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Microbial degradation of recalcitrant organics from radioactive waste using indigenous cultures of naphthalene degrading bacteria

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Nuclear energy generation and some medical research facilities produce effluents containing radioactive materials. These often consist of radioactive heavy metals and complex mixtures of hazardous organic compounds such as polycyclic aromatic hydrocarbons (PAHs) and irradiated surfactants. The organic compounds therein are recalcitrant and potentially harmful to living organisms in the environment. The nuclear industry has not presented an environmentally favourable solution for treatment and disposal of these compounds. Microbial treatment represents a more environmental friendly approach since it achieves complete mineralization of the compounds and can be performed under natural temperature and pH conditions. In this study, radioactive wastewater with radioactivity of 0.677 Bq was collected from a sump at a radioisotope processing facility in Cape Town, South Africa. The wastewater sample was extracted by solid phase extraction. Samples were analyzed for the presence of PAHs and derivatives by HPLC against the PAH standard containing 16 priority PAHs. Identification of compounds in wastewater was based on the retention time match against calibration standards. All 16 priority PAHs were detected in the sample with concentrations ranging from 0.001mg/L to 25.1mg/L. The results have shown that there are high levels of PAHs in the wastewater exceeding the World Health Organisation (WHO) recommended maximum values for safety. The results also provide insight into the distribution pattern of PAHs in radioactive wastewater. Biodegradation studies were conducted in batch reactors using synthetic wastewater containing the controlled amounts of irradiated organics in order to determine degradation rate parameters. An indigenous culture of aromatic compound degrading bacteria obtained from a landfill site was used in the batch studies.

1. Introduction

Nuclear energy is an important component of the world's energy supply. Globally, 17% of the overall electricity supply comes from nuclear power. To address the problem of increasing energy demand with the rapid increase of the world population, nuclear energy is becoming more and more important as an alternative energy source (Purushotham et al, 2000). Additionally, nuclear energy is regarded as a greener

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alternative to the burning of fossil fuels for energy generation due to its low CO_2 output. The major draw back of nuclear energy generation is the production of substantial amounts of radioactive waste discharged as a mixture of metallic radionuclides and hazardous organic compounds (Ismagilov et al, 2000). These are mainly generated by the so called nuclear industry which includes power generating facilities, radioisotope manufacturing facilities, and medical facilities.

Other than the nuclear industry, radioactive wastes are also produced by non-nuclear activities such as processing of raw materials containing naturally occurring radionuclides with low levels of radiation, research facilities, and laundry facilities for the radiation research laboratories (IAEA, 1994). Wastes produced in these facilities are classified as irradiated compounds and are usually toxic and not easily degradable by mesophilic bacteria in conventional wastewater treatment plants. The organic component is typically comprised of polycyclic aromatic compounds (PAHs) and chlorinated biphenyls from process water and surfactants from laundry wastewater. The PAHs represent a unique class of hydrocarbons with a pyrogenic nature and a complexity of assemblages. They are also common in petrochemical pollution (Sarma *et al*, 2004).

Irradiated organic compounds produced from the non-nuclear industry are recalcitrant and represent a significant risk to the environment. Treatment and conditioning techniques of organic radioactive waste are therefore required to obtain a product that can be stored or disposed of more safely. Techniques currently used include physical and chemical treatment and they result in the shortcoming of production of secondary waste that requires further treatment before disposal (IAEA, 2004). Microbial treatment represents a more environmental friendly approach since this may achieve complete mineralization of the organics to CO_2 and water and can be carried out under natural temperature and pH conditions (IAEA, 2004). It has been demonstrated that biological treatment of radioactive organic waste is a desirable alternative to physical and chemical treatment since it does not produce secondary radioactive waste (Stringfellow et al, 2004).

The aim of this study is to perform the biological removal of recalcitrant organics found in nuclear and radioactive waste. Naphthalene was used as a model compound to represent the common organic pollutants in radioactive waste.

2. Materials and Methods

2.1 Characterization of radioactive wastewater

Radioactive wastewater was collected from radioisotope processing facility belonging to iThemba Labs (Cape Town, South Africa). The radioactivity of the samples was determined to be 0.677 Bq, enough to inhibit the growth of mesophilic bacteria from activated sludge processes (Lee *et al*, 2004). Pre-treatment of wastewater samples was carried out by solid phase extraction (SPE) and concentrated by Dry Vap concentrator (Rotavapor RII, BUCHI, Switzerland). The concentrated wastewater sample was then analysed by HPLC against a standard mixture of 16 priority PAHs. Identification of compounds in wastewater samples was based on retention time match against calibration standards. The calibration standards were also used for quantification of identified compounds.

