Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

ISSN: 0972-6268	Vol. 11	No. 3	pp. 385-392	2012

Original Research Paper

Bioremediation of Caffeine-Contaminated Soil by Immobilized Yeast – A Laboratory Based Study

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Nat. Env. & Poll. Tech.
Website: www.neptjournal.com
Received: 20/2/2012
Accepted: 5/3/2012

Key Words:
Caffeine contaminated soil
Immobilization
Biostimulation
Bioaugmentation
Yeast species

ABSTRACT

The aim of this work was to ascertain the efficacy of immobilised yeast for the remediation of caffeine in soil under laboratory condition. Biostimulation with inorganic nutrients and bioaugmentation with caffeine utilizing single and mixed yeast cultures viz., Mixed culture 1, Mixed culture 2, Mixed culture 3 and Mixed culture 4 were employed as remedial options for the removal of caffeine from contaminated soil. To promote caffeine removal, biowaste materials (wheat bran, sawdust, peanut hull powder) were used as biocarriers for immobilization of caffeine degrading yeast species following the method of physical adsorption. We constructed laboratory biopiles to compare the treatment bioaugmentation with bioaugmentation combined biostimulation using single and mixed cultures of immobilized yeast. Maximum removal of caffeine was found to be 98.3 % after 24 days in the biostimulated soil which was bioaugmented with sawdust immobilized yeast mixed culture No. 4 consisting of three yeast species viz., *Trichosporon asahii, Candida tropicalis* and *Candida inconspicua*. Dehydrogenase activity in the soil was remarkably enhanced to 639 µg TPF g^{-1} soil and microbial numbers were also increased for the soil treated under the same conditions described above. Phytotoxicity assay confirmed the reduction of caffeine toxicity in the contaminated soil after treatment. Thus, sawdust immobilized mixed yeast culture No. 4 could serve as potential tool for the remediation of caffeine from contaminated soil.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine), a purine alkaloid is a key ingredient of many drinks especially tea and coffee. This acts as a stimulant of the central nervous system and occurs naturally in leaves, seeds and fruits of more than 60 plant species, including cocoa beans, tea leaves, kola nuts, guarana seeds, coffee beans, etc. (Steffen 2000). Excessive consumption of caffeine through beverages results in a number of health problems like adrenal stimulation, irregular muscular activity, cardiac arrhythmias, osteoporosis and increased heart output (Schuh & Griffiths 1997). Apart from health effects, caffeine degradation is important from environment point of view. The presence of caffeine in soil affects soil fertility as it inhibits seed germination and growth of seedlings (Friedman & Waller 1983, Batish et al. 2008). Caffeine is considered as a xenobiotic compound (Spiff & Uwakwe 2003).

Some bacteria and fungi, viz., *Pseudomonas* sp. (Woolfolk 1975, Gummadi et al. 2009), *Serratia* sp. (Mazzafera et al. 1994), *Stemphyllium* sp. (Kurtzman & Schwimmer 1971), *Klebsiella* and *Rhodococcus* sp. (Madyastha & Sridhar 1998), and *Aspergillus* and *Penicillium* sp. (Hakil et al. 1998), have been reported as the potential degraders of caffeine. But reports are scanty regarding the potentiality of yeast as caffeine degrader.

The use of microorganisms for degradation of toxic compounds in freely suspended state is limited owing to their inherent disadvantages such as small particle size, possible clogging and low mechanical strength of the biomass (Godjevargova et al. 2004). Immobilized cell technology has been successfully used to degrade contaminants (Baskaran & Nemati 2006). Immobilized cell systems have the potential to degrade toxic chemicals faster than conventional treatment systems due to their high mechanical strength and high cell concentration (Jianlong et al. 2001, Beshay et al. 2002) and they are less likely to be adversely affected by predators, toxins or parasites, in comparison to free cells (Prabua & Thatheyus 2007).

