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Isolation, characterization and preservation of toluene degraders from soil subjected to biofiltration studies

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ABSTRACT

A microbial community of a soil differential biofilter reactor treating toluene vapors was investigated in this work. Strains were isolated and checked for toluene-degradation activity. Following basic primary identification, the secondary identification of the isolated strains was carried out using 16S rRNA and 18S rRNA gene-sequence comparison which showed that they belonged to 4 genera: *Pseudomonas*, *Stenotrophomonas*, *Aspergillus*, and *Ochrobactrum*. This work will help the current biofiltration researchers in selecting the appropriate toluene degraders in developing biofilm based biofilter reactors for the effective treatment of toluene.

KEYWORDS

Toluene degraders;
Biofilter;
Soil.

INTRODUCTION

The biodegradation effectiveness of a biofilter hugely depends on the microbial population present in the biofilter bed[7]. Bacteria and fungi are the most dominant groups of microorganisms contributing to the biodegradation of organic pollutants in a biofilter. However, bacteria are more advantageous than fungi due to their rapid growth rate and degradation rate[1,12,16]. The biodegradation mechanisms taking place in a biofilter are normally aerobic and are considered energetically favourable[9]. Hence it is essential to understand the biodegradation capacity of different microbial species present in natural environments like soil, water, etc., In addition, it is also important to find an organism which can adapt to the non-natural system which uses the pollutant as the sole energy source. Moreover, to develop a pure biofilm reactor for treating particular gaseous pollutants (e.g. toluene), these studies are prerequisite. The current work will discuss the isolation of toluene degraders from soil which was subjected to biofiltration environment and will also discuss the primary and secondary characterization of different isolated toluene degraders along with the methods used in preserving the isolated strains.

MATERIALS AND METHODS

Isolation of toluene degraders

A differential biofilter reactor (Figure 1), developed by Beuger and Gostomski (2009) was setup with 8.65 g (wet weight) of soil. An inlet toluene concentration of

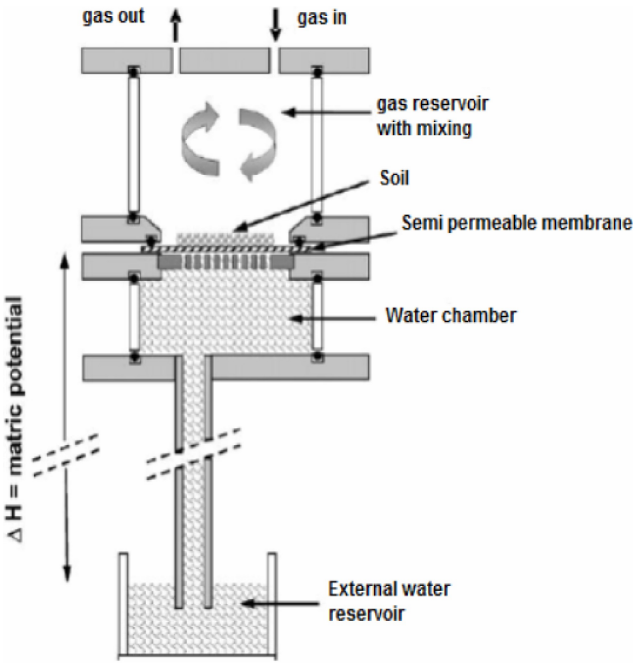

Figure 1 : A cut-away section of the differential biofilter reactor with water content control

sample of this soil was used for a serial dilution. A standard serial dilution procedure[10] was followed and a 1 ml sample from 10^{-5} dilution was used to inoculate the agar plates using the spread plate method[2]. A control agar plate without any sample was also used in the experiment. A minimal salt medium[15] with 1.5% agar was used to prepare all the agar plates. Toluene was used as a sole carbon and energy source for growth with the MS medium. Plates were incubated at 30 °C in a 5 litre glass desiccators containing a 100 mL beaker with 1% liquid toluene in Vacuum Pump Oil (vpo)[15,17] with an approximate head space toluene concentration of 430 ppm. The plates were observed every day for growth and a fresh 1% liquid toluene in vpo was replaced daily.

