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Mycoremediation of Benzo[a]Pyrene by Pleurotus ostreatus Isolated from Wayanad District in Kerala, India

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ABSTRACT

Benzo[a]pyrene (BaP) is a ubiquitous environmentally significant compound which due to its recalcitrant nature is often referred as persistent bioaccumulative toxin. The present study was carried on to determine the degradation potentials of three natural isolates of the white rot fungi Pleurotus ostreatus towards BaP, of which isolate PO-3 showed promising results. The factors affecting biodegradation efficiency of the isolate in liquid culture were also investigated. An increase in the BaP concentration in the medium resulted in the decrease in the extent of degradation and the fungal biomass formation. Addition of co-substrates improved the degradation efficiency of the isolate and the highest degradation (52.3%) was mediated by glucose amendment in the media. Organic nitrogen sources proved to be superior nitrogen supplements and yeast extract supported the maximum utilization of BaP (57.32%). Inorganic nitrogen sources though supported the fungal growth, were insignificant in enhancing degradation. Surfactants improve the bioavailability of the otherwise recalcitrant compounds. Incorporation of Tween 80 facilitated an elevated level of BaP removal (62.14%) as compared to the control and other ionic and non ionic surfactants. Thus, Pleurotus ostreatus PO-3 can significantly degrade the toxic BaP with certain alterations in the biodegradation strategies and can be a potential candidate for the reclamation of sites polluted with polyaromatic hydrocarbon.

KEYWORDS: *Benzo[a]pyrene, Pleurotus ostreatus, degradation, co-substrate, surfactant.*

INTRODUCTION

Benzo[*a*]pyrene (B*a*P), a potent carcinogen is a pentacyclic high molecular weight polyaromatic hydrocarbon (HMW PAH) with high recalcitrant nature[1]. B*a*P is commonly formed during the pyrolysis and incomplete combustion of biological material and organic compounds and is found in various concentrations in coal tar, petroleum, and oil-based fuels. From contaminated site, B*a*P enters into the food chain and metabolized to its ultimate genotoxic form which then interacts with nucleic acids and proteins forming highly reactive macromolecular adducts resulting in B*a*P induced toxicity, mutagenesis and carcinogenesis in mammals[2]. In contrast to the mammalian system, some algal, fungal and bacterial species are shown to degrade B*a*P co-metabolically, alone or in a co-culture[3].

Microbial degradation is one of the major processes in treatment of PAH contaminated soil. Although several bacterial and fungal species are potentially involved in PAH oxidation, degradation of these contaminants is limited by their low water solubility[4, 5]. Fungi display several advantages such as penetration

of the hyphae and excretion of oxidative enzymes in the polluted sites.

White rot basidiomycetes fungi like *Phanerochaete chrysosporium, Trametes versicolor, Cirnipellis stipitaria* and *Pleurotus ostreatus* have the ability to efficiently degrade lignin, a natural aromatic polymer having structural similarity to most PAHs by the synthesis of lignin modifying enzymes like lignin peroxidises (LiP), manganese peroxidases (MnP), laccases and other oxidases[4]. Thus, the non selective nature of these enzymes has prompted the suggestion that these fungi might be able to degrade HMW PAHs like B*a*P.

Previous works involving the PAH degradation was done with *Phanerochaete chrysosporium*, since under ligninolytic and non ligninolytic conditions it metabolized a wide variety of PAHs including the potent carcinogen B*a*P[6]. However other white rot fungi like *P. ostreatus* may be more promising than *P. chrysosporium* in its ability to mineralize HMW PAHs like B*a*P to CO_2. *P. ostreatus* differs from *P. chrysosporium* in PAH degradation mechanism; in that it does not exhibit lignin peroxidise activity[7]. Instead its lignin degradation ability is assumed to be correlated with production of high levels of laccase[8].