Biodegradation of caffeine in agricultural soil using liquid municipal biosolids as bulking agent has been reported (Topp et al. 2006). This bulking agent enhanced the aeration and microbial activity, and thereby increased the biodegradation rate of contaminants. Bioaugmentation and biostimulation can accelerate the rate of biodegradation in soil (Vasudevan & Rajaram 2001). In many cases, bioaugmentation was reported as a feasible strategy for contaminant removal and site remediation (Ruberto et al. 2003, Jacques et al. 2008). Addition of enriched indigenous microorganisms or exogenous microorganisms can degrade the toxic contaminants and offer higher tolerance to toxicity (Ellis et al. 2000, Barathi & Vasudevan 2003).

So far no work has been reported using yeast species for removal of caffeine from the soil. The objective of the present study was to compare the efficiency of biostimulation and bioaugmentation strategies in reducing caffeine contamination in soil using single and mixed culture of yeast under laboratory conditions. Biowaste materials were used as biocarriers to immobilize the yeast cells. Microbial population and dehydrogenase activity (DHA) were used as sensitive indicators to assess the caffeine remediation from soil.

MATERIALS AND METHODS

Chemicals: Chemicals used in the present study are of analytical grade procured from Himedia Limited, Mumbai, India and SRL Chemicals Limited, Mumbai, India.

Soil: Caffeine contaminated soil samples used for the experiments were collected from coffee cultivation area, Coffee Board, Yercaud, Tamil Nadu, India. During the collection of soil, surface layer was removed and soil samples were collected to a depth of 10 cm. The soil was air dried and sieved through a 2 mm mesh sieve, homogenized using shovels and then stored at 4°C in dark until required. The texture of the soil viz., clay, course sand, fine sand and silt, was analysed by international pipette method (Day 1965, Franzmeier et al. 1977). Moisture content was measured by drying 10-20 g soil at 105°C for 8 h. The pH was measured in 1:2:5 mixture of soil: water: 0.01M $CaCl_2$ solution using pH electrode. Total phosphorus was measured in $NaHCO_3$ extraction solution (0.5 M at pH 8.5) using a molybdenum-antimony colorimetric method and the total nitrogen was measured by MgO-alloy distillation method (Lu 1999). Total organic content was measured by following the methods of Miyazawa et al. (2000). The initial caffeine concentration in the soil was determined by chloroform extraction using UV-visible spectrophotometer.

Yeast and growth conditions: The yeast species were isolated from caffeine contaminated soil and identified to the species level as *Trichosporon asahii, Candida tropicalis* and *Candida inconspicua* using VITEK 2 compact yeast card reader with the software version: 03.01 from Council for Food Research and Development (CFRD), Kerala, India. Caffeine Liquid Medium (CLM) containing (g/L): K_2HPO_4, 0.8; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.1; $FeSO_4.7H_2O$, 0.005; yeast extract, 0.2; sucrose, 8.0 and caffeine, 10.0 was used as the growth medium, for inoculum preparation. The initial pH of the medium was adjusted to 6.5 and the temperature was maintained at 28°C.

Mixed culture of yeast used in the soil study: Mixed cultures of yeast species viz., Mixed culture 1 (*T. asahii* + *C. tropicalis*), Mixed culture 2 (*T. asahii* + *C. inconspicua*), Mixed culture 3 (*C. tropicalis* + *C. inconspicua*) and Mixed culture 4 (*T. asahii* + *C. tropicalis* + *C. inconspicua*) were prepared by mixing equal proportions of pure yeast cultures in caffeine liquid medium.

