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A novel approach on degradation of Benzo[a]pyrene by yeast consortium isolated from contaminated soil

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ABSTRACT

Benzo[a]pyrene (BaP), a high molecular weight polyaromatic hydrocarbon (HMW PAH) is commonly found in contaminated soils and considered as a potent carcinogen. Microbial degradation offers an effective approach to remove toxic pollutants. In this study, four types of yeast strains viz. Rhodotorula sp. NS01, Hanseniaspora opuntiae NS02, Debaryomyces hansenii NS03 and Hanseniaspora valbyensis NS04 isolated from BaP contaminated soil were used for the degradation of BaP. Identification of yeast isolates was done using 18S rRNA sequences. Out of four consortia studied, YC01, consisting of Rhodotorula sp. NS01, Hanseniaspora opuntiae NS02 and Debaryomyces hansenii NS03, was found to show a maximum BaP degradation of 76% within 6 days in aqueous medium under optimized conditions viz. pH 7.0, temperature 30 ºC, shaking speed of 130 rpm, an inoculum dosage 3% (w/v) and an initial BaP concentration of 50 mg/L. Degradation of benzo[a]pyrene by the consortium YC01 exhibited first order kinetics with half-life period of 3.03 days. The degradation products were monitored by Fourier transform infrared spectroscopy (FTIR), Gas Chromatography Mass Spectrometry (GC-MS) analysis. A possible pathway of BaP degradation by yeast consortium, YC01 was proposed. Significant role of enzymes like laccase, 1,2-dioxygenase, 2,3-dioxygenase, lignin peroxidase, manganese peroxidase and catalase were noted during the process of BaP degradation. To the best of our knowledge, this is the first report on the use of yeast consortium towards BaP degradation.

Keywords: Benzo[a]pyrene, Enzymes, GC-MS, Pathway, Yeast consortium.

INTRODUCTION

Benzo[a]pyrene (BaP), a homologue of benzene of five fused aromatic rings belongs to high molecular weight polyaromatic hydrocarbon (HMW-PAH) group, is regarded as an effective carcinogen [1-4].U.S. Department of Health and Human Services (1990) reported on BaP as a commonly found PAH in contaminated soils [5].It has been asserted that 60-90% of human cancers are caused by various environmental chemicals including PAHs [6]. BaP are formed in incineration processes and some energy-related chemical manufacturing processes, such as coal gasification, coal liquefaction and petroleum refining [7].They enter the environment as an unfinished combustion product of fossil fuels where it becomes bound to organic particles in aquatic and terrestrial sediments [8].Because of its low solubility and high recalcitrance in nature, BaP is a persistent pollutant, contaminating industrial sites around the world, and poses a serious threat to the environment and public health [9-13].The US-EPA (United States Environmental Protection Agency) has set a maximum contaminant level for BaP in environment (0.0002 µg/L). Majority of human exposure occurs by inhalation, dermal absorption, and ingestion during the consumption of char-broiled foods and smoking etc. [14].

While there are many physico-chemical methods for remediation of BaP include chemical oxidation, photolysis, incineration, landfilling, volatilization and adsorption. Application of these methods is limited due to certain drawbacks such as, high operating cost, long treatment times and the formation of toxic by-products etc. [15]. As a

result, degradation of BaP through biological method is receiving serious attention nowadays. Microbial biodegradation is one of the most significant natural processes that can influence the fate of pollutants in both aquatic and terrestrial environments.

While there are many reports on BaP degradation by microorganisms including bacteria, fungi and algae such as *Rhodococcus* sp., *Pleurotus ostreatus*, *Trichoderma* sp., *Ochrobactrum* sp., and *Selenastrum capricornutum*[16-20], there are relatively few publications that have reported the successful degradation of BaP using yeasts [21].The use of microbial consortia is considered to be more stable and effective than the use of single organisms, because of their diversity and synergetic effect of metabolic activity that occur in microbial consortia[22-24].

Table 1: Reported works on biodegradation of Benzo[a]pyrene (BaP) by microbial consortia

Microbial Consortia	Concentration of BaP	Removal efficiency	References
Trametes versicolor ATCC 42530, *Lentinustigrinus* CBS 577.79	22±1 mg/Kg	23% in 180 days	[25]
Brevibacterium sp., *Delftia* sp., *Dietzia* sp., *Gordonia* sp., *Kocuria* sp., *Naxibacter* sp., *Microbacterium* sp.	100 mg/L	23.6% in 30 days	[26]
Pedobacter sp., Bacillus sp., Paenibacillus, sp., Uncultured Sphingobacterials bacterium clones & Proteobacterium clones	1190.8 ± 141.8 mg/Kg dry soil	36.6% in 56 days	[27]
Bacteroidetes, α-Proteobacteria, Actinobacteria, β-Proteobacteria and γ-Proteobacteria	50 mg/L	65.1% in 28 days	[28]
Bacillus sp. SB02, *Mucor* sp. SF06	50 mg/Kg	79.6% in 42 days	[29]
Stenotrophomonas maltophilia VUN 10,010 and *Penicillium janthinellum*VUO 10,201	50 mg/L	53% in 100days	[30]
Class of *Proteobacteria*, genera *Mycobacterium* and *Sphingobacterium*	10 mg/L	65 % in 2 weeks	[31]
YC01(*Rhodotorula* sp. NS01,*Hanseniaspora opuntiae* NS02, *Debaryomyces hansenii* NS03)	50 mg/L	76% in 6 days	Present study

Table 1 summarizes the reported works on biodegradation of BaP by various microbial consortia. From the table, it was evident that there is a need for a consortium, which can degrade BaP more effectively in less time. Hence, in the present study, the degradation of BaP by yeast consortium has been reported for the first time. The degradative role of various ligninolytic yeast enzymes towards BaP degradation has also been analysed.

MATERIALS AND METHODS

Chemicals

Benzo[a]pyrene (≥ 96 % purity by HPLC) were procured from Sigma-Aldrich (St. Louis, USA). A stock solution of BaP was prepared at a concentration of 1 mg/mL in chloroform. The required concentration of working solutions was prepared by diluting the stock solution. All other chemicals were of high quality and obtained from Hi-Media Laboratories (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).

Soil Sampling

The soil sample was collected from Katpadi [12.968085°N, 79.148646°E], Vellore, Tamil Nadu, India. Soil cores (5–20 cm) were brought to the laboratory in sterile plastic bags and stored at 4 °C until microbial isolation. The soil was air dried at room temperature and sieved at a particle size of <2mm.

Enrichment culture for isolation and screening of BaP degrading yeast strains

Yeast isolation was carried out from the soil sample using standard enrichment procedures. The experiments were conducted in sterilized 250 mL Erlenmeyer flasks to which the required concentration of working solution of BaP (0.1 mg/L) was added and allowed to evaporate overnight or till dry before inoculation [32].100 mL of mineral medium (MM) containing per litre of potassium dihydrogen phosphate 1 g, dipotassium hydrogen phosphate 1 g, sodium chloride 5 g, ammonium sulphate 0.3 g, magnesium sulphate 0.3 g and calcium chloride 0.02 g, at pH 6.8 ± 0.5 [33] was added to the same flask containing BaP. 1.0 g of the soil sample was added to the medium and incubated for 5 days at 28 ± 2 ºC in a rotary shaker (120 rpm). 10 mL of the enrichment culture was transferred every 5 days to a fresh sterile medium, incubated under same conditions and the concentration of BaP in the enrichment culture was increased from 0.1 to 1.0 mg/L in a step-wise manner. After 2 weeks of evaluation period, the pure yeast isolates obtained by spread plate were sub-cultured. The isolates thus obtained were named as NS01, NS02, NS03 and NS04 respectively. The isolates were maintained on yeast extract peptone dextrose (YEPD) agar slants containing yeast extract; 10 g/L, peptone; 20 g/L, dextrose; 20 g/L and agar; 20 g/L with BaP (1.0 mg/L) and stored at 4ºC.

Development and acclimatization of yeast consortium

Different combinations of yeast strains viz. NS01, NS02, NS03 and NS04 were used for the development of four yeast consortium (YC), viz., YC01, YC02, YC03 and YC04. The combination of yeast strains of the consortia were shown in Table 2.

