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Prospecting and Characterizing Efficient Phthalate Esters (PAEs) Utilizing Fungi from Contaminated Soil

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ABSTRACT

The present study was conducted during August 2020 involving twenty-nine fungi isolated from PAE-contaminated soils [viz. sanitary landfill, Bhalswa (SLF) and Centre for Protected Cultivation (CPCT), ICAR-IARI, New Delhi. The objective of the study was to qualitatively screen the isolates for enzyme production (laccase and esterase) and phthalate ester (PAE) tolerance limits. For laccase production, the isolates were inoculated on malt extract, while esterase production was determined using tributyrin agar. Furthermore, radial growth of isolates on plates supplemented with different concentrations (100, 500 and 1000 mg l^{-1}) of di-*n*-butyl phthalate (DBP) and di (2-ethylhexyl) phthalate (DEHP) was done to assess tolerance limits. Results revealed that, out of twenty-nine fungi, except for two which produced laccase (CDEHP1 and SDBP2), all the other isolates were able to produce esterase. Six isolates each for DBP and DEHP, were able to tolerate high concentration of PAEs (up to 1000 mg l^{-1}), and selected for further work. The results indicated that, at 14 days of inoculation, isolate SDBP4 showed highest radial growth at all concentrations of DBP. In case of isolates grown on plates supplemented with DEHP, radial growth was observed to be in the order SDEHP2>SDEHP1>SDEHP4>SDEHP5>SDEHP3>CDEHP6. Also, physicochemical properties of the contaminated soils were assessed. The study emphasizes on isolation and screening of efficient PAE-utilizing fungi for potent application in bioremediation of contaminated soils.

KEYWORDS: Phthalate esters, fungi, sanitary landfill, esterase, bioremediation

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Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

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1. INTRODUCTION

Plastics contain special class of chemicals called plasticizers (phthalate esters, PAEs) such as benzyl butyl phthalate (BBP), di-ethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), di (2-ethylhexyl) phthalate (DEHP) etc. which also contribute to environmental pollution (Mankidy et al., 2013, Zhao et al., 2021). Of these, DBP and DEHP are the most extensively produced and utilized PAEs globally. Being non- covalently bound to plastic surfaces, they are known to elute into surrounding environmental matrices, especially water and soil, thereby contaminating them. Though PAEs improve elasticity and malleability in plastic products, they are known to cause serious health issues in humans and animals (Staples et al., 1997, Li et al., 2016). Their pervasiveness in air, water, soil is a menace, causing important health concerns as a result of their estrogenic, teratogenic, carcinogenic and endocrine disruption properties (Chen et al., 2014, Ungewitter et al., 2017). They are also reported to lead to disorders in behavior and reproduction (Capela et al., 2019). Some of them can reduce the survivability of embryos, increase resorptions, increase abortion in animals like rats (Schmidt et al., 2012). Due to these alarming problems caused to humans, they have been categorized as 'priority pollutants' by the United States Environmental Protection Agency (USEPA) and the European Union (EU) (Anonymous, 1982, Peck and Albro, 1982, Anonymous, 2008).

Some studies have reported higher concentrations of DBP and DEHP in environmental matrices as compared to other PAEs (Zeng et al., 2009, Zeng et al., 2010, Gao and Wen, 2016). Sludges, atmospheric accumulation, soil amendments like agrochemicals and plastics used in mulches and greenhouses are potent sources of PAEs in arable lands (Net et al., 2015). They are taken by plants and thus, enter the food chains. Additionally, the plastics ending up in landfills elute DBP and DEHP into leachates, which in turn pollute water resources underground, thus increasing risks of human exposure (Sayyad et al., 2017). Furthermore, PAEs also affect fauna and flora grown in contaminated soils (Cartwright et al., 2000).

Therefore, degradation of PAEs is a good alternative to curb their accumulation. However, DBP and DEHP, due to their hydrophobicity and chemical structure, persist in environmental matrices for a long duration. Abiotic degradation takes longer time as compared to microbial degradation. Depending on soil type, abiotic degradation of DBP and DEHP were found to be in the range of 0.60–2.91% (insignificant) after 30 days of incubation (Xu et al., 2008, Zhang et al., 2020).