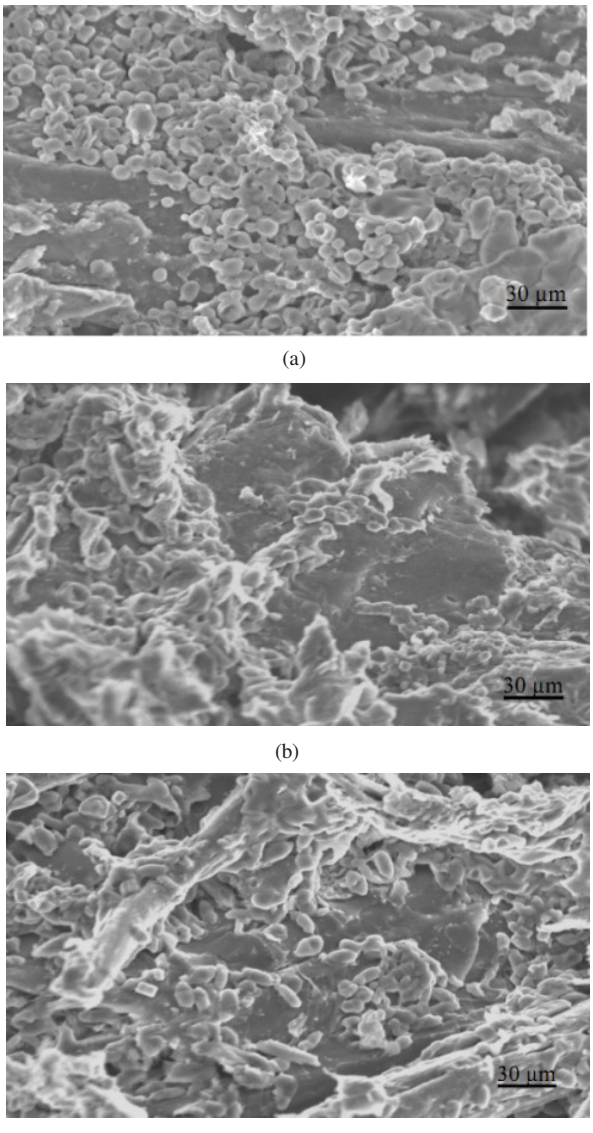

Fig. 1: SEM image (× 1500) showing the adhesion of *T. asahii* (a), *C. tropicalis* (b) and *C. inconspicua* (c) to the surface of sawdust following 48 h incubation and washing with 10 mM phosphate buffer (pH 7.0).

Biocarriers: The biowaste material i.e., wheat bran was collected from local market. Peanut hull and sawdust were obtained from respective mills and used as biocarriers. They were mechanically ground to obtain a particle size of approximately 1-2 mm. Before use, biocarrier materials (each 0.3 g) were sterilized by autoclaving at 15 lbs for 15 min. Each vial containing the sterile biocarrier material was inoculated with 1 mL 10 mM phosphate buffer containing yeast

Table 1. Experimental setup for treatment of caffeine contaminated soil.

S. No.	Soil Treatment	Principle
A1	Soil + sawdust	Control (Bioaugmentation)
A2	Soil + sawdust + nutrients	Control (Biostimulation + Bioaugmentation)
B1	Soil + sawdust immobilized *T. asahii*	Bioaugmentation
B2	Soil + nutrient + sawdust immobilized *T. asahii*	Biostimulation + Bioaugmentation
C1	Soil + sawdust immobilized *C. tropicalis*	Bioagumentation
C2	Soil + nutrient + sawdust immobilized *C. tropicalis*	Biostimulation + Bioaugmentation
D1	Soil + sawdust immobilized *C. inconspicua*	Bioaugmentation
D2	Soil + nutrient + sawdust immobilized *C. inconspicua*	Biostimulation + Bioaugmentation
E1	Soil + sawdust immobilized mixed culture 1	Bioaugmentation
E2	Soil + nutrient + sawdust immobilized mixed culture 1	Biostimulation + Bioaugmentation
F1	Soil + sawdust immobilized mixed culture 2	Bioaugmentation
F2	Soil + nutrient + sawdust immobilized mixed culture 2	Biostimulation + Bioaugmentation
G1	Soil + sawdust immobilized mixed culture 3	Bioaugmentation
G2	Soil + nutrient + sawdust immobilized mixed culture 3	Biostimulation + Bioaugmentation
H1	Soil + sawdust immobilized yeast mixed culture 4	Bioaugmentation
H2	Soil + nutrient + sawdust immobilized mixed culture 4	Biostimulation + Bioaugmentation

Table 2: Biomass production and caffeine degradation potential of free and immobilized *T. asahii* cells on various biocarrier materials (Initial caffeine concentration-10 g/L, pH-6.5, temperature-28°C, shaking speed-120 rpm).