Table 2: Yeast consortia consisting of three yeast strains

Name of Consortium	Combination of yeast strains
YC01	*Rhodotorula* sp. NS01, *Hanseniaspora opuntiae* NS02, *Debaryomyces hansenii* NS03
YC02	*Hanseniaspora opuntiae* NS02, *Debaryomyces hansenii* NS03, *Hanseniaspora valbyensis* NS04
YC03	*Rhodotorula* sp. NS01, *Hanseniaspora opuntiae* NS02, *Hanseniaspora valbyensis* NS04
YC04	*Rhodotorula* sp. NS01, *Debaryomyces hansenii* NS03, *Hanseniaspora valbyensis* NS04

A liquid culture of the yeast consortium was prepared by growing 1% (v/v) of three different yeast cultures in YEPD broth. The inoculated flasks were shaken thoroughly to dispense the inoculants and incubated at 28±2 ºC and 120 rpm in a rotary shaker. After 5 days the yeast consortium was transferred to a fresh sterile medium with a step-wise increase in the BaP from 1.0 to 10.0 mg/L for acclimatization studies. The acclimatized flasks were incubated under same conditions as stated above.

Gene sequencing and identification of the yeast isolates

Gene sequencing and identification of the yeast isolates was done at Acme Progen Biotech Pvt. Ltd., Salem (Tamilnadu) and Yaazh Xenomics, Mumbai (Maharashtra). Yeast cells were grown in YEPD broth (1.0 mg/L of BaP) for 48 h at 28 °C. Cells were harvested by centrifuging at 8400×g for 10 min. High molecular weight DNA was obtained from the yeast cells by phenol/chloroform extraction protocol [34]. The DNA samples were dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and were used for PCR amplification. The primers used were as follows: forward-UL18F:5'-TGTACACACCGCCCGTC-3' and reverse-UL28R:5'-ATCGCCAGTTCTGCTTAC-3'. PCR amplification was carried out for 35 cycles at following conditions: 30 s at 95 °C, 40 s at 60 °C, 40 s at 72 °C. The amplicon comprised of partial and complete sequences for the genes of 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA. The purified PCR products were characterized by partial and complete sequence analysis. DNA sequencing was done using the same primers as mentioned above. A BLAST (Basic Local Alignment Search Tool) program was implied for similarity search from the database available on the GenBank [35]. The phylogenetic analysis was performed using CLUSTAL W (DDBJ- DNA Databank of Japan). A phylogenetic tree was constructed by neighbour-joining method using MEGA 6.0 version software for displaying phylogeny [36].The assembled partial and complete 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA sequences of strain *Rhodotorula* sp. NS01, *Hanseniaspora opuntiae* NS02, *Debaryomyces hansenii*NS03, *Hanseniaspora valbyensis* NS04 were deposited in GenBank database under accession number KP300039, KT226114, KR872423 and KR872424 respectively.

Growth monitoring

The four yeast consortia viz., YC01, YC02, YC03 and YC04 were studied to find the best consortia based on their growth and BaP degradation efficiency. The growth of yeast was determined by measuring the dry weight of the biomass. Yeast culture acclimatized in YEPD broth [Optical density (O.D.) $_{600}$ =0.1] was added to series of flasks containing MM. The flasks were incubated at 28 ± 2 ºC for 6 days on a rotary shaker at 120 rpm. Samples were subcultured at regular intervals and the cell suspension was centrifuged at 8400 ×g for 10 min. Then, it was transferred into pre-weighed Petri dish and dried to constant weight at 105 ºC for 45 min and the dry weight of biomass was calculated. Based on the biodegradation results, YC01 with the combination of NS01, NS02 and NS03 was selected for further study. All the experiments were carried out in triplicate.

Effect of growth parameters on BaP degradation

To study the effect of pH (5.0–9.0), incubation temperature (10–40 ºC), shaking speed (90–150 rpm), inoculum dosage (2–5 %) and initial BaP concentration (10–50 mg/L) on growth and BaP degradation, the acclimated yeast consortium was added to Erlenmeyer flasks (100 mL) containing 25 mL of mineral medium supplemented with BaP (10 mg/L). Inoculum was prepared by growing the yeast in YEPD broth containing BaP for 48 h. The cells were then harvested, washed, and re-suspended in the required quantity of MM containing BaP (10 mg/L) as sole carbon and energy source. During optimization of each parameter, all the other parameters were kept constant. While optimizing pH, parameters such as temperature 28 ºC, shaking speed 120 rpm, inoculum dosage 2 % and initial BaP concentration 10 mg/L were kept constant, varying the pH values ranging from 5.0 to 9.0. A similar trend was followed for other parameters too. All the experiments were carried out in triplicates. An abiotic control for BaP and biotic control for yeast growth were maintained for each experiment.

Biodegradation of BaP

The required quantity of BaP stock solution was dispensed into Erlenmeyer flasks (100 mL capacity), containing sterile mineral medium (25 mL) and was inoculated with the yeast consortium. The experimental flasks were incubated on a rotary shaker (130 rpm) at 30 ºC for 6 days. The flasks were removed at required intervals for the analysis of residual BaP. Uninoculated flasks were maintained as control. The residual BaP in the culture medium was calculated using the formula:

$$\text{Residual BaP (\%)} = \frac{C_i - C_f}{C_i} \times 100 \quad (1)$$

where, C_i is the initial concentration BaP in the medium and C_f is the final concentration of BaP.

Kinetics studies on BaP biodegradation

The experimental data of the degradation kinetics of BaP were fitted with various kinetics models like zero order [37], first order [12] and second order [38], respectively.

Extraction and characterization of degraded products by GC–MS and FTIR

Residual BaP and the potential degradation products in the culture broth were monitored by GC–MS and FTIR analysis. Flasks were withdrawn at regular intervals for analysis of degradation products. Individual flasks were withdrawn at 0, 2, 4 and 6 days of incubation. The degradation products were extracted and analysed by GC–MS as described by Samanta et al., (1999) [39]. The cultures were centrifuged and the supernatant was collected to remove residual crystalline BaP. The pH of the supernatant was adjusted to 2.0 with 2 N HCl and sodium chloride (20 %, w/v) was added to achieve better separation of the aqueous and organic layers. The extracts were taken twice by using equal volumes of ethyl acetate. The solvents were 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 5% diphenyl and 95% dimethyl polysiloxane as stationary phase. The inlet temperature was 220 ºC; oven temperature was increased from 50 to 250 ºC at 10 ºC rev/min; the GC interface temperature was 250 ºC; carrier gas was nitrogen at a flow rate of 1.0 mL/min. Mass spectrum conditions had the ionization energy 70 eV, ion chamber temperature was maintained at 250 ºC with tungsten filament, which is used for the ionization of molecules. The obtained compounds were compared with respective 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.

Infrared spectra were obtained using an IR affinity-1 FTIR spectrophotometer (Shimadzu) using KBr. The extracted degraded products dissolved in ethyl acetate, were mixed with KBr and made in the form of pellets. The scanning range was kept from 4000-500 cm^{-1} and the spectral resolution was 4 cm^{-1}.

Enzyme assays

Cells grown in the presence of BaP were harvested at different time intervals (0, 2, 4 and 6 days). The yeast cells were grown in the MM with 50 mg/L BaP as carbon and energy source. The cultures were centrifuged (10,000 rpm, 15 min) at 25 ºC and rapidly washed 3 times with 50 mM phosphate buffer (pH 7.0). Cells were disintegrated with a probe-type sonic oscillator for 10 min. The extract was centrifuged at 10,000 rpm at 4 ºC for 30 min to remove whole cells and large debris. The collected supernatant was used immediately for the enzyme assays following the standard protocol where the activities of 1,2-dioxygenase and 2,3-dioxygenase were assayed using catechol as the substrate [4,40].Catalase [41], lignin peroxidase [42], manganese peroxidase (MnP) [43] and laccase [44] activities were also assayed. One unit of activity was defined as the amount of enzyme that oxidizes 1.0 mmol of substrate per minute and the activity was expressed in U/L.

