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Biodegradation of naphthalene by bacteria isolated from petroleum contaminated soil

M.F. Tawfiq[1], **A.R. Abdullah**[1] and **A.F. Merican**[2]

[1]Department of Chemistry and [2]Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

ABSTRACT Environmental isolates of *Pseudomonas* sp., *Aeromonas* sp. and *Acinetobacter* sp. were tested for their ability to grow and degrade naphthalene in liquid culture media. The experiments were carried out during 62 h incubation time, in which the three bacteria were incubated separately. Viable cell determination using plate count method showed that the bacteria grew well in media containing naphthalene at different concentrations. Residual naphthalene was quantified by GC-MS employing anthracene as internal standard. All bacteria were found to significantly removed naphthalene from the culture media. Bacterial growth was enhanced in the presence of naphthalene in the media. *Acinetobacter sp.* was able to remove 68 % of the naphthalene in the culture media during 62 h of incubation time.

ABSTRAK Isolat alam sekitar *Pseudomonas* sp., *Aeromonas* sp. and *Acinetobacter* sp. diuji untuk mengetahui kebolehan untuk bertumbuh dan mengdegradasi naftalena di dalam media pertumbuhan cecair. Eksperimen telah dijalankankan sepanjang 62 jam, di mana tiga bakteria telah diinkubasi secara berasingan. Penentuan jumlah sel hidup menggunakan kaedah kiraan plat menunjukkan bakteria-bakteria bertumbuh dengan baik dalam media yang mengandungi naftalena pada kepekatan yang berbeza. Naftalena yang tinggal ditentukan menggunakan GC-MS dengan anthacene sebagai piawai dalaman. Semua bakteria didapati berkeupayaan tinggi untuk mengeluarkan naftalena daripada media pertumbuhan. Pertumbuhan bakteria adalah baik di dalam kehadiran naftalena di dalam media. *Acinetobacter sp.* berkebolehan untuk mengeluarkan 68 % naftalena di dalam media pertumbuhan semasa 62 jam tempoh inkubasi.

(Environmental bacteria isolates, naphthalene, PAH, biodegradation)

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants having toxic, genotoxic, mutagenic and/or carcinogenic properties [1]. Contamination of the environment with PAHs is considered hazardous because of the toxic effect of specific PAHs [2]. PAHs enter the terrestrial and the marine environment from a wide range of natural and anthropogenic sources (forest fire, oil spill, petroleum industry) [3,4,5,6]. The fate of the PAHs is linked normally to abiotic and biotic processes, which includes volatilization, bioaccumulation, photo-oxidation, chemical oxidation and microbial activity. It is generally accepted that aerobic microbial oxidation dominate the process which is involved in PAHs removal from the environment [5,6,7].

Two types of PAHs could be classified, the high and low molecular weight PAHs. The persistence of high molecular weight PAHs such as fluoranthrene and benzo[a]pyrene is due chiefly to their hydrophobic properties and low water solubility, which are limiting factors for their elimination. Due to their relatively high solubility, which enables them to transport through out the microbial cell membrane, low molecular weight PAHs such as naphthalene, phenanthrene and anthracene more rapidly

degradable than high molecular weight PAHs [8]. Naphthalene is considered to be a primary irritant and the US Environmental Protection Agency (EPA) has classified naphthalene as a priority toxic pollutant [9,10]. Naphthalene and its methyl derivatives are considered one of the most acutely toxic compounds [11].

In this study, degradation of naphthalene using environmental isolates of *Pseudomonas* sp., *Aeromonas* sp. and *Acinetobacter* sp. as carbon and energy source was investigated. The growth of the bacteria on naphthalene was studied and compared with that of media without naphthalene. For each bacteria, batch culture experiments were used to quantify the amount of naphthalene remaining at each sampling time during incubation period.

MATERIALS AND METHOD

Bacteria

Environmental isolates of *Pseudomonas* sp., *Aeromonas* sp. and *Acinetobacter* sp., were from soil contaminated with used engine oil and diesel oil [12].