Inoculum	TPC (cfu/mL)		Caffeine degradation (%) ± standard deviation about the mean	
	at 24 h	at 48 h	at 24 h	at 48 h
Free cells	4.4×10^8	7.1×10^8	50 ± 3	70 ± 1
Cells immobilized in biocarriers				
Sawdust	5.6×10^9	9.1×10^9	69 ± 1	91 ± 2
Peanut hull	3.2×10^8	7.5×10^8	54 ± 3	75 ± 2
Wheat bran	4.5×10^9	8.4×10^9	65 ± 2	83 ± 1

cells suspension (4 g/L wet weight) obtained from 24 h YEPD broth culture. Vials were incubated for 48 h and their contents were washed by resuspending the carrier cultures in 2 mL phosphate buffer (pH 7.0) and the supernatants were drained. This was done to remove the yeast cells which were not sufficiently adhered to the carrier surfaces. After incubation period, replicate cultures were withdrawn and dried in a sterile hood in a stream of dry air at room temperature for 4 days.

Screening of different biocarriers for caffeine degradation: To screen the best biocarrier material for immobilization and caffeine degradation, *T. asahii* cells were chosen as it has already shown best performance in our previous study (Lakshmi & Das 2010). The yeast cells immobilized on various biocarriers were transferred into 50 mL of caffeine liquid medium containing 10 g/L caffeine. The pH of the medium was adjusted to 6.5 and incubated at 28°C on a rotary shaker at 120 rpm. At regular time intervals, samples were collected and degradation was monitored. The above procedure was also followed for studying the biodegradation of caffeine by free cells by inoculating free *T. asahii* cells (4 g/L wet weight) to the caffeine liquid medium containing 10 g/L caffeine and incubated under same conditions as stated above.

Table 3. Degradation of caffeine in soil by yeast species in various soil treatments after 24 days.

Soil Treatment	Caffeine degradation (%)
A1	NS
A2	NS
B1	66.3 ± 1.5
B2	75.7 ± 2.1
C1	63.9 ± 0.8
C2	72.4 ± 1.1
D1	60.7 ± 1.6
D2	70.8 ± 1.3
E1	70.4 ± 1.2
E2	82.8 ± 0.9
F1	67.6 ± 1.0
F2	77.4 ± 1.5
G1	64.9 ± 1.6
G2	73.6 ± 1.7
H1	85.9 ± 1.7
H2	98.3 ± 1.1

NS - not significant

Growth of free and immobilized yeast cells in different biocarriers were monitored by total plate count method.

Caffeine degrading yeast cells (single and mixed cultures) were suspended in phosphate buffer to get approximately 4

Table 4: Phytotoxicity study of treated and untreated caffeine contaminated soil.

Seed	Soil	Germination %	Root length (cm)	Shoot length (cm)
Raphanus sativus	Control[a]	100	3.5 ± 0.02	14.9 ± 0.03
	Untreated[b]	40	1.8 ± 0.03	4.5 ± 0.02
	Treated[c]	100	3.3 ± 0.03	14.2 ± 0.04
Cicer arietinum	Control[a]	100	5.6 ± 0.01	4.1 ± 0.01
	Untreated[b]	20	3.1 ± 0.01	1.7 ± 0.03
	Treated[c]	100	5.2 ± 0.04	3.5 ± 0.05
Abelmoschus esculentus	Control[a]	100	3.7 ± 0.04	6.5 ± 0.02
	Untreated[b]	20	1.9 ± 0.01	3.0 ± 0.02
	Treated[c]	100	3.0 ± 0.01	6.1 ± 0.04
Phaseolus mungo	Control[a]	100	4.4 ± 0.02	6.2 ± 0.03
	Untreated[b]	50	1.9 ± 0.02	3.7 ± 0.02
	Treated[c]	100	4.4 ± 0.03	6.1 ± 0.04
Beta vulgaris	Control[a]	100	6.1 ± 0.03	7.9 ± 0.02
	Untreated[b]	50	3.2 ± 0.04	5.8 ± 0.05
	Treated[c]	100	5.5 ± 0.02	7.7 ± 0.03