RESULTS

Screening of BaP degrading yeasts consortium

The yeast consortium (YC01, YC02, YC03, and YC04) were initially screened for BaP degradation with an initial concentration of 10 mg/L. Fig. 1a shows comparative BaP degradation percentage by four different yeast consortia. YC01 showed the highest BaP degradation (59%) on compare to other three consortia (YC02- 49%, YC03- 55%, YC04- 51%). Based on the screening results, the yeast consortium, YC01 was selected for further biodegradation studies.

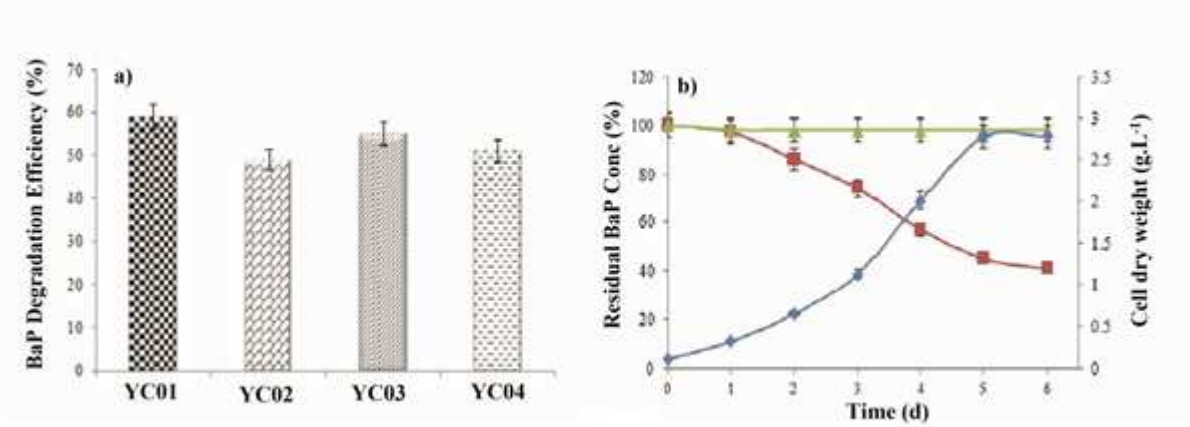

Figure 1:(a) Biodegradation efficiency of different yeast consortium and (b) Growth of yeast consortium, YC01 in mineral broth and BaP degradation (%), cell dry weight (♦), residual BaP concentration (■) and abiotic control (▲)

The growth pattern of yeast consortium YC01 was studied with respect to BaP degradation (Fig. 1b). The YC01 was found to utilize BaP as sole carbon and energy source. The metabolism of BaP by yeast consortium YC01 was assessed by the increase in the cell dry weight. There was a lag phase on the 1st day where the consortium growth was scanty, producing a cell dry weight of only 0.32 g/L. Later the growth initiated well from day 2 onwards and continued up to day 5, after which stationary phase was attained on day 6. Fig. 1b showed a positive correlation with an increase in yeast biomass to the BaP degradation percentage. No significant difference in the BaP concentrations was noted in case of abiotic controls.

Identification and phylogenetic analysis of yeast isolates of consortium YC01

Partial 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene regions were amplified and sequenced using designed primers for polymerase chain reaction (PCR). Sequence analysis of 18S rRNA gene of the strains NS01, NS02, NS03 and NS04 allowed as to confirm their closest relatedness to *Rhodotorula* sp. GM5 (GenBank accession no: KF543865; 99%), *Hanseniaspora opuntiae* isolate GK01 (GenBank accession no: KC870065; 98%), *Debaryomyces hansenii* strain NRRL Y-7426 (GenBank accession no: JQ689041; 99%) and *Hanseniaspora valbyensis* strain NRRL Y-1626 (GenBank accession no: JQ689026; 97%) in similarity search using BLAST program. Therefore, the four isolated yeast strains were named as *Rhodotorula* sp. NS01, *Hansenia sporaopuntiae* NS02, *Debaryomyces hansenii* NS03 and *Hanseniaspora valbyensis* NS04 respectively. The sequences were deposited in GenBank database under accession number KP300039, KT226114, KR872423 and KR872424 respectively. The sequence and phylogenetic positions of yeast consortium of the strains was also performed by neighbour-joining algorithm using MEGA 6.0 software [36] and presented as Fig. 2.

Optimization of the growth parameters for BaP degradation by yeast consortium YC01

The optimum range of the five significant growth parameters: pH 5.0–9.0 (Fig. 3a); temperature 10–40 ºC (Fig. 3b); shaking speed 90–150 rpm (Fig. 3c); inoculum dosage 2–5% (Fig. 3d) and initial BaP concentration 10–50 mg/L (Fig. 3e) affecting degradation were determined in separate experiments. The yeast consortium YC01 showed a maximum cell dry weight and degradation efficiency at pH 7.0, temperature 30 ºC, shaking speed 130 rpm, inoculum dosage 3% g dry weight/L and initial BaP concentration of 50 mg/L, which were noted as optimal growth conditions. Under optimized conditions, the yeast consortium, YC01 was able to degrade 76% of 50 mg/L BaP at the end of 6days.

Kinetics of BaP degradation by yeast consortium

The experimental data for the degradation kinetics of BaP was fitted with various kinetic reaction models (Fig. 4) and the best fit was found was seen with first order model. The degradation rate constant (k), half-life periods ($t_{1/2}$) and regression values (R^2) were also calculated with respect to the optimal concentration (50 mg/L) and were presented in Table 3. The half–life period of BaP was recorded as 3.03 days. The rate constant is calculated as 0.229 day^{-1}. The regression coefficient (R^2) as 0.952.

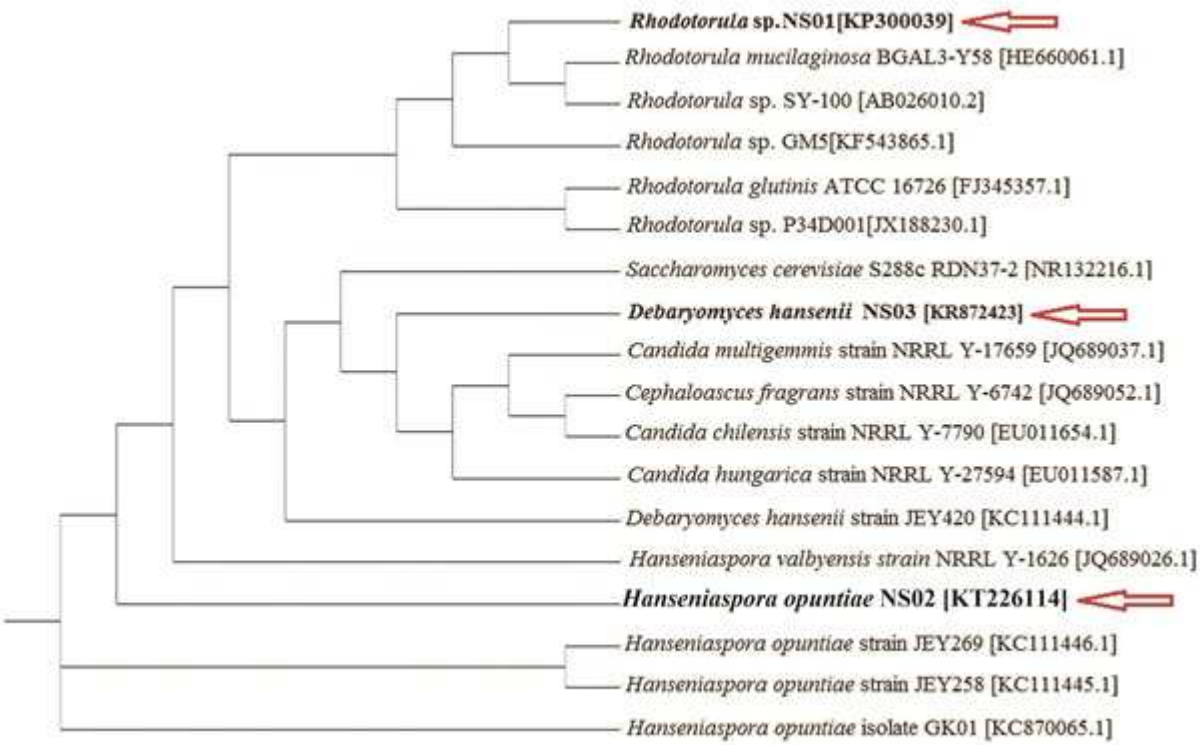

Figure 2: Phylogenetic relationships of yeast consortium, YC01 isolated in this study and related species. Phylogenetic tree of partial and complete gene sequences of 18S rRNA, ITSI, 5.8S rRNA and 26S r RNA was generated by neighbor-joining method. GenBank accession number of each organism used is given in parentheses