Microbial degradation is an effective strategy to degrade PAEs from soils, and microorganisms produce different kinds of enzymes for degradation, of which, laccase and esterase are well- explored (Kim et al., 2008, Prasad and Suresh, 2012, Huang et al., 2019, Sun et al., 2022). Several microorganisms have been isolated from various environmental matrices like wastewater, activated sludge, freshwater, marine water etc., which can utilize or breakdown PAEs. However, only few studies report the use of soils, especially those from agricultural, dumpsites and sanitary landfills, including leachates, for isolation of efficient DBP and DEHP degrading microorganisms (Latorre et al. 2012, Pradeep and Benjamin, 2012, Pradeep et al., 2013, Ahuactzin-Pe´rez et al., 2014, Wang et al., 2015b, Zhao et al., 2016, Xu et al., 2020, Feng et al., 2021). Scanty documentation is also available related to the screening process of fungal isolates for DBP and DEHP degradation or the testing of PAE tolerance limits, which aids in the selection of efficient isolates. Thus, the present study aimed at isolating and screening promising major PAEs (DBP and DEHP) utilizing fungi from contaminated sites, viz. sanitary landfill, Bhalswa (SLF) and Centre for Protected Cultivation (CPCT), ICAR- IARI, New Delhi.

2. MATERIALS AND METHODS

2.1. Physicochemical properties of contaminated soils

Soils from CPCT, ICAR- IARI, New Delhi (28° 62' N, 77° 15' E) and SLF, Bhalswa, New Delhi (28° 73' N, 77° 15' E) were acquired and their physicochemical properties were analyzed using standard procedures at Division of Microbiology, ICAR- IARI, New Delhi (28° 63' N, 77° 15' E) in August, 2020. pH and EC were estimated using digital pH meter and conductivity bridge respectively. Organic carbon (%) was estimated using Walkley and Black (1934) method. Total nitrogen and sulfur were estimated using CHNS analyzer (Model EA 3000). Available phosphorus and potassium were estimated by method of Olsen et al. (1954) and Hanway and Heidal (1952). Standard procedure of Lindsay and Norvell (1978) was employed for determination of micronutrient content.

2.2. Isolation of PAE utilizing fungi by enrichment technique

Sterile basal salt (BS) medium was prepared with the following constituents (g l^{-1}): K_2HPO_4 (1.0), $MgSO_4$ (0.4), NaCl (1.0), NH_4Cl (0.5), $CaCl_2$ (0.3), $FeSO_4.7H_2O$ (0.003), pH 6.5.Fifty grams of each soil were added separately into the conical flasks and shaken well. Both DBP and DEHP were added to medium aseptically at 50 mg l^{-1} each and incubated at 30 °C for 14 days at 150 rpm. Aliquots of ten ml soil suspensions were transferred to fresh medium and after two weeks incubation, the process was repeated thrice. The samples were plated out on BS agar plates containing 100 mg l^{-1} PAEs individually, using serial dilution technique. The PAE stock solution was prepared in equal volumes of

ethanol and added to plates to attain desired concentrations. To improve the solubility of PAEs, Tween 80 (400 mg l^{-1}) was also added to medium. The isolates were inoculated and maintained on potato dextrose agar plates and used in further experiments.

2.3. Qualitative screening of fungal isolates

2.3.1. Laccase activity (Modified method of Hankin and Anagnostakis, 1975)

The isolates were inoculated on agar plates supplemented with 2% malt extract and 0.1% peptone followed by incubation at 32 °C for a week. After incubation, using a sterile cork borer, a hole was made at the centre of the plate. The bottom of the hole was sealed using molten agar solution. Freshly prepared solution of one ml guaiacol [2-methoxyphenol; (1% v/v) in 95% ethanol] was added to the hole and the plates were incubated in darkness for 12 hours. The plates with purplish red colour on the reverse side were considered positive for laccase activity.