2.2 Culture media

Microorganisms were isolated from soil collected from a landfill (Chloorkop, Johannesburg, South Africa) and from mine water exposed to background radiation (Harmony Gold mine, South Africa). Initial cultures were obtained by inoculating 100 mL of sterile nutrient broth (autoclaved at 121°C, 2 atm for 15 min.) with 1g of soil or 1 mL of mine water in a 250 mL Erlenmeyer flask. The flask was incubated for 48 hrs on a Orbital Environmental Shaker operated at 120 rpm and 28±2.0°C. Enrichment cultures were obtained by sub-culturing a 2% (v/v) of 48hrs culture medium in mineral salt medium (MSM) containing naphthalene as the only carbon and energy source. The MSM contained 10 mM NH_4Cl, 30 mM Na_2HPO_4, 20 mM KH_2PO_4, 0.8 mM Na_2SO_4, 0.2 mM $MgSO_4$, 50 µM $CaCl_2$, 25 µM $FeSO_4$, 0.1 µM $ZnCl_2$, 0.2 µM $CuCl_2$, 0.1 µM NaBr, 0.05 µM Na_2MoO_2, 0.1 µM $MnCl_2$, 0.1 µM KI, 0.2 µM H_3BO_3, 0.1 µM $CoCl_2$, and 0.1 µM $NiCl_2$ (Roslev *et al*, 1998). The enrichment procedure was repeated 3 times to allow for high degree of selection of efficient naphthalene degrading organisms.

2.3 Analytical Methods

To determine naphthalene degradation rate, naphthalene at different concentrations was dissolved in 2 mL of methanol and added into 500 mL of a sterile MSM in 1L Erlenmeyer flasks. 2% (v/v) of enrichment culture was used as inoculum and incubated on a rotary shaker at 28±2.0°C. At certain intervals, 5 mL aliquot samples were withdrawn from the bioreactor aseptically. The sampled aliquots were centrifuged and the supernatant analyzed for naphthalene concentrations using a Waters Model 2695 (HPLC) equipped with a Photodiode Array (PDA) detector (Johannesburg, SA).

2.4 Culture characterization

16S rRNA fingerprinting method was used to obtain DNA sequences of pure isolated bacteria. Genomic DNA was extracted from the pure cultures using a DNeasy tissue kit (QIAGEN Ltd, West Sussex, UK) as per manufacturer's instructions. The 16S rRNA genes of isolates were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers pA and pH1 (Primer pA corresponds to position 8-27; Primer pH to position 1541-1522 of the 16S gene) (Coenye et al. 1999). An internal primer pD was used for sequencing (corresponding to position 519-536 of the 16S gene). Sequences obtained were matched against known microbial DNA sequences from the NCBI (National Centre for Biotechnology Information) genomics database.

3 Results and Discussion

3.1 Naphthalene Degradation Studies

The wastewater analysed in this study contained all the 16 priority PAHs in the range 0.001-25 mg/L (Table 1). Among the concentrations shown in Table 1, acenaphthene (detected at 25.1 mg/L) was the most abundant. All the PAHs in the wastewater samples from the radioisotope manufacturing facilities exceeded the WHO limit of 0.05µg/L indicating the need for further treatment before final disposal to the environment. Firstly, results from the biodegradation experiments using mixed cultures from landfill soil are shown (Figure 1). These were carried out in the range of 30-60 mg/L. Naphthalene was completely degraded under the low initial concentration of 30-60mg/L

Table 1: Concentrations of PAHs in radiioactive wastewater samples

Compound	**Concentration (mg/L)**
Naphthalene	1.654
Acenaphthylene	0.001
Acenaphthene	25.101
Fluorene	0.942
Phenanthrene	0.390
Anthracene	0.695
Fluoranthene	0.000
Pyrene	0.014
Benzo(a)anthracene	0.019
Chrysene	15.305
Benzo(b)fluoranthene	0.057
Benzo(k)fluoranthene	0.005
Benzo(a)pyrene	0.048
Benzo(ghi)perylene	0.047
Dibenzo (ah)anthracene	0.006
Indeno(1,2,3-cd)pyrene	0.438

within 15 hours of incubation. In contrast, naphthalene degradation was not complete at when large amounts of naphthalene were added (200-500 mg/L) (data not shown). Since the solubility of naphthalene is only 30mg/L, it was expected that the actual concentration of naphthalene in water could not exceed this value, thus the rate of degradation could be a function of the rate of dissolution of naphthalene from the solid phase into the bulk liquid. Notably, the level of naphthalene measured in liquid solution never exceeded the theoretical solubility limit. The lower measured values in this experiment were attributed to low solubility of naphthalene in the highly saline mineral medium used for culture growth. No significant degradation was observed in cell free controls, thereby ruling out the significance of abiotic naphthalene removal processes in the experiments.

In the second set of experiments, for the batch cultures were inoculated with bacteria from mine water. The experiments were also conducted in the range 30-60 mg/L naphthalene to compare the performance with the landfill soil cultures. The performance of the mine water bacteria was much slower than the performance of landfill soil bacteria as indicated by 29 hours required to completely degradate 60 mg/L in the mine water bacteria batch experiments (Figure 2). The lower degradation rate in the mine water culture may be due to a limited number of phenolic ring degrading species as confirmed later by the wider biodiversity tests using the 16S rRNA fingerprinting.