Intermediates of BaP identified by GC-MS measurement

The GC-MS spectra of BaP and its metabolites formed during the degradation by yeast consortium YC01 was recorded at different time intervals viz, 0, 2, 4 and 6th day and are shown in Fig. 5. In Fig. 5, the day 0 sample showed a high intense peak of retention time (RT) of 25.6 min in the positive ion mode, which confirmed the presence of the parent compound, BaP.

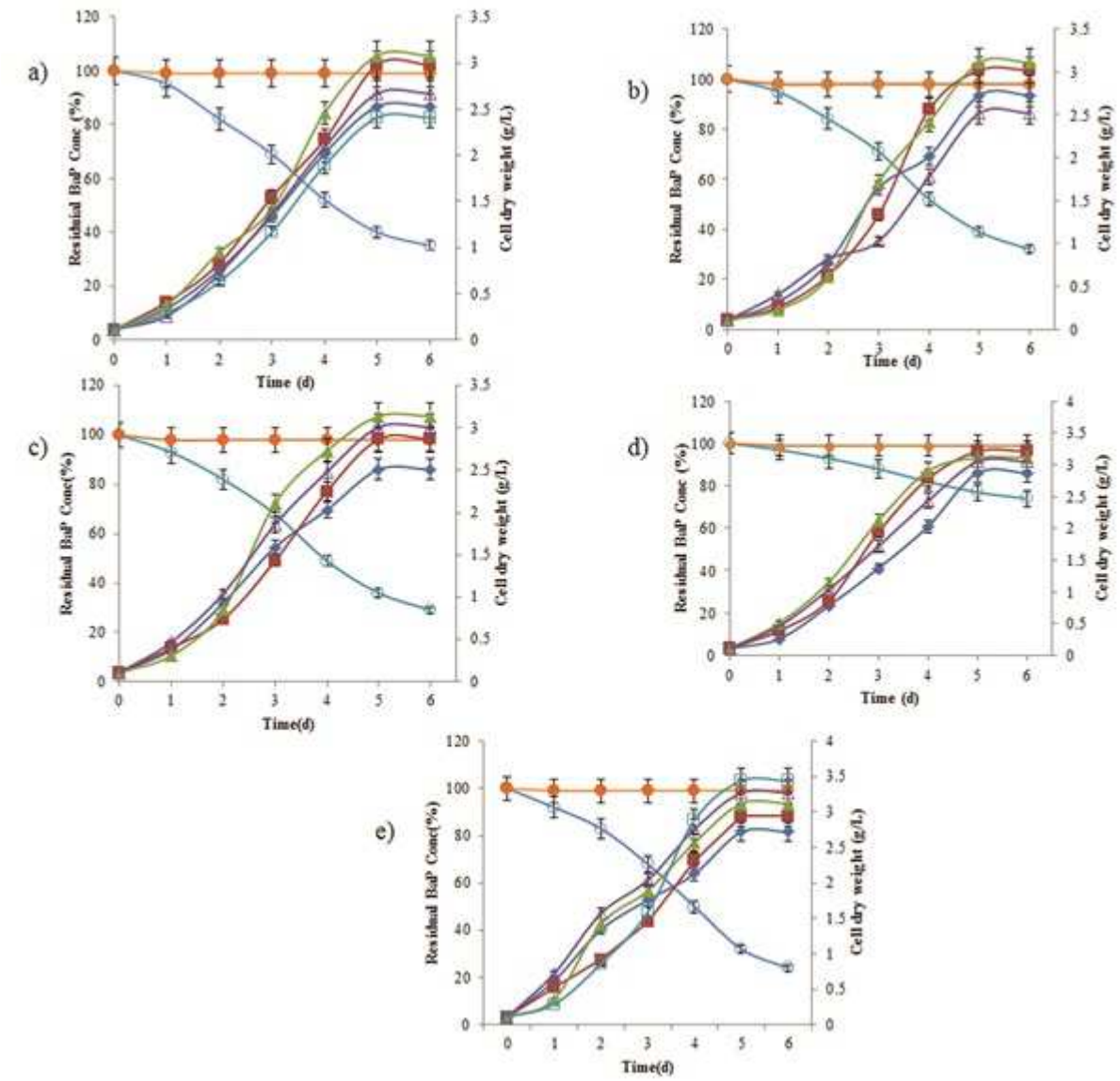

Figure 3: The effect of culture conditions on growth and degradation by consortium YC01 which was grown in mineral medium with concentration of BaP (50 mg/L) for a period of 6 days. The effect of pH, temperature, shaking speed, inoculums dosage and initial BaP concentration were evaluated. 3(a). Effect of pH- 5.0(♦), 6.0(■), 7.0(▲), 8.0(△), 9.0(□), residual BaP conc (%) at pH 7.0 (○). 3 (b). Effect of Temperature- 10 °C (♦), 20 °C (■), 30 °C (▲), 40 °C (△), residual BaP conc (%) at 30 °C (○). 3(c). Effect of shaking speed- 90 rpm (♦), 110 rpm (■), 130 rpm (▲), 150 rpm (△), residual BaP conc (%) at 130 rpm (○). 3(d). Effect of inoculum dosage (w/v) - 2% (♦), 3% (■), 4%(▲), 5% (△), residual BaP conc (%) at 3% inoculum dosage (○). 3(e).Effect of initial BaP concentration (mg L^{-1}) -10 (♦), 20 (■), 30 (▲), 40 (△), 50 (□) residual BaP conc (%) at 50 mg/L (○), (●) abiotic control