2.3.2. Test for esterase activity (Kumar et al., 2012)

Sterilized tributyrin agar plates containing tributyrin (10 g l^{-1}) and peptone (5 g l^{-1}) were prepared and inoculated with fungal isolates. Isolates producing clear zones around colonies were considered positive for esterase activity.

2.3.3. Testing for phthalate tolerance limits

The plugs of fungal isolates were inoculated at the centre of BS agar plates supplemented with various concentrations of respective PAEs (100, 500 and 1000 mg l^{-1}). The isolates showing considerable establishment of mycelia at the end of 7 days were tested positive for growth.

2.4. Assessment of growth on different concentrations of PAEs (Ahuactzin-Pérez et al., 2014)

Radial growth is an efficient way to quantitatively screen the fungal isolates for utilization of different concentrations of DBP and DEHP. The fungal isolates were inoculated at the centre of plates amended with DBP and DEHP at various concentrations (100, 500 and 1000 mg l^{-1}). Using a ruler, the radial growth (mm) of the isolates was recorded at 7 and 14 days. The experiments were conducted in triplicates and analysed using one- way ANOVA.

3. RESULTS AND DISCUSSION

3.1. Physicochemical properties of PAE contaminated soils

The physicochemical properties of soils previously contaminated with PAEs, viz. SLF and CPCT are provided in table 1. It was found that both SLF and CPCT soils were alkaline in nature. However, CPCT (8.71) was more alkaline as compared to SLF (7.83). SLF contained higher organic carbon than CPCT. Furthermore, N, P, K and S were multiple folds higher in SLF as compared to CPCT. The micronutrients like Fe, Mn, Zn and Cu were extremely high as compared to CPCT soil. The trend was concurrent with the study by Oshoma et al. (2017), who concluded that nutrient composition of dumping site soil was very high as compared to a closely situated arable soil. Similarly, Amaral et al. (2017) reported that landfill leachate had very high concentrations of nutrients, especially N and Fe. Furthermore, SLF soil had higher PAEs (DEHP: 64.77–93.8 mg kg^{-1} soil and DBP: 6.71–28.7 mg kg^{-1} soil) than CPCT soil (7.29–13.81 and 2.40–7.55 mg kg^{-1} soil) respectively (to be published data). These physicochemical properties greatly depend on the type of wastes dumped, topography and rainfall of the locality Oshoma et al. (2017).

Table 1: Physicochemical properties of PAE contaminated soils

Properties	CPCT	SLF
pH	8.71	7.83
EC (ds m^{-1})	0.70	2.70
OC (%)	0.33	1.20
Total N (%)	0.308	1.432
Available P (%)	1.013	3.079
Available K2O (%)	0.025	0.120
Total S (%)	0.064	0.142
Fe (mg kg^{-1})	4.35	44.60
Mn (mg kg^{-1})	6.40	24.30
Zn (mg kg^{-1})	1.13	12.02
Cu (mg kg^{-1})	1.32	13.32
Selected PAEs (phthalate esters)		
DEHP (mg kg^{-1} soil)	7.29-13.81	64.77-93.80
DBP (mg kg^{-1} soil)	2.40-7.55	6.71-28.70

3.2. Isolation of PAE utilizing fungi

A set of twenty-nine PAE- utilizing fungi were isolated from both SLF and CPCT soils., out of which, sixteen were DBP- utilizing, while thirteen were DEHP- utilizing isolates (Figure 1). Among the twenty-nine isolates, twelve were from CPCT soil (7-DEHP and 5-DBP utilizing) and seventeen were isolated from SLF (6-DEHP and 11-DBP utilizing).The greater number from SLF samples can be attributed to the high levels of nutrients, facilitating the