[a]Control - Garden soil without caffeine; [b]Untreated-caffeine contaminated soil without treatment
[c]Treated-caffeine contaminated soil after treating with mixed culture of sawdust immobilized yeast ± S.E.M.

g/L wet weight of cell concentration and mixed with 100 g of sawdust for each treatment and incubated for 48 hours to get the cells immobilized.

Dehydrogenase activity and microbial population in soil: The microbial populations in soil were determined by TPC (Total Plate Count) method. Nutrient agar medium was used for bacteria and YEPD medium for yeast count. Microbial dehydrogenase activity (DHA) in the soil was determined using 2,3,5-triphenyltetrazolium chloride (TTC) method. Cultures (1 mL) were mixed with 2 mL of TTC solution (4 mg/mL), 2 mL anoxic water and 2 mL tris-HCl (pH 8.4) in a 50 mL Erlenmeyer flask. The mixture was incubated at 37°C for 3 h on a shaker with H_2SO_4 to terminate the reaction, then extracted with chloroform and absorbance was measured at 487 nm (Liang et al. 2009).

Experimental design and treatments: Experimental units consist of 1 L plastic trays. A total of 16 trays were used in the study. A summary of treatment and control trays tested are presented in Table 1. Each treatment was carried out in triplicate. For each tray, 1 kg of soil and the corresponding material (sawdust immobilized yeast cells) were added and mixed thoroughly. Soil moisture content was maintained at 10-20 % by daily addition of distilled water. Nutrients application was designed to optimize levels for caffeine degradation by yeast species. Inorganic nutrients such as $(NH_4)_2SO_4$ and K_2HPO_4 were added to the treatment units to give final C:N:P ratio of 100:10:1. Each unit was thoroughly mixed every alternate day to allow good aeration. The trays were incubated at 25-30°C.

Sampling and instrumental analysis: Soil samples were collected on alternate days and stored at 20°C and thawed 3 days before analysis. Residual caffeine in soil was extracted using chloroform. The degradation of caffeine was expressed as the percentage of caffeine degraded in relation to the amount of the remaining fractions in the appropriate control samples. Caffeine concentration was estimated by using UV-visible spectrophotometer (Hitachi U-2800). Absorbance was measured at 253 nm. Percentage of caffeine degradation was calculated as follows:

$$\text{Caffeine degradation (\%)} = \frac{(a-b)}{a} \times 100$$

Where *a* is the initial caffeine concentration and *b* is the residual caffeine concentration.

Phytotoxicity assay: Seeds of raddish (*Raphanus sativus*), chick pea (*Cicer arietinum*), lady's finger (*Abelmoschus esculentus*), black gram (*Phaseolus mungo*) and beet root (*Beta vulgaris*) were used to assay the phytotoxicity of caffeine in soil after various treatments (Table 1). Soil without caffeine, collected from garden was selected as control. The phytotoxicity assays were carried out using plastic cups. Ten seeds of each variety were planted in triplicates. Toxicity effects were measured in terms of germination percentage and shoot/root length after 10 days.

RESULTS AND DISCUSSION

Soil properties: In the present study, the texture of the raw soil was classified as a course sandy loam and physicochemical characteristics were noted as follows: course sand, 12%; fine sand, 69%; clay, 11%; silt, 8%; pH, 6.5; moisture, 14%; organic matter, 7.62%; total nitrogen, 75.7 mg/kg; total phosphorus, 45.4 mg/kg; and caffeine, 5.3 g/kg of moist soil. For the optimal growth of microorganisms, inorganic nutrients such as $(NH_4)_2SO_4$ and K_2HPO_4 were supplemented to the treatment units to give final C:N:P ratio of 100:10:1.