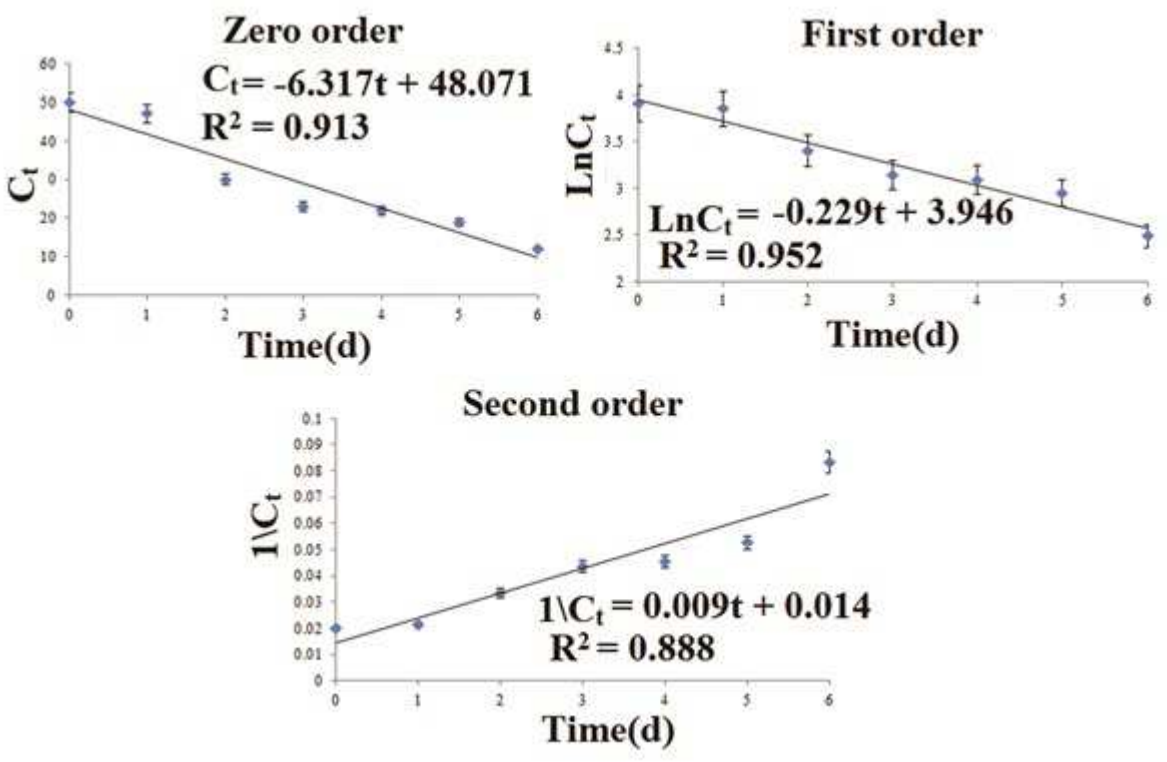

Figure 4: Kinetic plots of BaP degradation by consortium. a. Zero order, b. First order and c. Second order reaction model

On the 2nd day, the onset of biodegradation of BaP by yeast consortium was observed by the presence of new peak at RT of 19.7 min, which corresponded to the first metabolite M1 along with reduced intensity of parent peak. On the 4th day, two other new peaks of RT 17.9 and 23.7 min along with a reduced intensity of M1 metabolite was noted. These peaks were designated as two new metabolites, M2 and M3 respectively. On the 6th day, two more new peaks of RT 15.9 and 21.3 min, which corresponded to two more new metabolites, M4 and M5 were observed. Reduced intensity of the parent compound along with the newly formed metabolites confirmed the degradation of the parent compound and its transformation as metabolites by the yeast consortium YC01. The mass spectra of BaP and its biodegradation metabolites are given in Fig. 5.

Table 3: The kinetic parameters for the degradation of Benzo[a]pyrene at 50 mg/L by consortium, YC01

Kinetics Model	Parameters	Consortium YC01
Zero order	Regression equation	C_t = -6.317t + 48.071
C_t-C_o=Kt	K (day-1)	6.317
$T_{1/2}$= *Co/2Ko*	$T_{1/2}$	3.958
	R^2	0.913
First order	Regression equation	lnC_t= -0.229t + 3.946
$lnC_t = K_1t + lnCo$	K (day-1)	0.229
$T_{1/2}$=*ln2/K*$_1$	$T_{1/2}$	3.026
	R^2	0.952
Second order	Regression equation	$1/C_t$ = 0.009t + 0.014
$1/C_t=1/C_o + K_2t$	K (day-1)	0.009
$T_{1/2}$=*1 / Co K*$_2$	$T_{1/2}$	2.222
	R^2	0.888

R^2 = Regression coefficient; K= Degradation rate constant; $T_{1/2}$=Half-life period

Possible BaP degradation pathway by yeast consortium

A possible BaP biodegradation pathway by YC01 is proposed and shown in Fig. 6. The degradation of BaP initiated with attack on the benzene ring of the parent compound forming benzene, 1, 1', 1'', 1'''-(1,2-ethanediylidene) tetrakis which is designated as M1 (m/z=334). This was further degraded, giving rise to two possible metabolites 1,3-benzodiol,o-(3-methylbut-2-enoyl)-o'-(4-nitrobenzoyl), M2 (m/z = 341) and 1, 3-benedio,o-(3-metylbut-2-

enoyl)-o'-pivaloyl, M3 (m/z = 276) in which two closed benzyl ring connected with enol groups were observed. From the structure, it was seen that, M3 was more simplified structure than M2,which confirmed the degradative ability of YC01.The degradation pathway proceeded further where M2 gave rise to phenol,3,5-bis-(1,1-dimethyl) as M4 (m/z = 206) and M3 gave rise to sarcosine, N-(3-methlbut-2-enoyl)-ethylester (M5) (m/z = 184).