The purpose of our investigation is to study the capacity of the selected *P. ostreatus* to utilize B*a*P by the production of ligninolytic enzymes, determination of the cultural conditions affecting the level of B*a*P degradation and thus draw conclusion about applicability of this fungal strain in bioremediation of PAH polluted sites.

MATERIALS AND METHODS

The present study was conducted during the period from 15.03.2011 to 3.04.2012 at the Department of Microbiology, Genohelix Biolabs, Chamarajpet, Bangalore, Karnataka, India. The present research findings are a part of the Ph.D research of the first author admitted to Karpagam University, Coimbatore, Tamil Nadu, India.

Chemicals and reagents:

HPLC grade B*a*P (98% pure) was obtained from Spectrochem Pvt. Ltd., Mumbai, India and other fine chemicals were from SRL Chemicals, India. All the chemicals used were of the highest purity available and of analytical grade.

Sample collection and processing:

Fruiting bodies of the Basidiomycetes samples were collected from 3 different locations- decomposing log near the boating point of the Banasura Sagar reservoir (11°40'12" N, 75°57'28"E), wooden bench on the bank of Pookot Lake (11°32'30" N, 76°1'37"E) and bark of dead coffee plant from Kuruva Island (11°49'18" N, 76°5'32"E) located in Wayanad district, Kerala, India, using pre-sterilized zip lock covers and sterile scalpels. Following sampling, all the samples were transported to the laboratory in Bangalore, where they were processed.

The log sample, the bench sample and the bark sample were designated as PO-1, PO-2 and PO-3 respectively. Soil and litter were removed from each sample and sections of the fungal gills were plated on Malt extract agar (MEA) and incubated at 28°C for 7 days under darkness. Repeated plating was done until pure culture obtained. The pure cultures were maintained in the same media at 8°C until used.

Identification of the isolates:

PO-1, PO-2 and PO-3 were identified by the protocol described by Petersen and Hughes, 2003[9].The distinctive features of *P. ostreatus* identification were shape of the fungal cup, arrangement of the gills and stem, spore print and shape of the spores.

Selection of the best B*a*P degrading isolate:

To study the potency of pure culture of fungal

species for the degradation of the B*a*P, the carbon free mineral salt medium was used with the following composition (g/L): $(NH_4)_2HPO_4$; 05, KH_2PO_4; 0.8, K_2HPO_4; 0.3, $MgSO_4.7H_2O$; 0.3, $CaCl_2.2H_2O$; 0.055, $ZnSO_4.6H_2O$; 0.004, $CuSO_4$; 0.07, Yeast extract; 0.2, Thiamine; 1ml (2mg/ml) at pH 6±0.2.

Primary screening:

Acetone solution containing B*a*P (1µg/ml) was mixed with molten mineral salt agar and plated. Following solidification, Plugs of fungal mycelia (6 mm) previously grown on MEA were then inoculated at the centre of the plates. Parallel plates without B*a*P were also inoculated in the same manner and acted as controls. Both the test and the control plates were incubated at 28°C for 15 days, with the diameter of mycelial extension been recorded every 5 days interval.

Secondary screening:

Acetone solution containing B*a*P (1µg/ml) was placed in 250 ml conical flasks overnight. After the acetone had become completely volatile, 50 ml sterile liquid mineral salt medium was added to each conical flask and two fungal plugs were inoculated in the solution under aseptic condition. An uninoculated flask served as the "control". Incubation of all the flasks, including the control was done at 28°C, 120 rpm in the dark under ambient air for 30 days, following which the degradation efficiency was determined.

ANALYTICAL METHODS:

Extraction of residual B*a*P:

The B*a*P extraction for fungal cultures was performed using a modified method proposed by Capotorti *et al.,* 2004[10]. Before liquid-liquid extraction, the culture was filtered through a Whatman no 41 filter paper (Whatman International Ltd., Maidstone, England) to separate mycelia from culture filtrate. The filtrate was then extracted twice with equal volumes of dichloromethane (DCM), shaken vigorously and centrifuged.