Identification of the toluene degraders

Isolated toluene degraders were individually subjected to Gram staining technique[6] as a primary identification step and were observed both in phase contrast and scanning electron microscopy. As a secondary identification technique, all the isolated toluene degraders were individually sub-cultured in Luria Bertani (LB) agar plates[3] and then the over-night cultures were sent to Eco Gene Ltd (Auckland, New Zealand) for

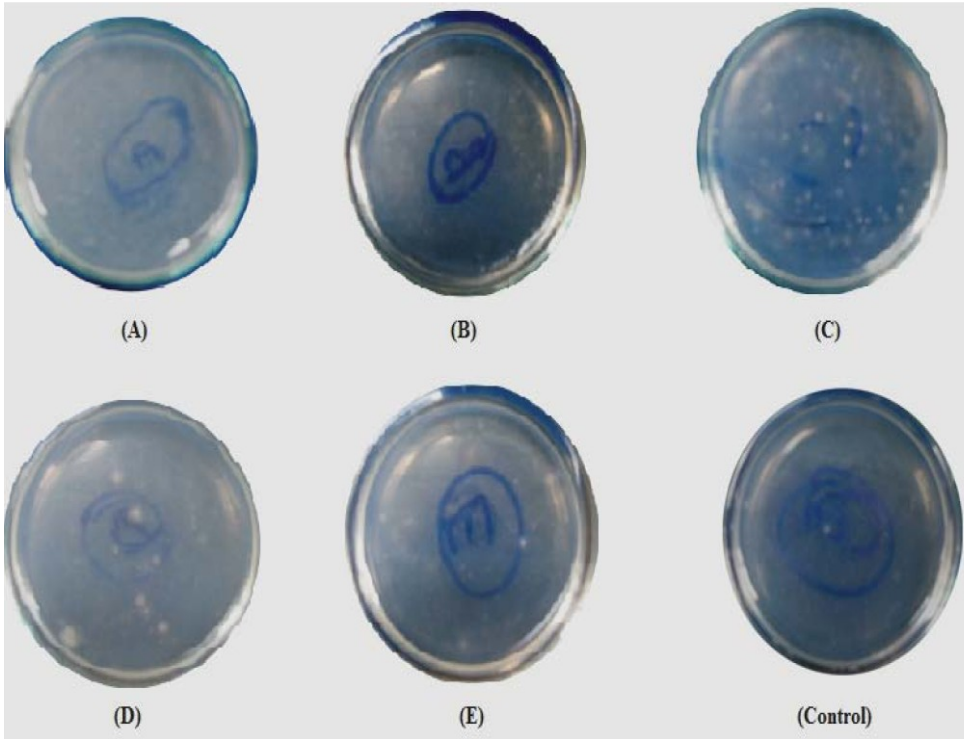

Figure 2 : Growth of isolated toluene degraders on MS agar plates (A-E) along with control in desiccator equilibrated with toluene-vpo mixture containing 1% toluene. The plates were photographed after 2 weeks of incubation

180 ppm (average) was maintained in the reactor. At steady state an outlet concentration of 132 ppm (average), a steady EC of 40 $g.m^{-3}.h^{-1}$ was observed after 30 days. Following this, the reactor was dismantled in a sterile environment and the soil was removed. A 1 g

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16s rDNA and 18s rDNA analysis.

Preservation of the toluene degraders

Following the secondary identification of the isolated toluene degraders, they were sub-cultured in duplicates. One set of grown plates were stored in -4 °C for short term preservation and the other set of grown plates[18] were used to prepare 100 mL liquid cultures in LB media. 5 mL of the overnight culture was added to a 5 mL of 40% glycerol in a 15 mL sterile screw cap centrifuge tube and stored in both -20 °C and -80 °C deep freezers for long term preservation.

RESULTS AND DISCUSSIONS

Isolation

Colonies were observed in plates 1, 4 and 5 after 8 days whereas in plates 2 and 3 colonies were seen after 13 days. This difference can be attributed to the different toluene degrading metabolisms involved in different species, growth rates, induction pathways and possibly the initial concentration in the serial dilution. In addition the response of microorganisms in a synthetic medium is always different when compared to a natural media like soil, compost etc.[13]. However, the control plate displayed no growth and hence the contamination issue was eliminated though it is not most important in the current scenario. Figure 2 shows the colonies in five agar plates along with the control agar plate subjected to toluene as a sole carbon and energy source. Based on visual identification, a total of six different colonies were picked and sub-cultured (purified) further in LB agar plates. The streak plate technique was adopted and the experiment was repeated to obtain pure individual isolates of the toluene degraders.

Primary identification

Four out of the five isolated toluene degraders were found to be rod shaped organisms in which three were Gram negative and one was Gram positive. The fifth isolate was found to be a fungus based on the morphology. The shapes (morphology) of all five isolates were further studied through scanning electron microscopy (SEM) and compared to the results obtained from phase contrast microscopy following the Gram staining experiment. Figure 3 compares both the phase contrast and SEM results of 5 toluene degrading strains which were subjected to primary identification technique. The phase contrast images clearly shows whether the isolated organism is gram positive or negative and the SEM images clearly shows the size & shape of the isolated organisms.