Chemicals

All chemicals and media were of the highest grade available. $MgSO_4$, $CaCl_3$, KH_2PO_4, $FeCl_3$, K_2HPO_4, $(NH_4)_2SO_4$, NaOH and $NaHCO_3$, were from BDH chemicals Ltd, Pool, England. Na_2O_4S were purchased from Darmstandt, Germany and NaCl was from Sigma Chemical Co., Louis, USA. Agar Bacteriological (Agar No.1), tryptone and yeast extract were from OXOID Ltd., Basingstoke, Hampshire, England.

Naphthalene and dichloromethane (DCM) were from R&M Marketing, Essex, England. Hexane (99.8% GC grade) was from Mallinckrodt Baker, Phillipsburg, NJ08865 USA. Anthracene was purchased from BDH Chemicals Ltd., Poole, England.

Growth media

Bacterial growth on naphthalene was carried out using basal salt media (BSM) having the following composition (per 500 ml): 0.5 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.01 g $FeCl_3$, 0.5 g $(NH_4)SO_4$. pH of the media was adjusted to 7.0 – 7.2 with 1N NaOH prior to sterilization. Microbial enumeration was done on Luria-Bertani (LB) agar plate using total plate count method [13]. Numbers of cells obtained were evaluated as cfu/ml. LB media consist of (per 500 ml): 5 gm Tryptone, 5 gm NaCl, 2.5 gm yeast extract. For preparing agar plates, 2% (w/v) of agar bacteriological (OXOID) was added to LB media.

Preparation of inocula and starter culture

To prepare the starter culture, a loop-full of fresh bacterial culture was inoculated into a 250 ml sterilized triple baffled shake flask (Bellco, USA) containing sterilized LB media. The flask was then incubated at 37 °C for 24 h in a rotary shaker incubator (Innova 4900, New Brunswick Scientific, USA) at 100 rpm. The bacteria were inoculated into experimental flasks (see below) when the absorbance of the culture reached A_{660} of 0.1.

Bacterial growth on naphthalene

All experiments were carried out in triplicate set for checking the consistency of the data. Growth experiments were performed separately with 3 different bacteria in 170 ml BSM media in a 500 ml triple baffled shake flask (Bellco, USA) to boost the shaking efficiency. For each bacteria (i.e. each set of experiment), 3 types of flasks were prepared: Control 1 [BSM + bacterial strain], Control 2 [BSM + bacterial strain + acetone] and Sample [BSM + bacterial strain + naphthalene dissolved in acetone]. The same volume of acetone that was used to dissolve solid naphthalene was spiked into each Control 2 flasks. For each type of flask, triplicates were made. In addition, at least 2 uninoculated flasks were used as negative controls (BSM + naphthalene dissolved in acetone) in each set of experiments to account for any volatilization of naphthalene during the incubation. For bacterial growth analysis naphthalene concentrations of 1, 5, 10, 20 and 30 ppm were used. For GC-MS analysis, naphthalene concentration in all sample flasks was maintained at 30 ppm (note that the maximum naphthalene solubility in water is 31.0 mg/L). All flasks were incubated in a rotary shaker (Innova 4900, New Brunswick Sci., USA) at 150 rpm, 37°C in the darkness for 62 h. The sampling times were 0, 4, 8, 18, 22, 48 and 62 h. At each sampling time, samples were taken for viable cells count using plate count method and GC-MS analysis. All experiments were done in

triplicates, and controls were run to account for volatilization of naphthalene.

Analytical procedure

At each sampling time, triplicate from batch cultures were carefully sampled out. Liquid-liquid extraction was performed using dichloromethane (DCM) to extract residual naphthalene from the culture media [14]. The internal standard (anthracene) was added to the extracted sample to quantify the naphthalene residual [15]. Quantification by GC-MS was evaluated as peak area ratio of standard naphthalene to internal standard [16], which derived from a calibration curve at naphthalene standards concentrations of 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.55 and 1 ppm. Internal standard concentration was maintained at 1 ppm for all standard concentrations. GC-MS analysis was performed using a Shimadzu QP 5000 GC-MS (Shimadzu, Tokyo, Japan). Helium gas was used as the carrier gas (flow rate 1.5 ml/min). A DB-1 (100% dimethyl polysiloxane) capillary column (30 x 0.25 mm) was attached to the GC-MS. Temperature program for naphthalene quantification was 60°C for 2 min, with increment of 30°C/min until it reached 280°C and hold for 5 min.