The mycelia were oven dried (40°C for 24 hr) and weighed. The mycelia were then homogenized with 10 ml of DCM. Following homogenization, the homogenate was centrifuged at 3000 rpm for 15 min. The cell free supernatant was collected and mixed with the original broth filtrate organic extract.

The DCM from the extract was evaporated at 40°C in a hot air oven. Once concentrated the extract was subjected to High Performance Liquid Chromatography analysis.

High Performance Liquid Chromatography analysis:

HPLC analysis was carried at Genohelix Biolabs, Bangalore-560019, India. The condensed sample was filtered through 0.25 µ nitrocellulose membrane filter. The B*a*P standard was obtained from Spectrochem Pvt. Ltd., Mumbai, India. The working standard solution was prepared in acetonitrile: water (80:20 v/v), with concentration of 10 µg /ml. The reverse phase HPLC system used was from Waters, USA, model number- 2487, with Dual λ absorbance UV detector and binary pump system (model number-1525). 20 µl of the eluate containing 0.1 µg of the standard B*a*P was injected. The mobile phase used was acetonitrile: water (80:20 v/v); with a flow rate of 1 ml /min. A reverse phase, YMCA Triart C-18 column of 3 µm (150 x 4.6 mm) was used.

The concentration of the B*a*P stock standard solution was determined at 254 nm. Area under the absorbance peak was used to estimate the percentage of degradation using a formula:

$$[(C_i-C_f)/C_i]*100$$

Where, C_i is the initial concentration of B*a*P and C_f is the final concentration of B*a*P.

Inoculum preparation:

The isolate of *P. ostreatus* showing highest B*a*P utilization was inoculated in Malt extract broth

(2 agar plugs, 6mm each) and incubated at 28°C under shaking for the development of the mycelia balls. Following the fungal growth, the mycelial mass was carefully filtered and washed 3-4 times with sterile distilled water and stored at 4°C in physiological saline.

Effect of different B*a*P concentrations, co-substrates, nitrogen sources and surfactants:

B*a*P was taken as five concentrations (1, 5, 10, 25 and 50µg /ml of the mineral salt medium). In order to stimulate the level of degradation, the carbon free mineral salt medium with the known concentration of B*a*P was supplemented with co-metabolic substrates like glucose, mannitol, sucrose and maltose (0.01% w/v). Influence of nitrogen supplementation was checked by amending the liquid medium with various organic and inorganic nitrogen sources like peptone, beef extract, tryptone, yeast extract, urea, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, $NaNO_3$ and $NaNO_2$ (0.1% w/v). Effect of surfactants was studied by incorporation of 0.05% (v/v) of Tween 20, Tween 40, Tween 80, Tween 100, Triton X-100 and 0.05% (w/v) sodium lauryl sulphate (SLS) and sodium deoxycholate (SDC). Growth and degradation conditions were maintained same as above.

Statistical analysis:

Effect of each parameter was studied in triplicate and the data are graphically presented as the mean ± S.D. of triplicates (n = 3). ANOVA was performed using Microsoft Excel 2007. *P* values < 0.05 were considered significant with a confidence limit of 95%.

RESULTS AND DISCUSSION

Xenobiotic chemicals are continuously released into the biosphere resulting in significant risk to human health due to their toxicity and persistence in the environment. PAHs are a ubiquitous large class of hazardous organic chemicals consisting of 3 or more fused benzene rings in linear, angular, and cluster arrangements[11]. Therefore, many PAHs including phenanthrene, acenaphthene, acenaphthylene, fluoranthene, pyrene, benzo[*a*]pyrene and benzo[*a*]anthracene are listed in the priority pollutant list of the United States Environmental Protection Agency[12].