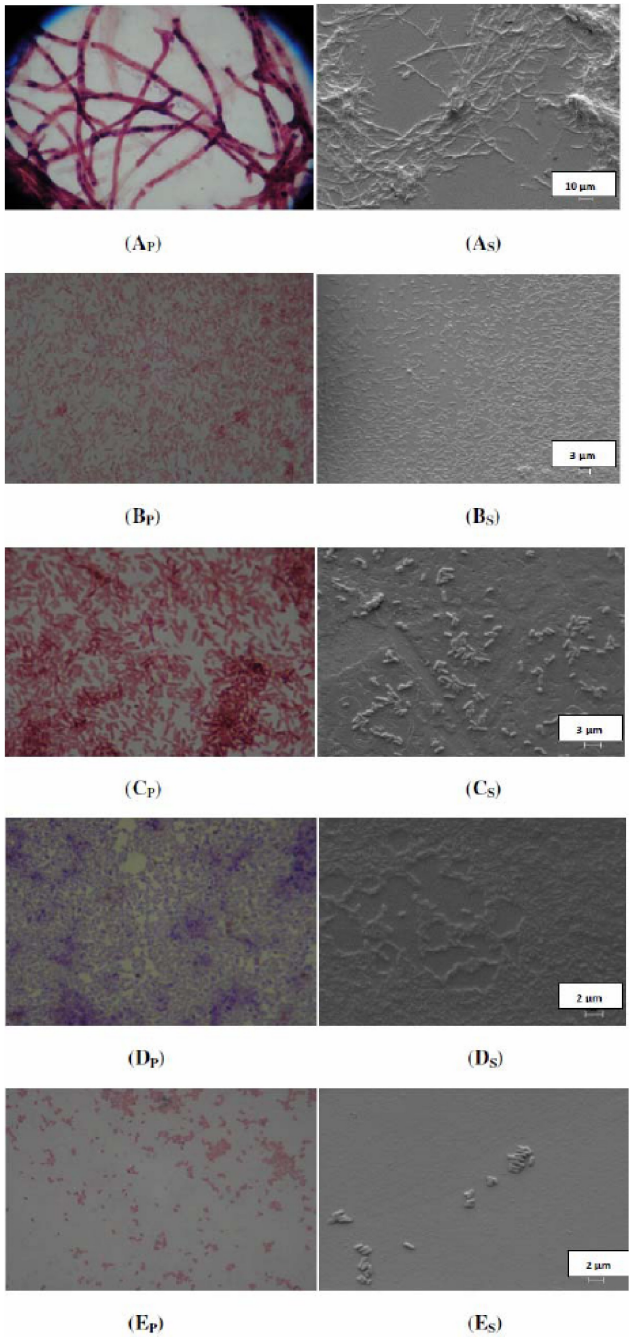

Figure 3 : A_P-E_P are the phase microscopy images (100X) of five different toluene degraders subjected to gram staining. A_S-E_S are the corresponding SEM images. (A_S: 500X; B_S to E_S: 1000X) of those five isolates.

Secondary identification

Following the primary identification, the five isolates were subjected to taxonomical identification through amplification and sequencing of the 18s rDNA for the fungus and 16s rDNA for the four bacterial isolates. The sequences were compared with the database of known 18s rDNA and 16s rDNA sequences through blast search for identification (Figure 4).

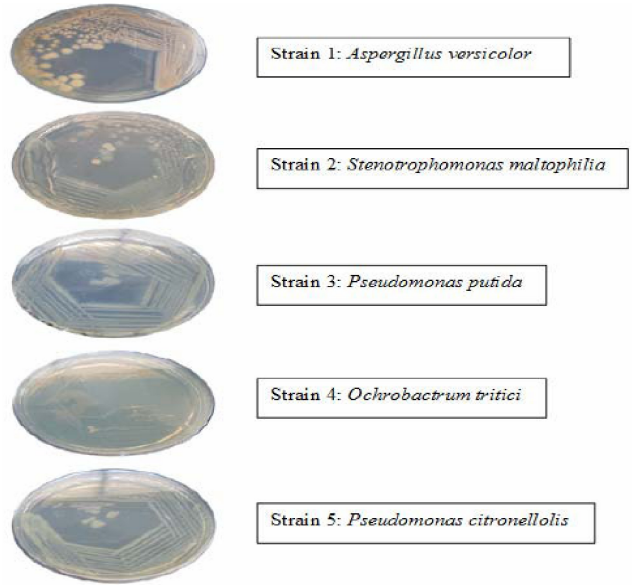

Figure 4 : Five different toluene degrader strains identified using 18s rDNA and 16s rDNA sequencing studies by Eco Gene Ltd (Auckland, NZ)

Out of these five identified toluene degraders, three were already reported in literature and the other two were not reported elsewhere as potential toluene degraders. However, only *Pseudomonas putida* was studied extensively and reported to follow the toluene degradation (TOD) catabolic pathway to degrade toluene[4,8,11] and the pathways used by the other isolated strains to degrade toluene are unknown. In the TOD pathway, toluene is first oxidized to cis-toluene dihydrodiol through the action of toluene dioxygenase (TDO). Cis–toluene dihydrodiol is dehydrogenated to form 3-methyl catechol which is cleaved at the ortho position and then converted in three steps to form acetaldehyde and pyruvate before entering the tri-carboxylic acid (TCA) cycle (Figure 5). TABLE 1 summarises the maximum toluene biodegradation percentage reported for the three potential toluene degraders isolated along with the other two isolated (non-reported) ones.