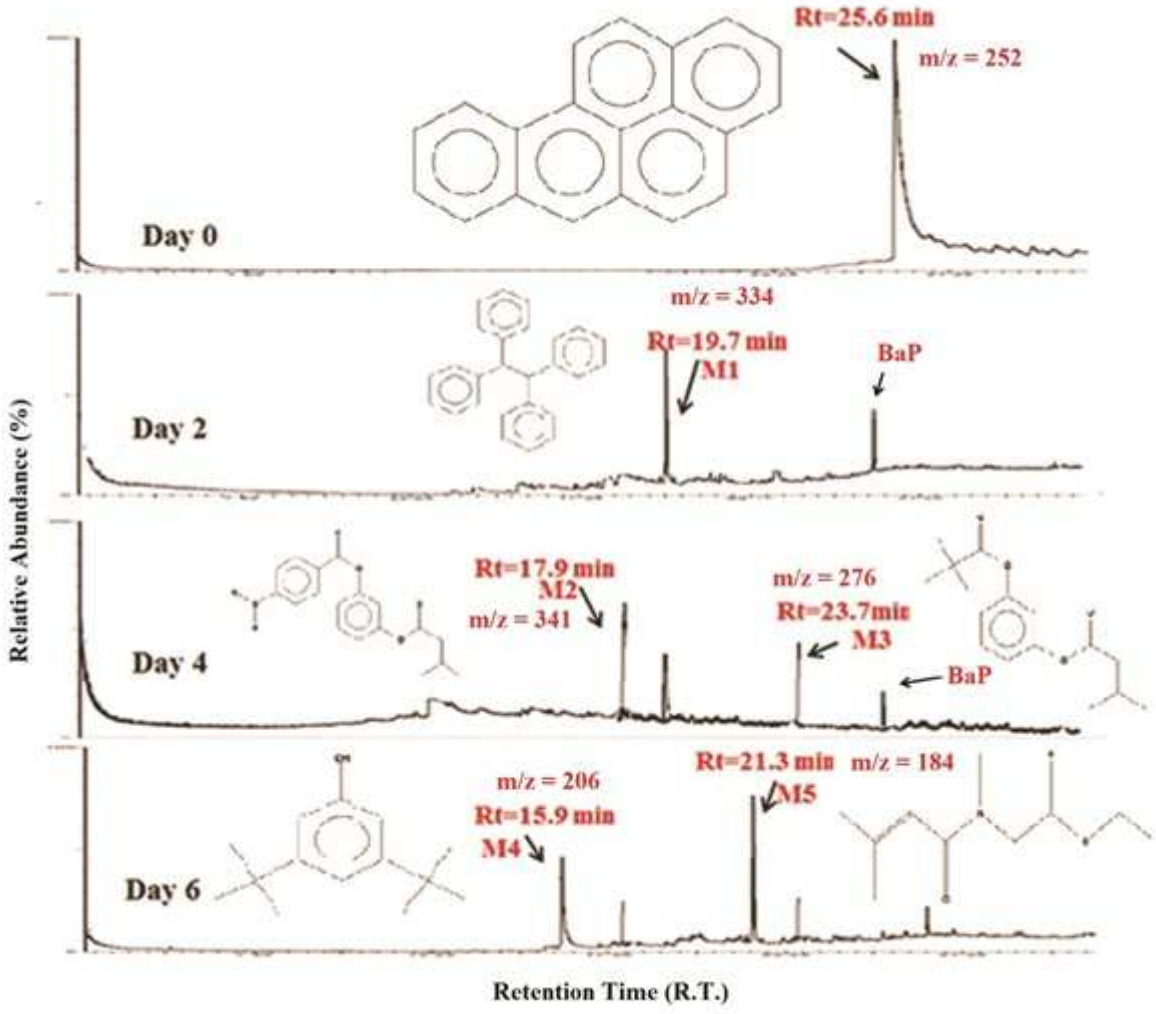

Figure 5: GC-MS analysis of BaP and its degradation products at 2, 4 and 6 days

Fig. 7(a, b) illustrates FTIR spectra of BaP before and after degradation by yeast consortium, YC01. The FTIR spectrum of pure BaP (Fig. 7a) showed the characteristic absorption peaks at 3028.24 cm^{-1} corresponds to =C-H stretch in aromatic hydrocarbon. The absorption peaks at 1467.83 cm^{-1} and 1176.58-1346.31 cm^{-1} represents C-C stretch in aromatic benzene rings. Strong peaks at between 871.82-671.23 cm^{-1} corresponds to =C-H bend. A comparison of FTIR spectra of BaP before and after degradation (Fig. 7b) by yeast consortium, YC01 revealed the complete destruction of the aromatic ring structure of the parent compound, which was confirmed by absence of bands corresponding to benzene ring. The presence of new bands at 2983.88 and 1450.47 cm^{-1} pertaining to C-H stretch of methyl group were noted confirming the linearized structure of the formed metabolites. Sharp peaks at 1757.15, 1735.93 and 1236.37 cm^{-1} represents C-O-C stretch in esters and the presence of band at 1043.49 cm^{-1} corresponds to CH-OH in cyclic alcohols also supported the results. The absorption bands at 837.40 and 846.75 cm^{-1} showed CH deformations that confirmed the effective BaP degradation by the yeast consortium, YC01.

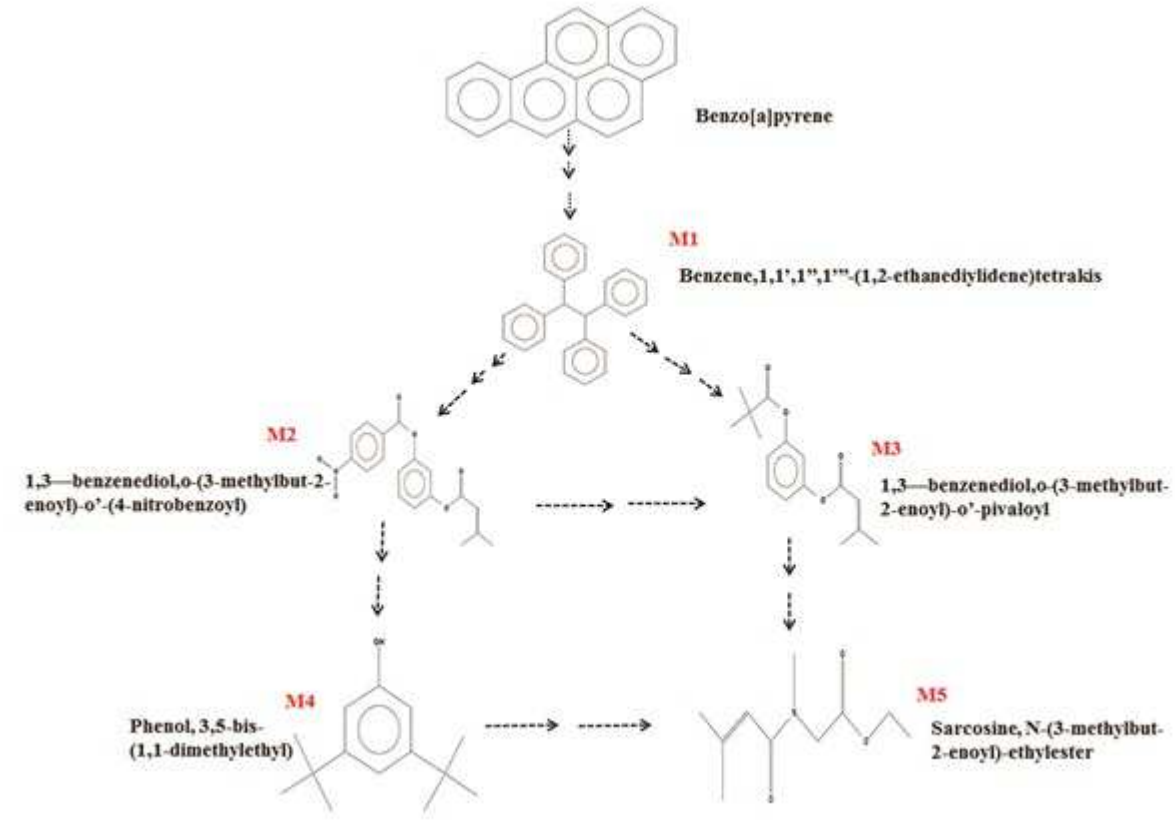

Figure 6: The proposed BaP microbial degradation pathway by yeast consortium. The complete arrows represent products identified by GC-MS. The doted arrows represent transient metabolites, which were not detected in GC-MS

Role of enzymes in BaP degradation

Table 4 represents the role of enzyme activities recorded in the culture supernatant and cell lysate collected on the 2nd, 4th and 6th day of BaP degradation by yeast consortium, YC01. Several enzyme such as 1, 2-dioxygenase and 2, 3-dioxygenase, lignin peroxidase (LiP), manganese peroxidase (MnP), laccase and catalase were found to play an important role towards BaP degradation by YC01.

Table 4: Enzyme activities (u/mL) in the culture supernatant and cell free extracts of YC01

Enzymes	Day 2		Day 4		Day 6	
	A	B	A	B	A	B
1,2-Dioxygenase	8.01±0.09	3.57±0.04	6.36±0.06	3.02±0.04	5.89±0.05	2.51±0.03
2,3-Dioxygenase	0.56±0.02	0.20±0.01	1.69±0.04	0.79±0.03	0.38±0.02	0.10±0.01
Laccase	5.02±0.28	2.04±0.66	7.05±0.66	3.17±0.33	3.07±0.34	1.88±0.05
Lignin Peroxidase	1.18±0.04	0.04±0.04	1.38±0.04	0.09±0.04	0.96±0.04	0.05±0.04
Manganese Peroxidase	2.66±0.02	0.17±0.62	2.89±0.02	0.67±0.44	1.74±0.21	0.39±0.54
Catalase	0.70±0.07	0.23±0.08	1.86±0.03	0.24±0.08	1.10±0.03	0.18±0.09

A: Enzyme activity in the culture supernatant; B: Enzyme activity in the cell lysate

On the initial phase of BaP degradation, 1, 2-dioxygenase and laccase had a profound activity both in the supernatant and in the cell lysate whereas the reverse trend was observed for other degradative enzymes studied. 1,2-dioxygenase activities reduced with increase in time whereas other enzyme activities were less on the day 2 but was found to be increased on the 4th day and gradually decreased over increased incubation time as the substrate was completely utilized by YC01. Enzyme activities were found to be high in the cell lysate, which confirmed the predominant role of the extracellular enzymes.