RESULTS AND DISCUSSION

Petroleum contaminated soil or sediments have proved to be a rich source of PAH-degrading bacteria [17, 18]. The environmental isolates *Pseudomonas* sp., *Aeromonas* sp. and *Acinetobacter* sp., were capable of utilizing naphthalene as the sole source of carbon and energy. The bacteria were incubated separately with various concentrations of naphthalene 1, 5, 10, 20 and 30 ppm, respectively. Figures 1, 2 and 3 demonstrate the growth of *Pseudomonas* sp., *Aeromonas* sp. and *Acinetobacter* sp. at various naphthalene concentrations in liquid culture, respectively. Increasing the substrate concentration leads to the increase in bacterial growth. This could be related to the bioavailability of the substrate. Several authors have reported that growth on naphthalene depends on the mass transfer of the substrate to the aqueous phase [19, 20, 21]. The bioavailability and biodegradability of low molecular weight PAHs such as naphthalene, depends mainly on the complexity of the chemical structure as well as physico-chemical properties of these compounds [22, 23].

The results showed that the three isolates grew in all 5 different concentrations of naphthalene during 62 h incubation time with *Acinetobacter* sp. exhibited better growth. However, among the three isolates, *Aeromonas* sp. did not show significant growth (Figure 2). Microbial population declined after 50 h of incubation time for *Aeromonas* sp. and *Acinetobacter* sp. (Figures 2 and 3). This decline may be due to natural decay, a buildup of toxic by-products, and/or consumption of readily utilizable carbon. However, the available data are insufficient to identify the cause. Agar plates for negative control (BSM + naphthalene dissolved in acetone); (without bacteria) did not show any growth (data not shown). Therefore we conclude that the control experiments remained essentially sterile throughout the incubation period.

The results of naphthalene degradation experiments for the *Pseudomonas* sp., *Aeromonas* sp. and *Acinetobacter* sp. bacteria are shown in Figures 4, 5 and 6, respectively. Residual naphthalene was quantified by area ratio based on a GC-MS calibration curve (data not shown).

All three bacteria were capable of degrading naphthalene at 30 ppm in BSM media within 62 h. It is not surprising that all three bacteria were able to degrade small amount of naphthalene (30 ppm). Furthermore, it is very likely that one of the most important requirements for the degradation behavior involves the ability to degrade naphthalene at low concentration [24, 25].

The results showed that the initial 30 ppm concentration of naphthalene was almost degraded within 48 h of incubation. The contribution of naphthalene volatilization was estimated from control flask to be approximately 0.103 ppm/h.

After 50 h of incubation, all of the bacteria showed significant degradation of naphthalene. So the level of naphthalene possibly utilized by *Acinetobacter* sp. was calculated to be approximately 20 ppm, *Aeromonas* sp approximately 18 ppm and *Pseudomonas* sp. approximately 17 ppm within the incubation period. GC-MS experiments were also

performed to detect naphthalene intermediates (Figure 7). Several peaks were observed. The identification of these peaks were based on the mass spectrum database (National Standard and Technology, USA) presence in the GC-MS software package. The detection of peak P4 (Figure 7A) suggest that naphthalene was converted to intermediate. P4 was identified as either cyclohexane or cyclohexene. Naphthalene biodegradation produced intermediates such as salicyclic acid and catechol [6] which are six carbon ring compound. Salicyclic acid or catechol could not be detected directly by GC-MS. Further experiments involving derivatization procedure is required in order to test for the presence of these intermediates.