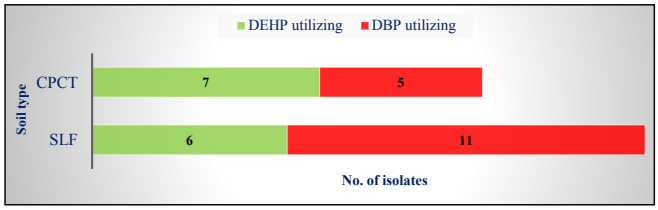

Figure 1: Number of fungi isolated from each soil type

build-up of a higher population. They were named after the type of soil isolated from and PAE-utilized, as CDEHP1, CDEHP2, CDEHP3, CDEHP4, CDEHP5, CDEHP6 and CDEHP7; CDBP1, CDBP2, CDBP3, CDBP4, CDBP5 isolated from CPCT soil, while SDEHP1, SDEHP2, SDEHP3, SDEHP4, SDEHP5 and SDEHP6; SDBP1, SDBP2, SDBP3, SDBP4, SDBP5, SDBP6, SDBP7, SDBP8, SDBP9, SDBP10, SDBP11 isolated from SLF soil (Figure 2a and b). Latorre et al. (2012) isolated five efficient DEHP degrading bacteria from landfill leachate by enrichment culture technique. Pradeep and Benjamin et al. (2012) used soil from municipal waste treatment and isolated three efficient DEHP- degrading fungi. Thus, it was evident that such niche are good habitats for PAE-degrading microorganisms. Feng et al. (2021) isolated several DBP-degrading bacteria from PAE-contaminated soils under greenhouses located in Guangzhou, China. Thus, PAE-polluted landfill soils, leachates and greenhouse soils can be good sources for isolation of efficient PAE utilizing microorganisms.

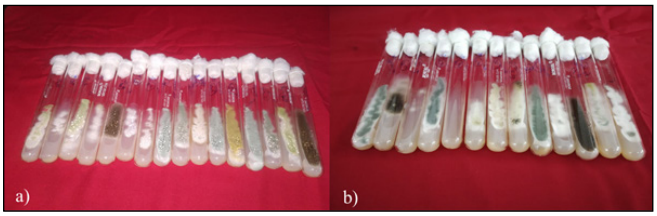

Figure 2: PAE- utilizing fungi isolated from contaminated soils: a) DBP- utilizing b) DEHP- utilizing

3.3. Screening for laccase and esterase activity

Measurement of enzymatic activities revealed that, out of 29 isolates, 27 were able to exhibit zones around the colonies in tributyrin agar, thus, testing positive for esterase activity (Table 2; Figure 3a). However, two isolates SDBP2 and CDEHP1 produced purplish red color at the reverse side of plates on malt extract agar, supplemented with 1% guaiacol, thereby testing positive for laccase (Table 2; Figure 3b). However, none of the other isolates produced laccase. Different microorganisms have been reported to employ various enzymes like esterase and laccase for degradation of PAEs (Huang et al., 2019, González-Márquez et al., 2021). In the present study, it was evident that esterase was more important for phthalate degradation as compared to laccase; similar trend was recorded by Hwang et al. (2012), who concluded that esterases play a bigger role than laccase in PAE degradation. Sun et al. (2022) observed that initially, esterase activity in DEHP degradation was more prominent when matched with laccase activity.

3.4. PAE tolerance limit

Sixteen-DBP and thirteen- DEHP were inoculated on BS agar plates with various concentrations of PAEs (100, 500 and 1000 mg l^{-1}). It was found that although all the isolates showed good growth at lower concentrations of PAEs (100 mg l^{-1}), not all isolates exhibited good growth at higher concentrations (500 and 1000 mg l^{-1}). In case of DBP, six

Table 2: Qualitative screening of PAE- utilizing fungal isolates

Activity	Isolates showing presence of activity/ growth		No. of isolates
	DEHP	DBP	
Laccase	CDEHP1	SDBP2	2
Esterase	SDEHP1, SDEHP2, SDEHP3, SDEHP4, SDEHP5, SDEHP6, CDEHP2, CDEHP3, CDEHP4, CDEHP5, CDEHP6, CDEHP7	SDBP1, SDBP3, SDBP4, SDBP5, SDBP6, SDBP7, SDBP8, SDBP9, SDBP10, SDBP11, CDBP1, CDBP2, CDBP3, CDBP4, CDBP5	27
PAE tolerance limits in concentrations			
100 (mg l^{-1})	All isolates	All isolates	29
500 (mg l^{-1})	SDEHP1, SDEHP2, SDEHP3, SDEHP4, SDEHP5, CDEHP6	SDBP4, SDBP6, SDBP7, SDBP8, SDBP9, SDBP10	12
1000 (mg l^{-1})	SDEHP1, SDEHP2, SDEHP3, SDEHP4, SDEHP5, CDEHP6	SDBP4, SDBP6, SDBP7, SDBP8, SDBP9, SDBP10	12