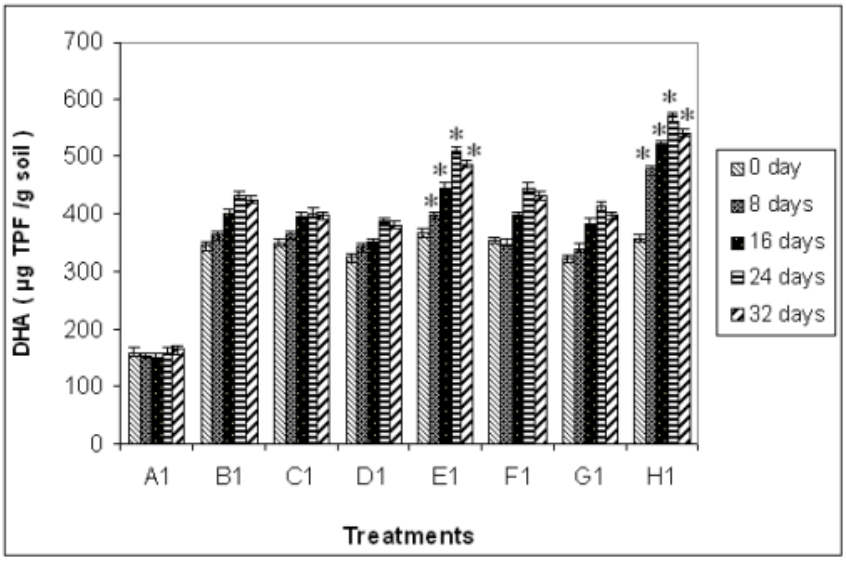

Fig. 2: Changes in dehydrogenase (DHA) activity during caffeine degradation in bioaugmented soil treatments. Values in each column labeled with an asterisk (*) indicated significant (p<0.05) differences between treatment A1 and other treatments in the same sampling time.

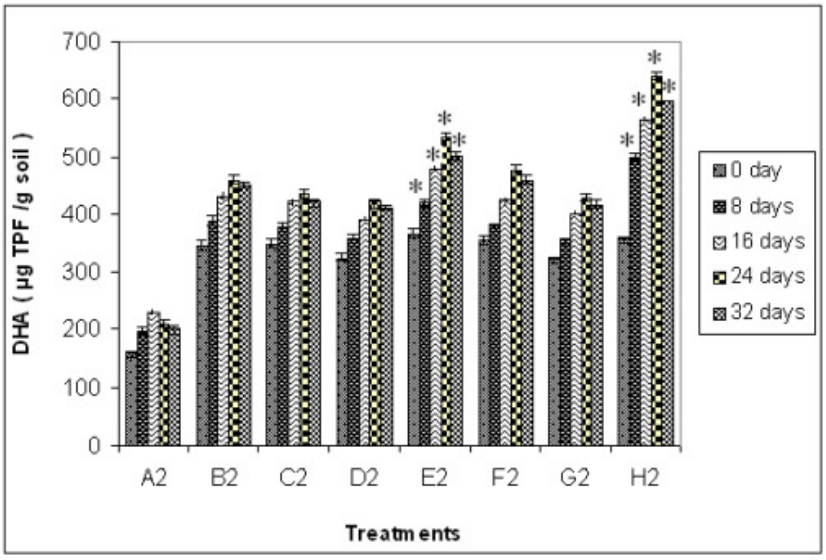

Fig. 3: Changes in dehydrogenase (DHA) activity during caffeine degradation in biostimulated combined bioaugmented treatments in soil. Values in each column labeled with an asterisk (*) indicated significant (p<0.05) differences between treatment A2 and other treatments in the same sampling time.