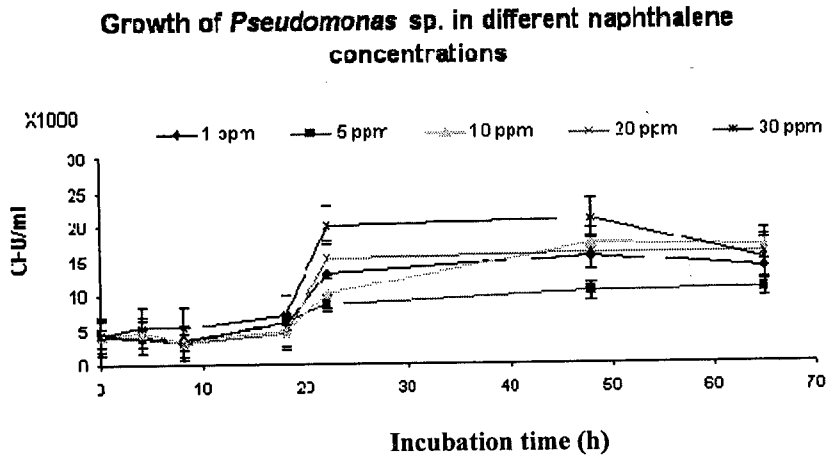

Figure 1 The growth curve of *Pseudomonas* sp., obtained from plate count method, when incubated in different concentrations of naphthalene (1, 5, 10, 20 and 30 ppm, respectively). Error bar was calculated by average of triplicates.

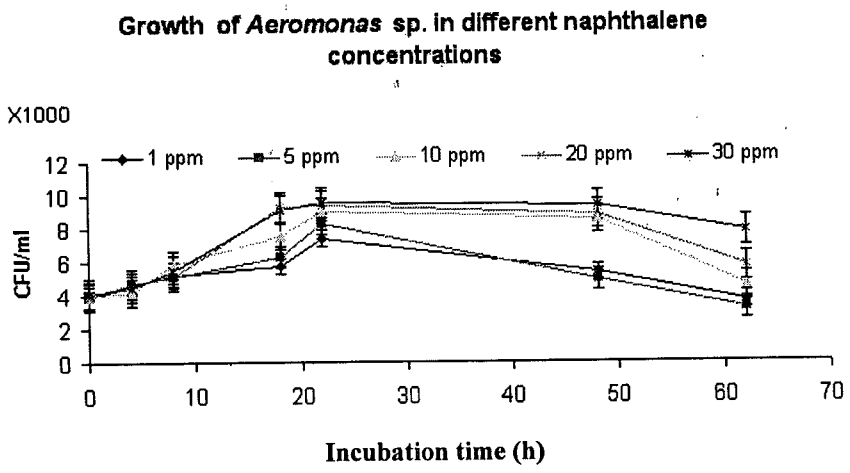

Figure 2 The growth curve of *Aeromonas* sp., obtained from plate count method, when incubated in different concentrations of naphthalene (1, 5, 10, 20 and 30 ppm, respectively). Error bar was calculated by average of triplicates.

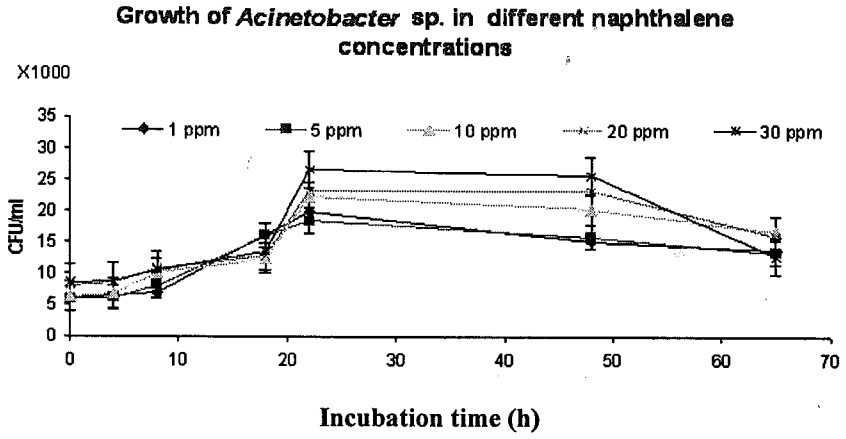

Figure 3 The growth curve of *Acinetobacter* sp., obtained from plate count method, when incubated in different concentrations of naphthalene 1, 5, 10, 20 and 30 ppm, respectively. Error bar was calculated by average of triplicates.