Mycoremediation is a process by which fungi degrade or transform hazardous organic contaminants to less toxic compounds[13]. White-rot and litter-decomposing fungi are potential candidates for the treatment of contaminated soils because of their high capability to degrade a wide range of xenobiotics not only in liquid culture but also in contaminated soil[8]. Fungi display several advantages such as penetration of the hyphae and excretion of oxidative enzymes in the polluted sites. Attempts have therefore been made to apply these fungi to the bioremediation of soils contaminated with compounds not sufficiently degradable by soil microorganisms.

Degradative potentials of the isolates:

When evaluated for their potency to degrade 1µg/ml of B*a*P in both solid and broth media, isolate PO-3 demonstrated the highest degradation, followed by PO-1 and PO-2.In the mineral salt agar media spiked with the given concentration of B*a*P, the maximum hyphal extension was seen for PO-3, suggesting that this isolate could use the B*a*P as its carbon source better than the rest two isolates. In the broth, maximum biomass yield and lesser residual B*a*P levels correlates the results obtained for the plates. In comparison to all the above results, the controls demonstrated the highest fungal growth **(Table 1)**.

Effect of concentration of PAH:

When exposed to different concentrations of B*a*P, for the isolate PO-3, the rate of degradation and biomass formation was inversely proportional to the increase in the

concentration of the PAH **(Figure 1)**. Beyond 10µg/ml there was a drastic reduction in the degradation and biomass yield, hence this concentration was used for the rest of the study.

TABLE 1: Selection of the desired *P. ostreatus* isolate.

P. ostreatus Isolate	Fungal Hyphae Extension on Solid Media (mm)	Growth in Mineral Salt Broth	
		Biomass (mg/50 ml)	% Degradation
PO-1	11	41	22.7
PO-2	9	37	19.3
PO-3	23	53	31.4
Control	28	54	-

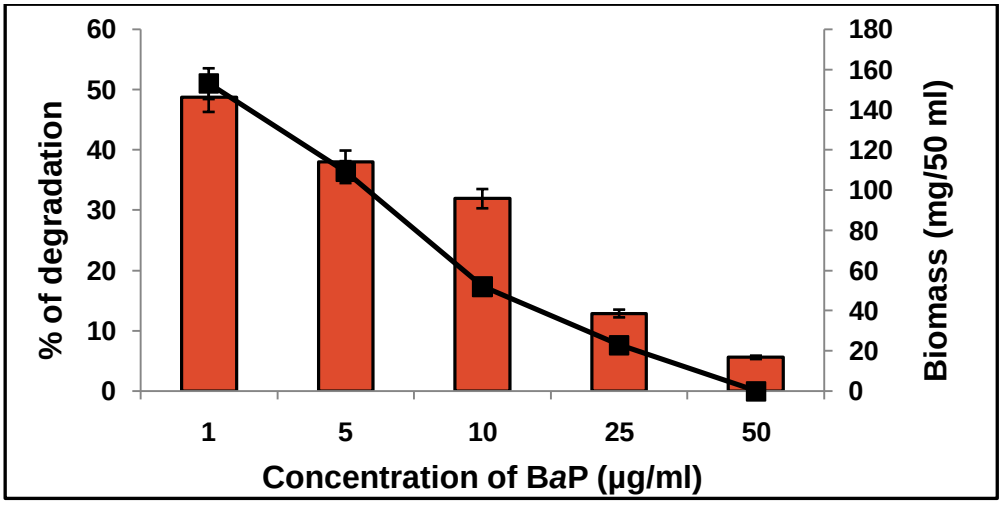

Figure 1: Effect of B*a*P concentration on its degradation and fungal biomass production. Data represent mean ± S.D. (n=3); *P* < 0.05.