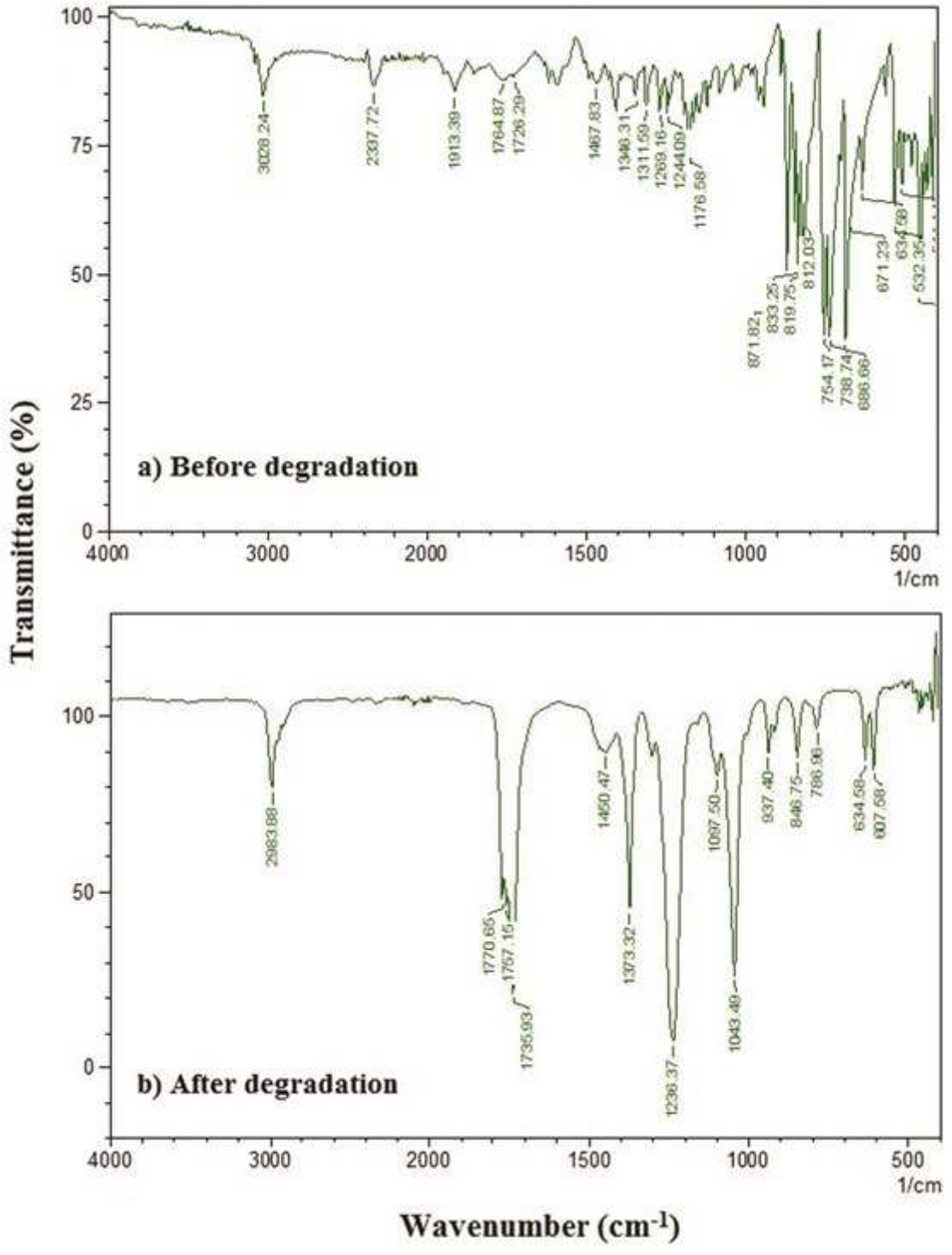

Figure 7: FTIR spectra of BaP biodegradation by yeast consortium at standard condition (a) before degradation and (b) after degradation

DISCUSSION

BaP has become a priority pollutant due to its mutagenic and carcinogenic properties. Bioremediation process employs the use of microorganisms to degrade and detoxify the pollutants present in the environment [45]. There are reports on microorganisms like bacteria and fungi which are capable of degrading BaP [16-20]. Reports are scanty on BaP degradation using yeast species [46].Hence, in the present study, the potentiality of yeast consortium, YC01 consisting of yeast strains viz. *Rhodotorula* sp. NS01, *Hanseniaspora opuntiae* NS02and *Debaryomyces hansenii* NS03 was tested for BaP degradation in detail.

Environmental factors such as, pH, incubation temperature, shaking speed, inoculum dosage and the initial concentration of the substrate greatly influence the growth of microbes and their degrading abilities [47].An optimized pH of 7.0 obtained in the present study was found to be similar to results obtained in other PAH biodegradation studies [48].In a recent study by Bacosa and Inoue (2015) [49], maximum degradation of mixed PAHs was achieved at 30 ºC and 120 rpm, which correlated with results of present study. The use of larger inoculum size increases the degradation efficiency of highly toxic compounds [50]. In our study too, the rate of BaP degradation was found to be maximum with an inoculum dosage of 3% (w/v), which may be possibly due to increased microbial proliferation.

The concentration of the target pollutant is an important factor, which affects the biomass production as well as the degradation rates. There are reports on BaP degradation using bacterial consortium which showed 23.6% degradation after 30 days of initial concentration of 100 mg/L [27]. BaP degradation using fungal consortium showed 23% degradation (22 mg/kg for 180d) as reported by Lladóa *et al.,* (2013)[25]. Inoculation of fungi-bacterial co-cultures showed BaP degradation of upto 53% at concentration of 50 mg/L in 100 days was reported [30]. Therefore, the results of the present study gains importance by confirming the potentiality of yeast consortium, YC01 which showed 76% BaP degradation within 6 days at 50 mg/L.

Based on the regression values, the kinetic analysis of BaP degradation by YC01 showed best fit with first-order kinetic model, which implied that, the BaP degradation was dependent upon the substrate concentration [2]. Kot-Wasik *et al.,* (2004)[12] reported photodegradation and biodegradation study of BaP showing very high half-life value of 85-433 days, whereas, the calculated half-life of BaP was found to be 3.03 days, which proved the astonishing potentiality of yeast consortium, YC01 on BaP degradation.

GC-MS based metabolite analysis has been done and the metabolites formed during the degradation of BaP by YC01 were identified. According to the reports available so far, most of the microbially induced BaP biotransformations are reported to be partially degraded, in which one of the benzene ring is cleaved forming dihydrodiols [31,51-53] and BaP-1,6, quinone [4]. In the present study, complete distortion of BaP molecule by the yeast consortium, YC01 has been noted. The results were also supported by FT-IR analysis [54]. It confirmed the degradation of the parent compound, which was transformed to more simpler intermediates for the utilization as carbon and energy source by yeast consortium YC01 for their metabolic needs. Based on the results, a possible hypothetical degradation pathway is proposed. To the best of our knowledge, this is the first report on BaP degradation using yeast consortium.

The enzyme activities of yeast consortium, YC01 were periodically monitored during BaP degradation process. The enzyme 1, 2-dioxygenase was found to play a predominant role in BaP degradation process. In case *Mycobacterium vanbaalenii* PYR-1, the involvement of mono and dioxygenases enzymes was reported to oxidise BaP during the degradation process [53]. The role of fungal dioxygenase system in transforming BaP to cis- or trans-dihydrodiol, and further, to dihydroxy BaP has been reported [55]. The role of other degradative enzymes, viz., lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase during BaP degradation was noted in the present study. Involvement of LiP and laccase enzymes were detected during BaP degradation by *L. theobromae* in liquid medium [56]. According to Harms *et al.,* (2011) [57], the enzymes viz. laccase and peroxidase are being required for the initial activation of BaP. It is likely that the respective enzyme catalyse was responsible for the later reactions of BaP-degradation. Involvement of catalase enzyme during BaP degradation was noted in the present study. Similar activity of catalase enzyme was reported in case of *Rhodopseudomonas palustris* CGA009 during BaP degradation [58].

CONCLUSION

Based on the results of the present study, it can be concluded that the yeast consortium, YC01 may serve as potential bioresource for remediation of BaP which could degrade 76% of BaP at a concentration of 50 mg/L within 6 days in aqueous environment.

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REFERENCES

[1] J. Schneider, R. Grosser, K. Jayasimhulu, W. Xue, D. Warshawsky, **1996**, *Appl. Environ. Microbiol.*, 62,13-19.
[2] Y. Zeng, P.K.A. Hong, D.A. Wavrek, **2000**, *Environ. Sci. Techn.*, 34,854-862.
[3] R.A. Kanaly, S. Harayama, K. Watanabe, **2002**, *Appl. Environ. Microbiol.*, 68, 5826-5833.