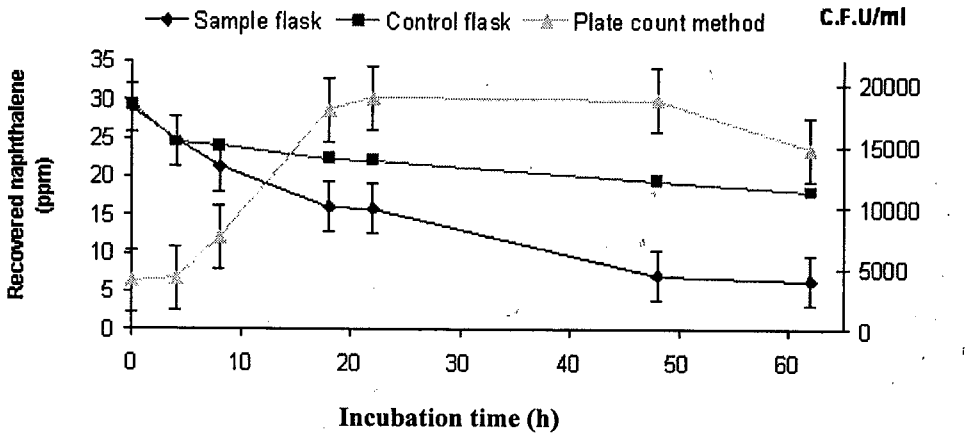

Figure 4 Removal of naphthalene and growth of *Pseudomonas* sp. in BSM media containing naphthalene at 30 ppm initial concentration (Sample flask). The control flask contains BSM media and naphthalene.

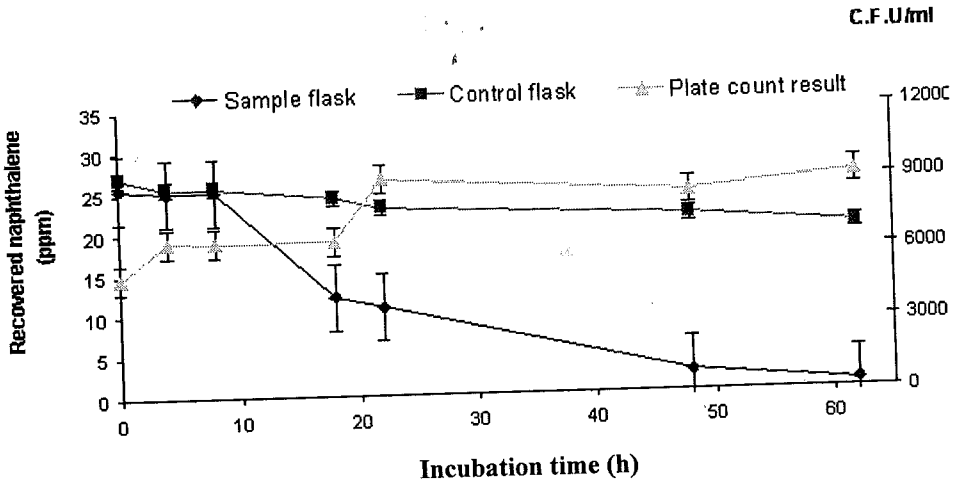

Figure 5 Removal of naphthalene and growth of *Aeromonas* sp. in BSM media containing naphthalene at 30 ppm initial concentration (Sample flask). The control flask contains BSM media and naphthalene.

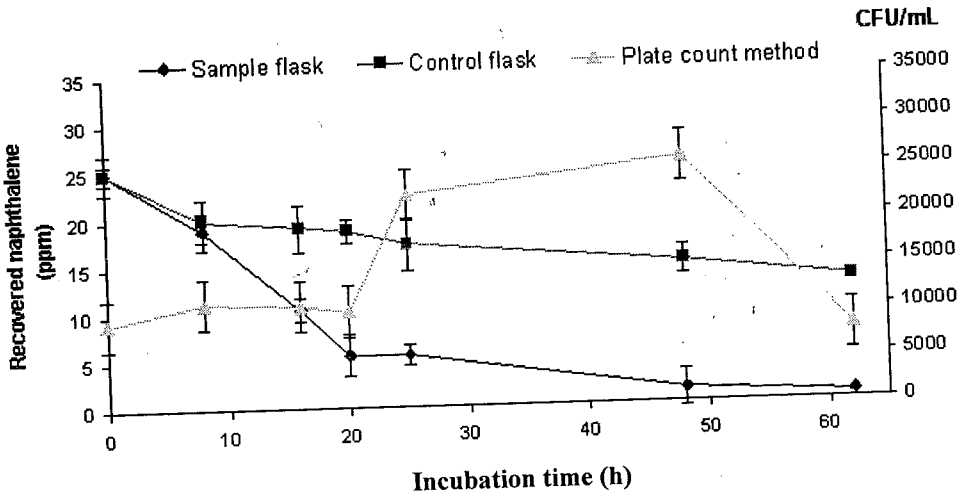

Figure 6 Removal of naphthalene and growth of *Acinetobacter* sp. in BSM media containing naphthalene at 30 ppm initial concentration (Sample flask). The control flask contains BSM media and naphthalene.