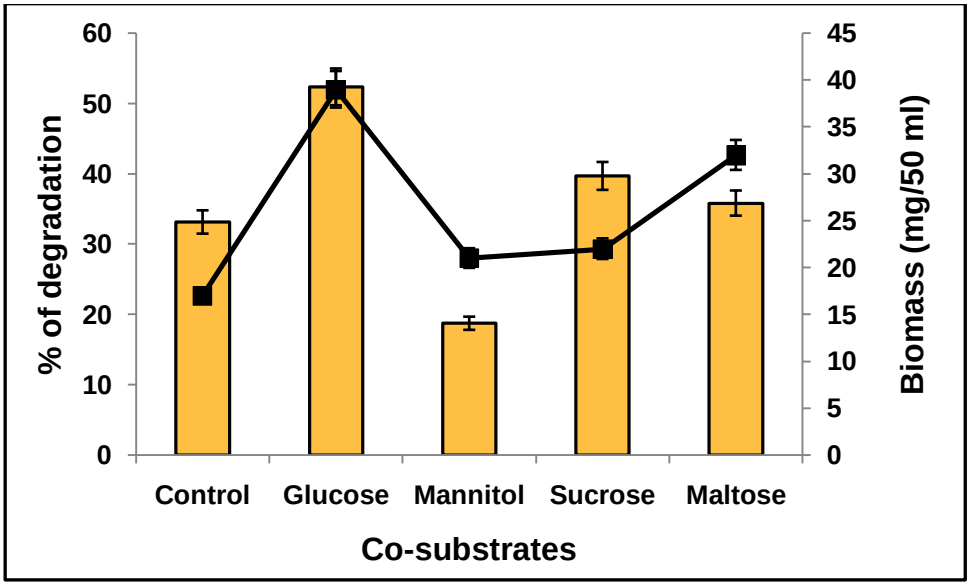

Figure 2: Effect of co-substrates on B*a*P degradation and fungal biomass production. Data represent mean ± S.D. (n=3); *P* < 0.05

Effect of co-substrates:

One metabolic barrier to the microbial degradation of organic compounds is the lack of catabolic enzyme induction. As B*a*P cannot be utilized as a carbon and energy source, a growth substrate must be supplied to initiate growth of the organism and to induce the production of catabolic enzymes[14].

Addition of co-substrates like glucose, maltose, mannitol and sucrose had a positive role to play in the degradation of B*a*P, wherein the monosaccharide glucose resulted in the highest degradation and biomass formation (52.3%, 39 mg/50 ml). All these substituted sugars resulted in better degradation and biomass formation as compared to the control, devoid of any easily metabolizable sugar **(Figure 2)**.

The results obtained from our study is at par with the works of previous workers where additions of starch, glucose and sodium succinate increased soil bacterial and fungal counts and accelerated the dissipation of phenanthrene and B*a*P. Decreases in phenanthrene and B*a*P concentrations were effective in soil supplemented with glucose and sodium succinate and starch. The results suggest that optimizing carbon source, C/N ratio, soil moisture and aeration conditions may be a feasible remediation strategy in certain PAH contaminated soils with large active microbial populations[15].

Earlier, transformation of B*a*P, by *Armillaria sp.* F022, having B*a*P as a source of carbon and energy, was investigated. Results indicated that the biodegradation of B*a*P has been limited because of its bioavailability and toxicity. In order to enhance the degradation process, five co-substrates were selected as co-metabolic carbon and energy sources. A 2.5-fold increase in degradation efficiency was achieved after addition of glucose. Meanwhile, the use of glucose as a co-substrate could significantly stimulate laccase production compared with other co-substrates and not using any co-substrate[16].