3.2 Culture Characterisation

After purifying and sequencing the rRNA genes from the soil and mine water bacteria, a total of 5 and 3 bacterial isolates were found, respectively. The rRNA sequences were isolated from bacteria with some tolerance to PAH toxicity and were thus candidate species for naphthalene degradation. Species identification was based on the match of

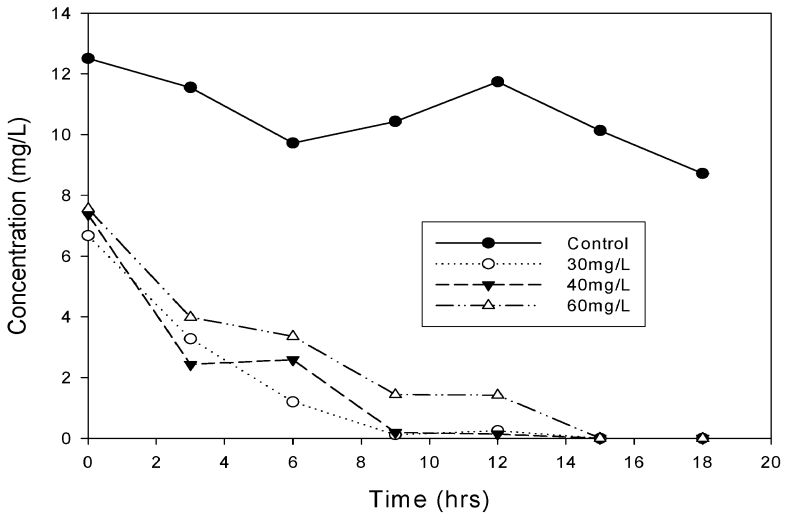

Figure 1: Naphthalene degradation at low initial concentrations by soil culture

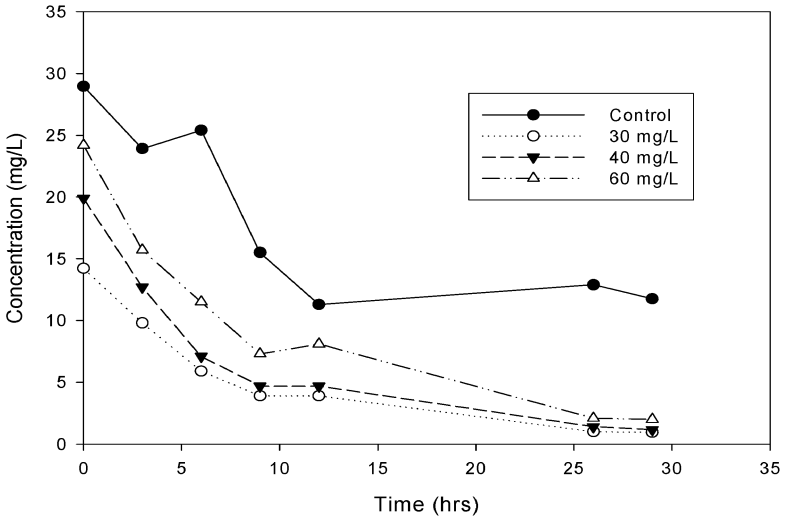

Figure 2: Naphthalene degradation at low initial concentration by mine water culture

16S rRNA with species in the GenBank. Hits were scored with 96% confidence and above, except for one that had 89% identity. The results are shown in Tables 2 and 3 (below). The bacteria from soil showed a wider biodiversity of known aromatic ring oxidizing species such as *Pseudomonas aeruginosa*, *Microbacterium esteraromaticum* and *Alcaligenes sp.* than in the culture from mine water – with only *Pseudomonas putida* as the known aromatic ring cleaving species. The wider biodiversity of aromatic ring degrading bacteria in the soil culture may be reason why the soil bacteria performed better than the mine water culture in degrading naphthalene.

Table 2: Characterisation of Naphthalene degrading bacteria isolated from landfill site.

Culture	16S rRNA ID	% Identity
A	*Microbacterium esteraromaticum*	98 %
B	*Achromobacter xylosoxidans*	99 %
C	*Alcaligenes sp.*	89 %
D	*Pseudomonas aeruginosa*	99 %
F	*Pseudomonas pseudoalcaligenes*	99 %

Table 3: Characterisation of naphthalene degrading bacteria isolated from mine water.

Culture	16S rRNA ID	% Identity
A	*Stenotrophomonas sp.*, *Stenotrophomonas maltophilia Strain* KNUC285	96 %
B	*Bacillus cereus, B. subtilis, B. thuringiensis*	99 %
C	*Pseudomonas putida, Pseudomonas taiwanensis*	99 %

4 Conclusion

Organics in radioactive wastewater from a radionuclide processing facility were characterized and were found to be predominated by PAH species. Naphathalene degrading bacterial species isolated from contaminated soil from a landfill site outperformed cultures from mine wastewater in degrading naphthalene, a PAH used as model compound for the PAHs found in radioactive wastewater. Degradation kinetics for the PAHs were affected by the rate of solubility of the solid phase.

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