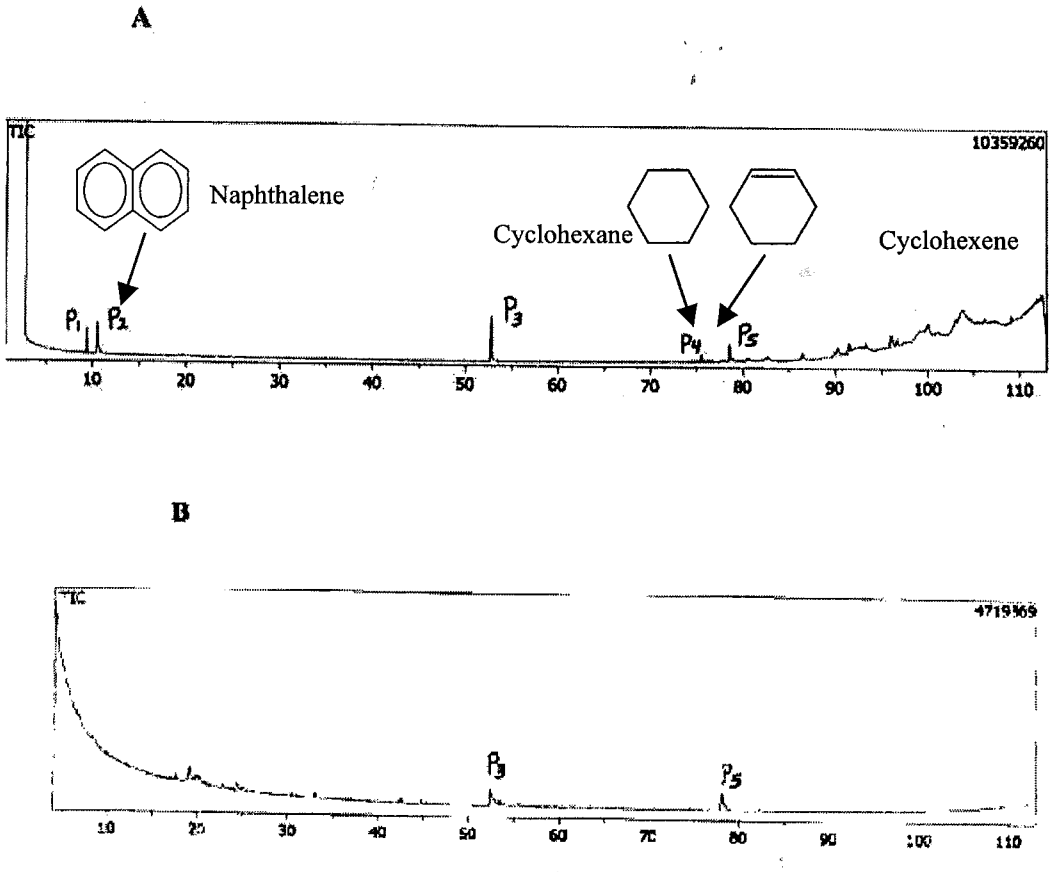

Figure 7 GC-MS chromatograms for BSM media + naphthalene + bacteria (A) and BSM media + bacteria (B). Note that peaks P3 and P5 are present in both chromatograms. Peak P1 represents the extraction solvent. Peak P4 may be the intermediate of naphthalene biodegradation.

CONCLUSION

The result of this study showed that natural bacteria isolated from petroleum contaminated soil, were capable of utilizing naphthalene as sole carbon and energy source. The three bacteria used in this study differed in their ability to utilised naphthalene in BSM media. Among the three, *Acinetobacter* sp. was found to utilize approximately 68 % of the naphthalene present in the media within 62 hours of incubation time. We found that the *Acinetobacter* sp. harbours large plasmids which may carry degradative genes responsible for its ability to degrade naphthalene (Merican, unpublished). Characterization of the plasmids will be published elsewhere.

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