Effect of nitrogen supplementation:

Nitrogen is a key component in many compounds necessary for life, such as amino acids and nucleic acids. However, the effect of available nitrogen has shown mixed results on white rot fungi's ability to express lignin degrading enzymes. In our study isolate PO-3 of *P. ostreatus* showed moderate to elevated levels of B*a*P degradation under the influence of different organic and inorganic nitrogen sources **(Figure 3)**. Yeast extract besides resulting in the maximum reduction in the concentration of the B*a*P also helped in the maximum fungal biomass formation (57.32%, 42 mg/50 ml). Yeast extract being the water soluble portion of autolyzed yeast contains vitamins, nitrogen, amino acids and carbon all required by the fungi to form biomass and secrete the oxidative enzymes. A previous study indicated that 0.2 g/L of yeast extract significantly enhanced the growth of bacterial consortium on PAHs like naphthalene and resulted in 78 % degradation[17]. Several other researchers have also shown that when co-substrates like yeast extract were present enhancement of B*a*P, pyrene and carbozole degradation occurred in soil[18]. *P. ostreatus* and several other white rot fungi were found to produce increased levels of lignolytic enzymes in the presence of nitrogen rich medium[19]. Another study showed that the white rot fungi *Irpex lacteus* was inhibited in its ability to produce LiP, MnP and laccase produced when in nitrogen limited liquid culture[20]. A study performed in liquid culture using the fungi *Lentinula edodes*, found increasing the level of nitrogen increased the level of laccase produced while decreasing MnP activity[21]. In contrast to our result a study showed that *P.*

chrysosporium was able to rapidly degrade PCP in nitrogen limited liquid culture[22].

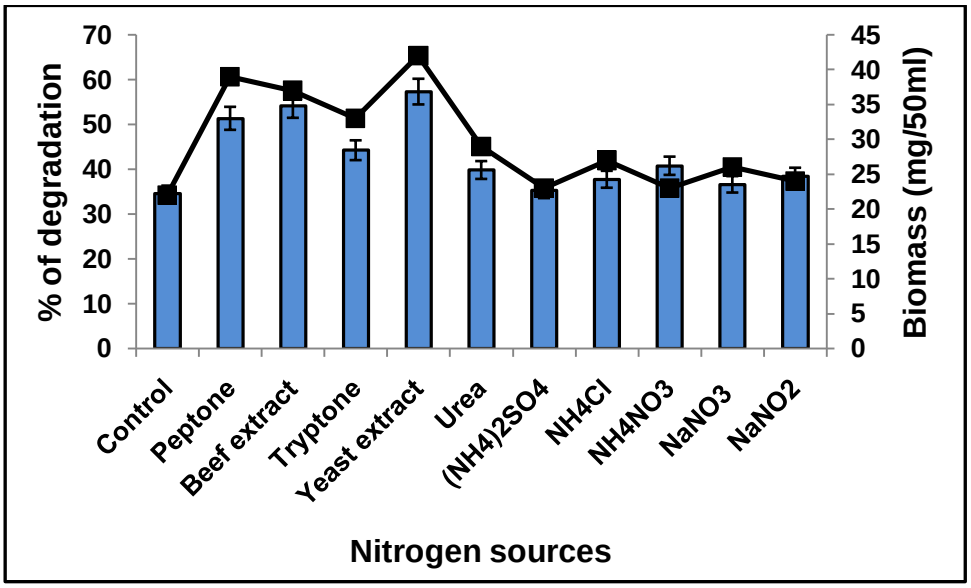

Figure 3: Effect of nitrogen supplements on B*a*P degradation and fungal biomass production. Data represent mean ± S.D. (n=3); *P* < 0.05

Effect of surfactants:

An important aspect of enhanced PAH degradation in soil by white-rot fungi is the bioavailability of these compounds for microorganisms. Bioavailability of HMW PAHs is generally low in soils due to their poor solubility in water and adsorption to soil particles[23].

The bioavailability of B*a*P in contaminated soils may be increased by the application of surfactants. Surfactants are a class of substances with both hydrophobic and hydrophilic groups and have roles of dispersion, emulsion and reduce the interface tension. The distribution role of the PAHs in the surfactant monomer-water, micelles is reportedly potent. It reduces the capillary tension of PAHs in the environment, improves its solubility in water and promotes bio-availability of PAHs[24]. Surfactants increase the bioavailability of organic contaminants by incorporation (solubilization) of the molecule into the hydrophobic core of micelles in solution.