Growth and caffeine degradation by free and immobilized yeast cells in broth culture: Biocarrier materials were screened to assess the caffeine degrading potential of free and immobilized yeast species in CLM. Sawdust immobilized *T. asahii* cells showed maximum growth and caffeine degradation (91 %) at 48 h compared to the free cells (70 %) (Table 2). Yeast cells immobilized in sawdust are shown in SEM images (Figs. 1a, b, c) Therefore, in this study we report for the first time that it has been possible to develop self immobilized yeast species in sawdust showing more caffeine degradation capacity compared to free cells. Catalytic stability can be greater for immobilized cells than free cells. Some immobilized microorganisms tolerate higher concentrations of toxic compounds compared to free cells, when the cell support carrier acts as a temporary sink for the excess toxin (Akay et al. 2005). Therefore, in the present study, use of sawdust as cell

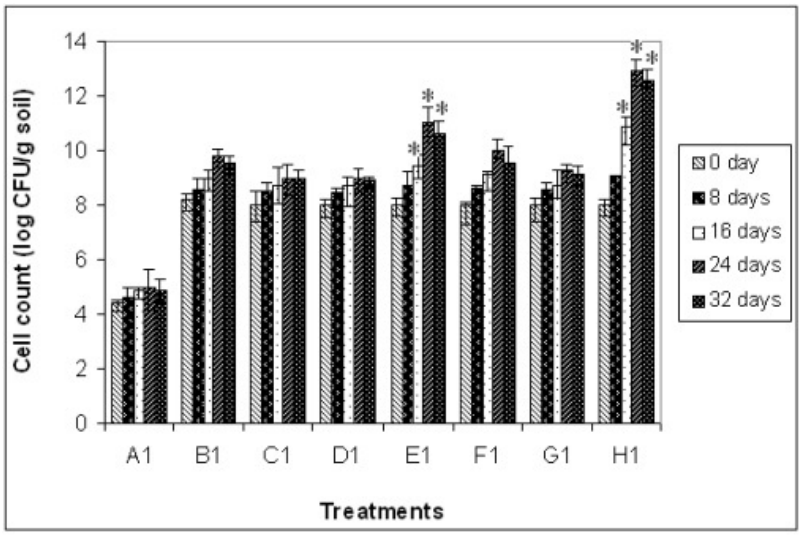

Fig. 4: Microbial growth during caffeine degradation in bioaugmented soil. Values in each column labeled with an asterisk (*) indicated significant (p<0.05) differences between treatment A1 and other treatments in the same sampling time.

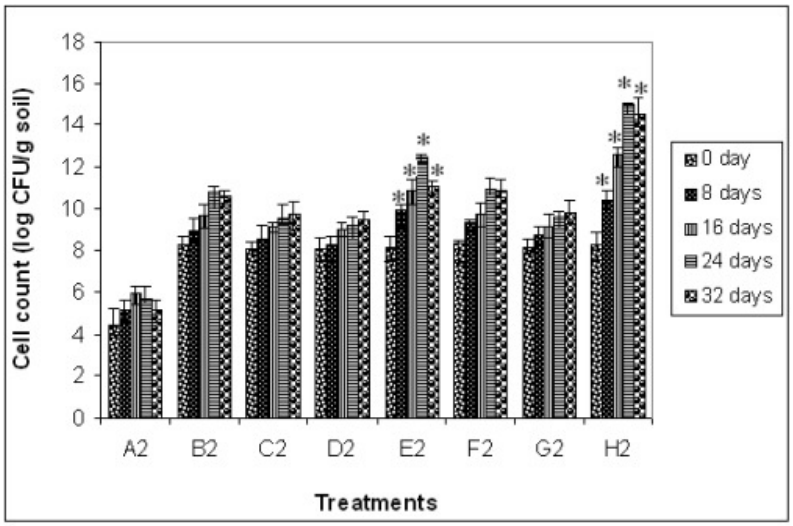

Fig. 5: Microbial growth during caffeine degradation in biostimulated combined bioaugmented soil treatments. Values in each column labeled with an asterisk (*) indicated significant (p<0.05) differences betweentreatment A2 and other treatments in the same sampling time.

support carrier could serve as potential decaffeination technique to be used readily towards caffeine degradation.

