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Evaluation on the Efficacy of Formulated *Pseudomonas* Cells in Degrading the Aromatic Hydrocarbon Toluene

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Abstract: In this study, the hydrocarbon-degrading bacterium *Pseudomonas* spp. isolate UTAR EPA2 was formulated with various combinations of formulative materials, comprising of clay-based carrier materials (bentonite (B) and kaolin (K)), enrichment materials (non-fat skimmed milk (N) and sucrose (S)), and a UV-protectant agent (para-aminobenzoic acid (P)). The efficacy of the formulated cells in degrading the aromatic hydrocarbon toluene (methylbenzene) and their subsequent cell viability after exposure to sunlight (natural UV irradiation) were investigated. Results indicated that degradation efficacy was slightly higher for kaolin-based formulated cells compared to bentonite-based formulations, especially after exposure to sunlight, although their percentages of degradation were not statistically different. Nevertheless, kaolin-based formulations have very low viable cell count especially in formulations with P (KP, KNP, KSP, KNSP). This suggested that aside from viable cells, the physical properties of the clays could have also contributed to the degradation of toluene. For storage purposes and applications in the field, we suggest that the bacterium is formulated with bentonite-based formulations especially using bentonite (B) clay singly, as relatively high percentage of toluene degradation and viable cell count was achieved with this formulation.

Key words: Cell viability, Formulation, Hydrocarbon-degrading bacterium, Toluene degradation, UV radiation

INTRODUCTION

Bioremediation using beneficial microbes to decompose or degrade toxic pollutants into less harmful compounds that do not affect the environment (Vidali, 2001), is one of the many sectors which benefits from the applications of microbial formulation. In this study, we attempt to study the possible degradation activity of a bacterium for the purpose of hydrocarbon bioremediation, specifically aromatic hydrocarbon. Aromatic hydrocarbons are important components of petroleum and its refined products. Among the most important aromatic petroleum hydrocarbons are benzene, toluene (methylbenzene), ethylbenzene, xylenes (dimethylbenzenes), and the polycyclic aromatic hydrocarbons (Chang, 2005). Aromatic hydrocarbons can support the growth of bacteria when they are present as the sole source of carbon and energy. Although aromatic hydrocarbons are not as readily biodegradable as are normal and branched alkanes, they are more easily degradable than the alicyclic hydrocarbons (Perry, 1984; Leahy and Colwell, 1990). In this study, the hydrocarbon toluene ($C_6H_5CH_3$) was used as the aromatic hydrocarbon substrate.

Formulation protects cells against harsh chemical and environmental conditions (Gentry, *et al*., 2004), and since most bioformulations are meant for field application, it is essential that suitable formulative materials are used to maintain cell viability under adverse environmental conditions (Brar, *et al*., 2006). The main compositions of a bioformulation include the carrier, enrichment, additive and the active materials (viable cells). These formulative materials applied singly or in combinations, help protect the active cells (Schisler, *et al*., 2004). In our study, bentonite and kaolin clays were selected as the carrier materials; non-fat skimmed milk (NFSM) and sucrose as enrichment materials; and para-aminobenzoic acid (PABA) as a UV protectant agent. Various combinations of the two clays with and without the incorporation of enrichment and additive materials were tested to develop the most compatible formulation, which may enhance the degradation process and sustain the cell viability of the hydrocarbon-degrading bacterium (UTAR EPA 2).

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MATERIALS AND METHODS

Isolate Preparation:

The bacterial isolate UTAR EPA2 was identified as *Pseudomonas aeruginosa* based on their sequence alignment when sequenced with specific primers Paer16SH (5'-AGG GCA GTA AGT TAA TAC CTT GCT G -3') and Paer16SIR (CCA CCT CTA CCG TAC TCT AGC TCA G). The bacterium was first cultured on Luria-Bertani (LB) agar (Pronadisa) and incubated at room temperature (27±2˚C) for 2 days prior to use. Two day-old cultures were used for all formulation exercises in this study.