As the B*a*P has a low water solubility (0.0038 mg/L, at 25°C)[25], the addition of surfactants may enhance its solubility to the organism. Among all the different surfactants used in our study, Tween 80 demonstrated the highest removal of B*a*P (62.14%). Tween 80 might have transformed the cell membrane structure and thereby promoted the permeation of ligninolytic enzymes from the cell into the medium as well as the solubility of the contaminants. Since all the surfactants were introduced in the mineral salt broth at very little concentration, they did not affect the synthesis of ligninolytic enzymes and the hyphal growth. The control without any surfactant demonstrated comparatively low level of degradation but mycelia development was not significantly affected **(Figure 4)**.

Similar to our result, PAHs were extensively degraded by *P. chrysosporium* from an artificially contaminated soil–slurry system in the presence of Tween 80. The surfactant was catabolized without any effect on the production of ligninolytic enzymes[26]. Likewise

enhanced PAHs degradation in artificially contaminated soil bioaugmented by *P. ostreatus* was observed using Tween 40, Tween 80 and Triton X-100 as surfactants[27]. In aged soils, Tween 80 exerted a significant stimulatory effect on the PAHs degradation by *P. ostreatus*[28].

CONCLUSION

In course of our study it was found that with the use of a liquid medium, cultural parameters have a profound effect on the B*a*P degradation by *P. ostreatus* isolate PO-3. Utility of B*a*P as the sole source of carbon greatly affected the success of the degradation. Use of glucose as a co-substrate, yeast extract as a nitrogen source and Tween 80 as a surfactant changed the recalcitrant nature of the PAH. The current findings clearly denote that *P. ostreatus* isolate PO-3 has a remarkable potency for the degradation of B*a*P. But in order to be exploited for environment clean up applications, a deep understanding of its enzymatic machinery has to be understood.

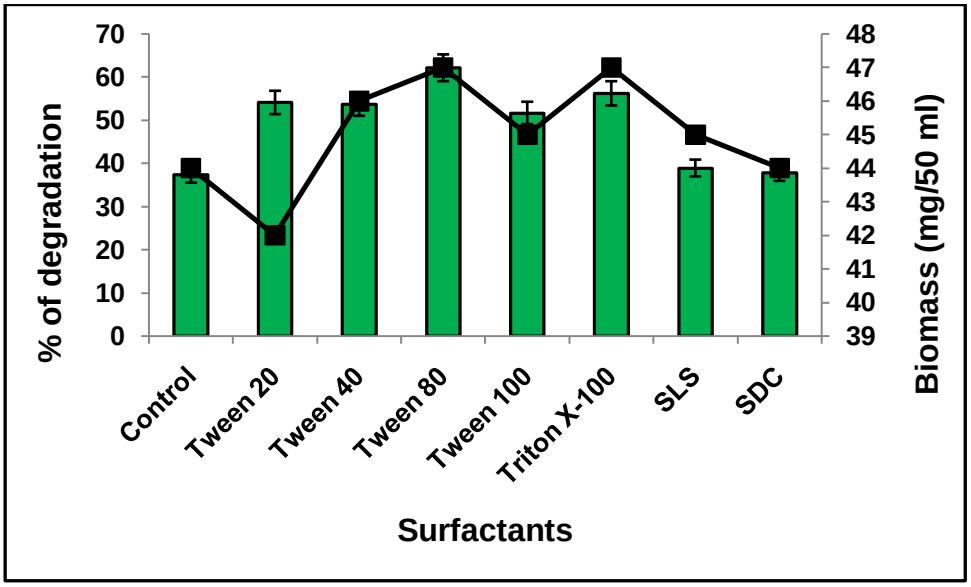

Figure 4: Effect of surfactants on B*a*P degradation and fungal biomass production. Data represent mean ± S.D. (n=3); P < 0.05

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