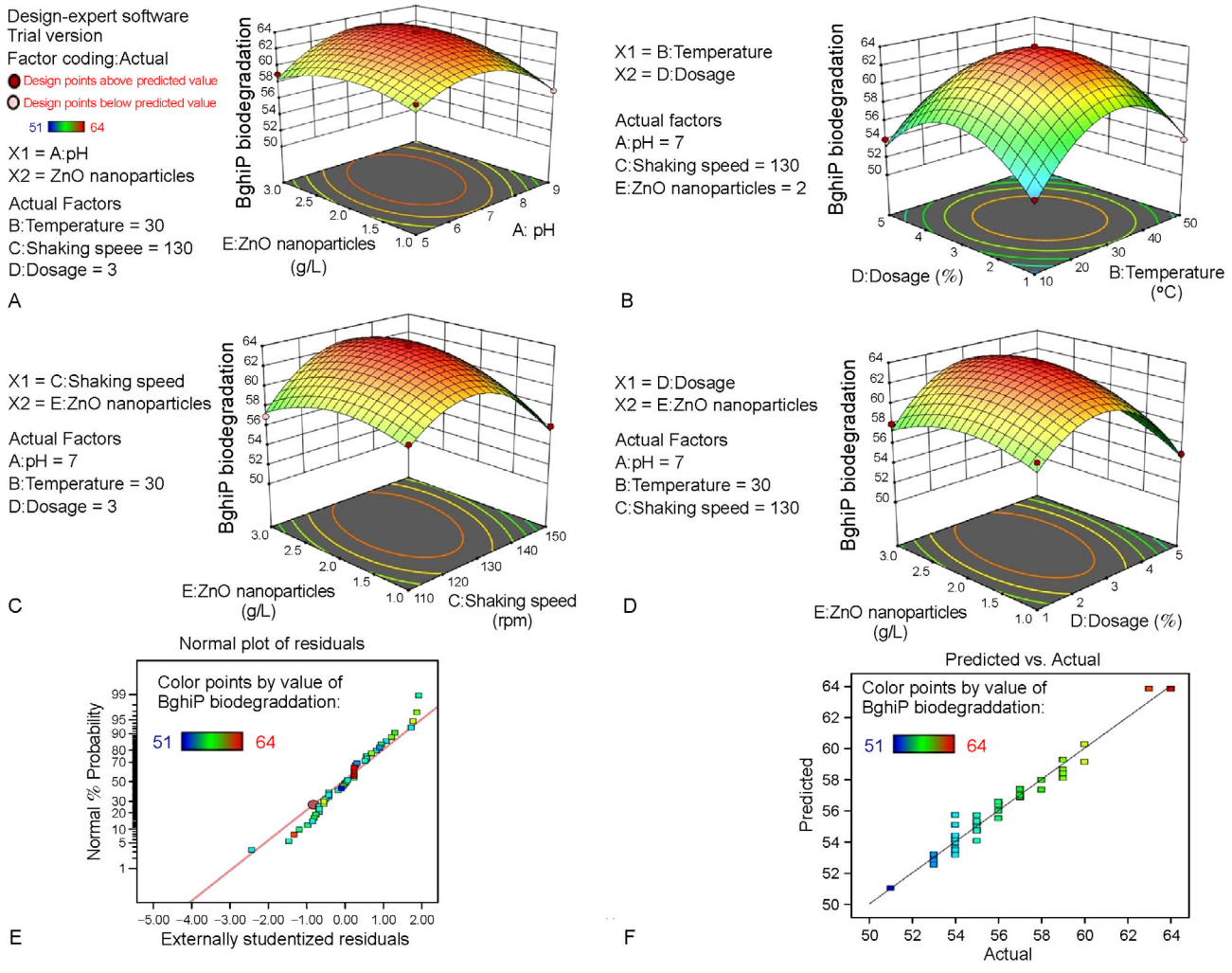

Figure 2 3-D interactions between the different variables for the response (BghiP biodegradation %). (A) pH vs. ZnO nanoparticles (AE); (B) Temperature vs. Dosage (BD); (C) Shaking speed vs. ZnO nanoparticles (CE); (D) D. Dosage vs. ZnO nanoparticles (DE); (E) Normal plot of residuals; (F) Predicted vs. actual plot.

compounds). These results suggest that the parental compound has undergone significant changes after degradation.