The strains *Ochrobactrum tritici* and *Pseudomonas citronellolis* are the first to be isolated from New Zealand soil and hence both the strains were deposited in the NZ culture collection maintained by International Collection of Microorganisms from Plants (ICMP), New

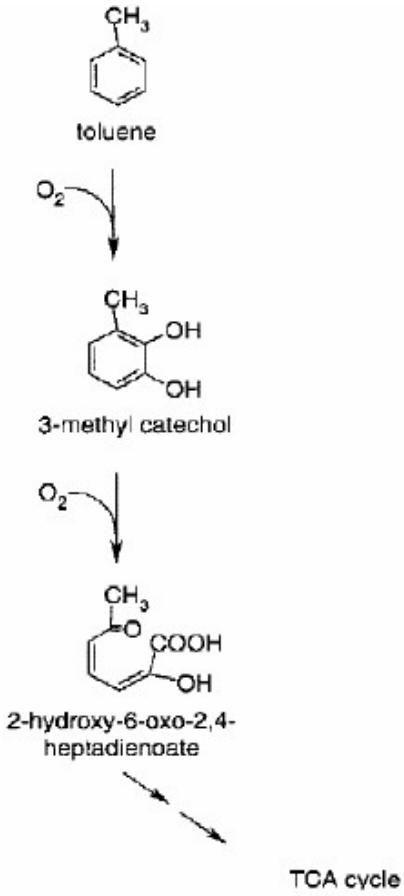

Figure 5 : TOD pathway followed by *Pseudomonas putida* during toluene degradation[14]

TABLE 1 : Reported maximum toluene degradation for the isolated strains

Toluene degrader	Maximum toluene Biodegradation percentage reported (%) (RE)	Reference
Stenotrophomonas maltophilia	83	(Lee et al., 2002)
Pseudomonas putida	95	(Men and Cheng, 2011)
Aspergillus versicolor	97	(Prenafeta - Boldú et al., 2012)
Ochrobactrum tritici	Unknown	-
Pseudomonas citronellolis	Unknown	-

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Zealand. ICMP accession number of 19448 and 19447 were given to these two strains *Ochrobactrum tritici* and *Pseudomonas citronellolis*. Later as per the request[19] from ICMP the other three isolated toluene degraders *Stenotrophomonas maltophilia*, *Pseudomonas putida* and *Aspergillus versicolor* were also deposited in ICMP with accession numbers 19446, 19449 and 19445. In addition to the deposition, all the five isolates were preserved in -4 °C for short term application and in -20 °C, -80 °C deep freezers for long term application.

CONCLUSIONS

Though toluene degraders are commonly found in soil and many people have already isolated and characterized them from soil, most of them are outside New Zealand. Since there are lots of time consuming procedures in purchasing pure cultures from microbial culture collections in and outside New Zealand, instead of purchasing the pure cultures, they were isolated from soil. The soil used was exposed to the outlet concentration of 132 ppm of toluene for nearly 30 days in the biofiltration reactor. Primary identification of the isolated toluene degraders through Gram staining and SEM analysis gave an insight into the morphology of the isolated species. Secondary identification using 16s rDNA and 18s rDNA amplification and sequencing studies helped to identify the taxonomy of five potential toluene degrading stains. Only *Pseudomonas putida*, *Stenotrophomonas maltophilia* and *Aspergillus versicolor* were reported as a toluene degrader in the literature and the other two isolates *Ochrobactrum tritici* and *Pseudomonas citronellolis* are novel toluene degraders. In addition, these newly reported toluene degraders were deposited in NZ culture collections maintained by ICMP, NZ. Following secondary identifications all the five toluene degraders were preserved under -4 °C, -20 °C and -80 °C for future experimentation to develop a pure biofilm reactor for toluene degradation.

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[17] Densities of toluene and vpo are nearly same (0.86 $g.cm^{-3}$ for toluene and 0.85 $g.cm^{-3}$ for vpo)

[18] Single colony was picked for inoculation.

[19] Our research group is the first to report these strains as potential toluene degraders in New Zealand.