[4] T. Hadibarata, R.A. Kristanti, **2012a**, *J. Environ. Manag.*, 111, 115-119.
[5] U.S. Department of Health and Human Services, **1990**, Toxicological profile for polycyclic aromatic hydrocarbons. *U.S. Department of Health and Human Services*, Washington, D.C.
[6] B. Mahadevan, A. Luch, J. Atkin, T. Nguyen, A.K. Sharma, S. Amin, W.M. Baird, **2006**, *Chem. Biol. Interact.*, 164,181-191.
[7] P.J. Tsai, H.Y. Shieh, L.T. Hsieh, W.J. Lee, **2001**, *Atmosph. Environ*., 35, 3495–3501.
[8] K.G. Wunch, T. Feibelman, J.W. Bennett, **1997**, *Appl. Microbio.Biotech.*, 47,620-624.
[9] R.M. Atlas, C.E. Cerniglia, **1995**, *Bioscience*, 45, 332-338.
[10] C. Chaineau, J. Morel, J. Oudot, **2000**, *J. Environ. Qual.*, 29:569-578.
[11] B. Guieysse, M.D.T.G. Cirne, B. Mattiasson, **2001**, *Appl. Microbiol. Biotechnol*.,56, 796-802.
[12] A. Kot-Wasik, D. Dabrowska, J. Namiesnik, **2004**, *J. Photoch. Photobio. Chem*., 168, 109-115.
[13] M. Couto, E. Monteiro, M. Vasconcelos, **2010**, *Environ. Sci. Pollut. Res*., 17, 1339-1346.
[14] A. Greenberg, C. His, N. Rothman, P.T. Strickland, **1993**, *Polycycl.Aromat.Comp*., 3, 101–110.
[15] S. Gan, E.V. Lau, H.K. Ng, **2009**, *J. Hazard. Mater*., 172, 532-549.
[16] D. Dean-Ross, J.D. Moody, J.P. Freeman, D.R. Doerge, C.E. Cerniglia, **2001**, *FEMS Microbiol.Lett.*, 204, 205-211.
[17] B.E. Andersson, S. Lundstedt, K. Tornberg, Y. Schnurer, L.G. Oberg, B. Mattiasson, **2003**, *Environ. Toxicol. Chem*., 22, 1238-1243.
[18] S.M.N. Chan, T. Luan, M.H. Wong, N.F.Y. Tam, **2006**, *Environ. Toxicol. Chem*., 25:1772-1779.
[19] T. Hadibarata, S. Tachibana, K. Itoh, **2007**, *Pak. J. Biol. Sci*., 10, 2535-2543.
[20] P. Arulazhagan, N. Vasudevan, **2011**, *Mar. Pollut. Bull*., 62, 388-394.
[21] A.E.L. Hesham, Z. Wang, Y. Zhang, J. Zhang, W. Lv, M. Yang, **2006a**, *Ann. Microbiol*., 56, 109-112.
[22] D. Zhao, C. Liu, L. Liu, Y. Zhang, Q. Liu, W.M. Wu, **2011**, *Int. Biodeter. Biodegr*., 65, 1244-1248.
[23] S. Mishra, S.N. Singh, V. Pande, **2014**, *Bioresource Technol*., 164, 299-308.
[24] M.I. Khan, J. Yang, B. Yoo, J. Park, **2015**, *J. Hazard. Mater*., 287, 243-251.
[25] S. Lladó, S. Covino, A.M. Solanas, M. Viñas, M. Petruccioli, A. Dannibale, **2013**, *J. Hazard.Mater*., 248, 407-414.
[26] C. García-Díaz, M.T. Ponce-Noyola, F. Esparza-García, F. Rivera-Orduña, J. Barrera-Cortés, **2013**, *Int. Biodeter. Biodegr*.,85, 311-322.
[27] J. Mao, Y. Luo, Y. Teng, Z. Li, **2012**, *Int. Biodeter. Biodegr*.,70,141-147.
[28] S. Sun, J. Jinghua, S. Guangdong, L. Ying, L. Zhipei, **2010**, *J. Environ. Sci*., 22,1576-1585.
[29] S.U. Dan, L.I. Pei-jun, F. Stagnitti, X. Xian-zhe, **2006**, *J. Environ. Sci*., 18, 1204-1209.
[30] S. Boonchan, M.L. Britz, G.A. Stanley, **2000**, *Appl. Environ. Microbiol*., 66, 1007-1019.
[31] R.A. Kanaly, S. Harayama, **2000**, *J. Bacteriol*., 182, 2059-2067.
[32] Z. Cui, G. Xu, W. Gao, Q. Li, B. Yang, G. Yang, L. Zheng, **2014**, *Inter. Biodeter. Biodegr*., 91, 45-51.
[33] C. Lin, L. Gan, Z. Chen, M. Megharaj, R. Naidu, **2014**, *Biochem. Eng. J*., 90, 1-7.
[34] H.R. Cheng, N. Jiang, **2006**, *Biotechnol. Lett*., 28, 55-59.
[35] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, **1990**, *J. Mole.Biolo*., 5, 403-410.
[36] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, **2013**, *Molec. Biol. Evol*., 30, 2725–2729.
[37] J. Wang, X. Quan, L. Han, Q. Yia, W. Hegemann, **2002**, *Wat. Res*., 36, 2288-2296.
[38] C. Capellos, B.H. Bielski, Kinetic systems: mathematical description of chemical kinetics in solution,Wiley-Inter science, New York, **1972**.
[39] S.K. Samanta, A.K. Chakraborti, R.K. Jain, **1999**, *Appl. Microbiol. Biotechn*., 53, 98-107.
[40] T. Nakazawa, A. Nakazawa, In: H. Tabor and W. Tabor, Eds., *Pyrocatechase* (*Pseudomonas*), Methods in Enzymology (Academic Press, New York,**1970**)518-522.
[41] R.V.G. Flores, E.P.S. Ceniceros, R.D.L. Gámez, C.B. García, J.L.M. Hernández, G.G. Lozano, C.N. Aguilar, A. Ilyina, **2014**, *Afri. J. Microbiol. Res*., 8, 2788-2800.
[42] X. Li, X. Lin, P. Li, W. Liu, L. Wang, F. Ma, K.S. Chukwukae, **2009**, *J. Hazard. Mater*., 172, 601-605.
[43] G. Annadurai, R. Babu, G. Nagarajan, K. Ragu, **2000**, *Biopro. Eng*., 23, 715-719.
[44] D.S. Arora, D.K. Sandhu, **1985**, *Enz. Microbiol.Techn*., 7, 405-408.
[45] B.K. Singh, **2009**, *Nat. Rev. Microbiol*., 7, 156-163.
[46] A.E.L. Hesham, S. Khan, X.C. Liu, Y. Zhang, Z. Wang, M. Yang, **2006b**, *Yeast*,23, 879-887.
[47] C.D. Elcey, A.A.M. Kunhi, **2010**, *J. Agric. Food Chem*., 58, 1046-1054.
[48] M. Kastner, M. Breuer-Jammali, B. Mahro, **1998**, *Appl. Environ. Microbiol*., 64, 359-362.
[49] H.P., Bacosa, C. Inoue, **2015**, *J. Hazard. Mater*., 283, 689-697.
[50] F. Guille´n-Jime´neza, E. Cristiani-Urbinab, J.C. Cancino-Dı´azc, J.L. Flores-Morenod, B.E. Barraga´n-Huertaa, **2012**, *Int. Biodeter. Biodegr*., 74, 36-47.
[51] M.D., Aitken, R.D., Stringfellow, N.C., Kazunga, S.H. Chen, **1998**, *Can. J. Microbiol*., 44, 743-752.
[52] S. Boonchan, M.L. Britz, G.A. Stanley, **1998**, *Biotechnol. Bioeng*. 59, 482-494.
[53] J.D. Moody, J.P. Freeman, P.P. Fu, C.E. Cerniglia, **2004**, *Appl. Environ. Microbiol.*, 70, 340-345.

[54] M.M. Bhat, S. Shankar, Shikha, M. Yunus, R.N. Shukla, **2011**, *Adv. Appl. Sci. Res.*, 2, 321-326.
[55] T. Hadibarata, R.A. Kristanti, **2012b**, *Bioresource Technol.*, 107, 314-318.
[56] H. Liu, C. Wang, Z. Zhang, W. Wu, Z. Hao, H. Sun, **2011**, *Environ. Sci.*, 32, 2696-2702. (In Chinese)
[57] H. Harms, D. Schlosser, L.Y. Wick, **2011**, *Nat. Rev. Microbiol.*, 9,177-192.
[58] M. Kanehisa, S. Goto, Y. Sato, M. Furumichi, M. Tanabe, **2012**, *Nucleic Acids Res.*, 40, D109-D114.