Process optimization

A three-level Box-Behnken Design with six central points was used to enhance the BghiP biodegradation using yeast consortium (YC04). Maximum BghiP biodegradation was found to be 63.83 ± 0.01 (%) at central values of all the factors viz., pH 7.0, at temperature 30°C of shaking speed 130 rpm in the presence of 2 g/L of ZnO nanoparticle using 3% inoculum dosages of yeast consortium YC04 after 6 days of incubation period. F-value of 51.70, R^2 of 0.9764, probability of < 0.0001 and coefficient of variation of 1.25% confirmed that the model is highly significant and the experiments are accurate and reliable. An adequate precision of 26.82 for response also validates the model. The lack of fit analysis was found to be not significant in the present case shown in supporting file (Table S1). Based on statistical significance, the second-order polynomial equation for the response can be written as:

$$Y = 63.83 - 0.0625A - 0.0625B - 0.3125C - 0.6875D + 0.6250E - 0.25AB - 0.50AC - 0.50AD + 1.00AE - 0.25BC - 1.00BD + 0.25BE - 0.25CD + 1.25CE + 1.00DE - 3.35A^2 - 6.02B^2 - 4.35C^2 - 5.02D^2 - 1.77E^2$$

Where, Y was representing BghiP biodegradation (%) as response and A, B, C, D and E were coded terms for the five test variables viz. pH, temperature, shaking speed, inoculum dosages and ZnO nanoparticle concentration respectively. The 3D plots showed significant influence on the response using yeast consortium YCO4 either independently or in interaction with each other (Fig. 2). Among all, D, E, AE, BD, CE, DE, A^2, B^2, C^2, D^2 and E^2 were found to be significant model terms ($p < 0.05$) (Supporting file Table S1). The

interaction between the variables pH vs ZnO nanoparticles (AE) was found to be significant as shown in Fig. 2A. The interaction between the variables BD (Temperature vs. Dosage), CE (Shaking speed vs ZnO Nanoparticles) and DE Dosage vs ZnO Nanoparticles) had also showed significant impact on BghiP biodegradation (%) using yeast consortium YC04 ($p < 0.05$) (Fig. 2B-D) (Table S1). Maximum BghiP biodegradation was observed at concentration of 2.0 g/L ZnO nanoparticles. Higher concentration of ZnO nanoparticles (3.0 g/L) was found to be toxic to yeast cells which resulted in the reduction of BghiP biodegradation. Similar report on crude oil degradation using *Bacillus licheniformis* was demonstrated by El-Sheshtawy and Ahmed (2017) in presence of produced biosurfactant in the medium and specific concentration of (Fe_2O_3 and $Zn_5OH_8Cl_2$) nanoparticles.

A statistical model was validated by executing point prediction tool of RSM from an optimum value of all the 5 variables A, B, C, D and E. The actual BghiP biodegradation ($63.83 \pm 0.01\%$) was in close agreement with the predicted value ($63.83 \pm 0.08\%$) indicating the validity of the model as shown in supporting file (Table S2). The normal plot for residuals and predicted vs actual plots were represented (Fig. 2e-f) respectively. Thus, BghiP biodegradation by YC04 was found to be increased from 62.0% to 63.83% in aqueous medium under optimized condition.

Kinetic studies

The kinetic data on degradation of BghiP (40 mg/L) was best fitted with the first order kinetic model in case of all set of conditions. The highest regression coefficient (R^2) values of (0.9708) of BghiP degradation was noted in case of biosurfactant producing YC04 in presence of ZnO nanoparticles as shown in supporting file Table S3. The calculated degradation rate constant (K) of BghiP is 0.17 d^{-1} and the theoretical half-life of BghiP is 4.076 days implied that the removal of BghiP by yeast consortium was time dependent process and degradation rate was directly proportional to substrate concentration (Jin et al., 2017).

Conclusions

This study investigated the potentiality of yeast consortium YC04 for the degradation of benzo [*ghi*] perylene using specific concentration of ZnO nanoparticles and produced biosurfactant in the growth media which may be used for remediation of BghiP contaminated sites. Process optimization of the growth parameters using Box Behnken design had significantly enhanced the BghiP degradation under optimized conditions. This is the first report on yeast mediated nanobioremediation of BghiP and optimization of the whole process using response surface methodology (RSM) which emphasized the novelty of our work. Further work on application of YC04 in nanobioremediation of BghiP from the real-world contaminated environment is underway in order to ascertain its relevance in pollution control

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Conflict of Interest

The authors declare no conflicts of interest.

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