Caffeine degradation in soil by yeast species: Table 3 shows the percentage of caffeine degradation obtained in various soil treatments. Caffeine concentrations in soil were found to be decreased for all the treatments after 24 days. Maximum caffeine removal (98.3 %) was found in Treatment H_2 soil biostimulated with nutrients and bioaugmented with sawdust immobilized mixed culture No. 4 consisting of three yeast species viz., *Trichosporon asahii, Candida tropicalis* and *Candida inconspicua*. In absence of yeast inoculum in the soil (Treatments A1 and A2), no caffeine degradation was noted. Use of bulking agent such as sawdust could lower the contaminated soil bulk density, increase porosity and oxygen diffusion which helped to form water stable aggregates. These enhanced the biodegradation of caffeine along with the mass transfer rate of water, oxygen, nutrients, substrate and microbial activity (Xu & Lu 2010). Similar results were found by Topp et al. (2006) who reported that the mineralization of caffeine using *Pseudomonas* sp. strain TH1, in the loam soil was accelerated by the addition of liquid municipal biosolids.

Dehydrogenase activity and microbial population: Dehydrogenase activity (DHA) is a measure of overall

microbial activity and consequently indicates whether stimulation or inhibition of the microbial communities present is occurring as a result of the selected remediation strategy (Xu & Lu 2010). Dehydrogenase activity is recognized as an indicator of microbial oxidative activities, which catalyse the oxidation of organic compounds (Casida 1977). Changes in soil DHA during caffeine biodegradation in various soil treatments are shown in Figs. 2 and 3. The highest soil DHA (639 µg TPF per g soil) was observed for the treatment H2 (soil treated with sawdust immobilized yeast mixed culture 4) after 24 days. Significant increase ($p<0.5$) in DHA was observed in treatments H1 and E1 (Fig. 2) and treatments H2 and E2 (Fig. 3), compared to the control treatments A1 and A2 respectively. Total microbial count was also found to be increased significantly ($p<0.5$) in treatments H1 and E1 (Fig. 4) and treatments H2 and E2 (Fig. 5). The highest microbial count of 3.66×10^{14}/g soil (14.56 log CFU/g soil) was obtained for the treatment H_2 after 24 days of incubation. DHA and microbial count was remarkably less in case of treatments B1, C1, D1, F1, G1 (Figs. 2 and 4) and treatments B2, C2, D2, F2, G2 (Figs. 3 and 5). The microbial count and DHA, almost in all the treatments were found to be decreased after 32 days of soil treatment. Data obtained for total dehydrogenase activity were found to be highly correlated with total microbial count.

Phytotoxicity assay: A summary of the results from the phytotoxicity assay is given in Table 4. For all five plant seeds, the germination % and the root and shoot lengths were reduced due to toxicity of caffeine in the contaminated soil. Similar effects on caffeine toxicity were observed in mung bean (*Phaseolus aureus*) (Batish et al. 2008) and in paddy (Smyth 1992). On the other hand, no germination inhibition was observed in seeds planted in soil obtained after treatment (H2) and the garden soil without caffeine. Reduction in caffeine toxicity in the soil after treating with sawdust immobilized yeast mixed culture 4 (Treatment H2) were noted in terms of germination and shoot/ root length. Values presented are the mean of the three experiments. Data were analysed by one-way ANOVA Test.

Therefore, the results of the present study demonstrated that the sawdust immobilized mixed culture of yeast (*T. asahii,C. tropicalis* and *C. inconspicua*) enhanced the degradation of caffeine in soil under laboratory conditions using the treatment (bioaugmentation combined with biostimulation). However, long-term monitoring experiments are needed to further examine the application of this method in the field scale.

ACKNOWLEDGEMENT

Authors acknowledge CFRD, Kerala, India, for identification of the isolated yeast species. We thank the Central Instrumentation Facility (CIF) in Pondicherry University, Puducherry, India for providing the facility for SEM analysis. Authors of this article would like to thank VIT University for providing Lab facility and financial support for the smooth conduct of the work.

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