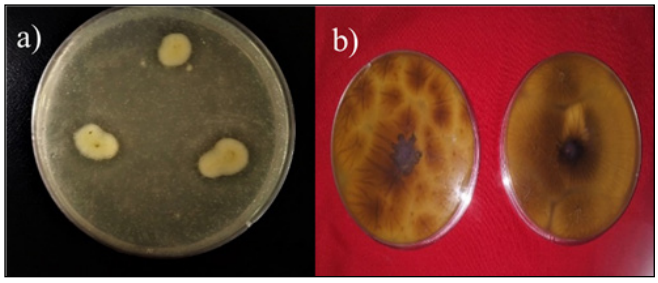

Figure 3: Qualitative screening for enzyme activity a) esterase (clear zones) b) laccase (purplish red)

isolates viz., SDBP4, SDBP6, SDBP7, SDBP8, SDBP9 and SDBP10 were able to grow on plates containing 500 and 1000 mg l^{-1} DBP, while others failed to grow at such high concentrations (Table2; Figure 4). Similarly, six isolates (SDEHP1, SDEHP2, SDEHP3, SDEHP4, SDEHP5 and CDEHP6) were able to tolerate higher concentrations of DEHP (500 and 1000 mg l^{-1}) (Table 2). In a parallel study, González-Escobar et al. (2020) investigated on DBP and DEHP tolerance limits (500 and 1000 mg l^{-1}) of endophytic bacterial isolates from *Liometopum apiculatum* and concluded that, out of 11 isolates, only 8 were able to grow on higher concentrations of DBP (1000 mg l^{-1}), while all the isolates could tolerate even high concentrations of DEHP (1000 mg l^{-1}). This variation could be due to difference in growth patterns, capacity to assimilate PAEs and fungal enzyme systems.

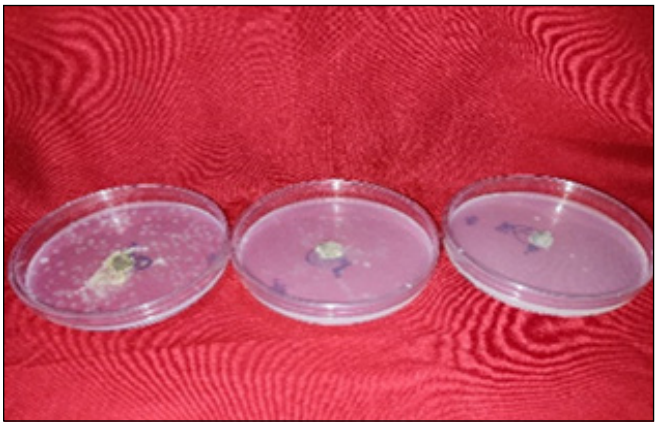

Figure 4: Fungal growth of isolate SDBP4 on various concentrations of DBP (100, 500 and 1000 mg l^{-1})

3.5. Radial growth of fungal isolates on various concentrations of PAEs

The six isolates for each PAEs exhibiting growth on higher concentrations (500 and 1000 mg l^{-1}) were assessed for radial growth (mm) on various concentrations of PAEs at 7 and 14 days. In case of treatments supplemented with different concentrations of DBP, the isolates showed good, but variable growth (Figure 5). The isolate SDBP4 recorded significantly higher growth at all concentrations of DBP as compared to other isolates. At the end of 14 days, the isolate attained a radial growth of 83.67, 78.1 and 72.11 mm at 100, 500 and 1000 mg l^{-1} DBP. However, isolate SDBP8 showed poor growth at all concentrations of DBP. In case of isolates growing on plates supplemented with DEHP, radial growth was in the order SDEHP2 > SDEHP1 > SDEHP4 > SDEHP5 > SDEHP3 > CDEHP6. Though there was no significant different in radial growth of SDEHP3 and CDEHP6 in plates supplemented with 100 mg l^{-1} DEHP, the isolate CDEHP6 showed significantly lower growth as

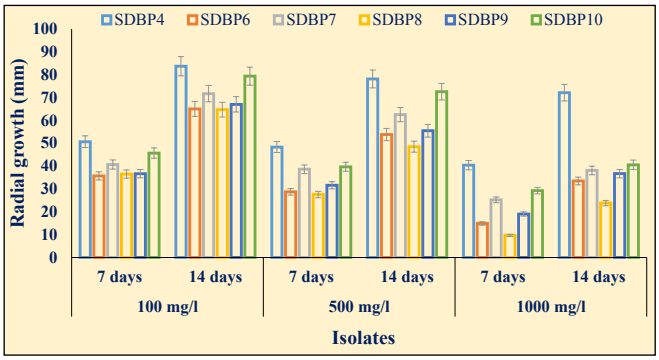

Figure 5: Radial growth of fungal isolates on various concentrations of DBP. Error bars represent standard error of means (p<0.05)

compared to SDEHP3 at 1000 mg l^{-1} at the end of study. The observations revealed that, with an increase in PAE concentration, the radial growth of the isolates reduced. This trend was also reported by Lee et al. (2004). However, a contrasting trend was reported by González-Márquez et al. (2015), who concluded that there was an increase in growth of fungal isolates with increase in concentration of PAE. Ahuactzin-Pérez et al. (2014) reported that growth of *Trichoderma harzianum* and *Neurospora sitophyla* did not significantly change when grown at similar concentrations of DBP (500 and 1000 mg l^{-1}). The variations largely depends on the location and chemical composition of type of soil used for isolation, differences at the strain level, phthalate tolerance limits, variations in enzyme systems. The results also revealed that across all concentrations of PAEs, radial growth of DBP- utilizing isolates was higher as compared to that of DEHP- utilizing ones. This may possibly be due to lesser complexity of short- chain PAEs like DBP in comparison with long- chained, more complex DEHP, influencing the assimilation/ mineralization by the microorganisms (Liang et al., 2008).

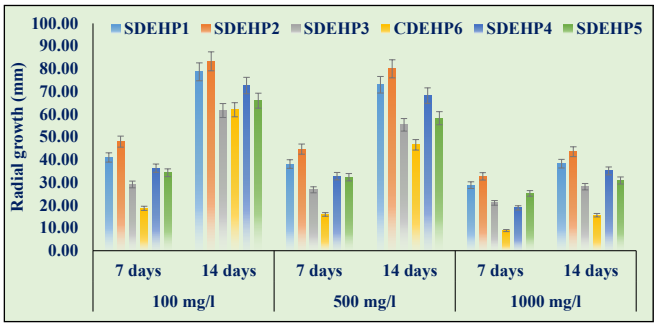

Figure 6: Radial growth of fungal isolates on various concentrations of DEHP. Error bars represent standard error of means (p<0.05)

4. CONCLUSION

Isolation of PAE- utilizing fungi, their screening for enzyme production (esterase and laccase) and tolerance

limits for two ubiquitous phthalates-DBP and DEHP. Among twenty-nine isolates efficiently growing on higher concentrations of DBP or DEHP, two isolates SDBP4 and SDEHP2 were found to be most potent and promising. These isolates can be further screened quantitatively for PAE degradation under mesocosm conditions with contaminated soil, followed by assessment of their degradative capacities in contaminated sites.

5. FUTURE SCOPE

Delivery systems and optimization of inocula can be taken up for further using these isolates in the bioremediation of PAE- contaminated soils. Since DBP and DEHP coexist in soil, a consortium of efficient strains can be developed, based on compatibility studies, and developed as a suitable formulation for soil application.

6. ACKNOWLEDGMENT

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