Formulation of Pseudomonas Cells:

The amount of clay, NFSM, sucrose, PABA and inoculum suspension for each of the formulation type was first optimized so that the formulation obtained is in granular form. From this, seven combinations of formulations were prepared for each clay type. The bentonite-based formulation comprised of 180 g bentonite clay mixed with 180 ml of inoculum suspension (15 $\log_{10}$ c.f.u. ml^{-1}, absorbance value of 0.200 at 600 nm). For bentonite-based formulations requiring a combination of enrichment materials such as NFSM (BN) and sucrose (BS), 15 g of NFSM and sucrose was incorporated, respectively. PABA was added into the bentonite-based formulations; bentonite (BP), bentonite with NFSM (BNP) and bentonite with sucrose (BSP), at a rate of 1.5 g for every 180 g of bentonite clay. The formulation containing all enrichment and additive materials tested (BNSP) was also prepared using similar compositions. No other additives were incorporated alongside the inoculum for formulation containing only bentonite clay (B). Kaolin-based formulations were achieved using 240 g kaolin clay mixed with 240 ml of inoculum suspension (15 $\log_{10}$ c.f.u. ml^{-1}, absorbance value of 0.200 at 600 nm). Formulations requiring NFSM (KN), sucrose (KS) and PABA (KP, KNP and KSP) were incorporated with 10 g of NFSM and sucrose, and 1.0 g of PABA, respectively. The formulation comprising NFSM and PABA (KNSP) was prepared using similar compositions, while the formulation comprising of just kaolin clay (K) was not incorporated with any other additives. In addition, bacterial free-cell suspension (FC) and formulations with just bentonite clay (BC) and kaolin clay (KC) without the bacterial cells, were also prepared as controls. Free-cell suspension was prepared as 180 ml broth suspension (15 $\log_{10}$ c.f.u. ml^{-1}, absorbance value of 0.200 at 600 nm). BC and KC was prepared by mixing 180 g bentonite clay (or kaolin clay) with 180 ml of sterile distilled water. All the granular formulations were then dried in an oven (Memmert) at 30±2˚C for 3 days, grind, sieved using a 1.0 mm sieve (Cole-Parmer) and stored in 500 ml glass Schott bottles at room temperature (27±2˚C) for subsequent experiments.

Efficacy Assessment:

Test tubes containing 20 ml of sterile deionized water supplemented with 1.0 ml of toluene were first prepared. For testings using bentonite-, kaolin-based formulations and free-cell suspension, 1.0 g, 2.0 g and 0.5 ml of the respective formulations (suspension) were added accordingly to the tubes. For sunlight (natural UV irradiation) treatment, formulations were placed into petri dish, exposed to sunlight for 6 h and then added into the test tubes. The amount of toluene degraded was quantified using the gas chromatography-mass spectrometer (GC-MS). Toluene was first extracted using acetone (Merck) and then by hexane (Merck), vortexed for 30 sec and 1 min, respectively, followed by 5 min of centrifugation (Sigma 3K30 Sartorius) at 9000 rpm and 4^0C. The top clear layer was collected for analysis.

The GC-MS analysis was conducted using the GCMS-QP2010 Plus Gas Chromatograph/Mass Spectrometer from Shimadzu, using the BPX5 chromatographic column with an internal diameter of 0.25 mm and length of 30 m, and helium gas as the carrier gas (flow rate of 1.38 ml min^{-1}) and temperatures preset at 60^0C for 5 min with a final temperature of 220^0C at the rate of 6^0C min^{-1}. The temperature of the induction port was preset at 240^0C while the temperature of the FID was pre-set at 315^0C with a split ratio of 1:80. The volume of toluene residue detected in the sample was determined based on a standard curve constructed using similar extraction and analysis conditions. The percentage of toluene degradation [(Vo–Vr)/Vo] x 100%, where Vo is the original volume of toluene added to the sample and Vr is the toluene residue detected in the sample after treatment. The assessment on degradation efficacy was conducted after a month of storage on a monthly interval for the next 6 months. The procedure was also repeated for all bioformulations exposed to 6 h of sunlight to determine the efficacy of formulated cells in degrading toluene after sunlight exposure. Hydrocarbon degradation for formulations exposed to sunlight was conducted at a monthly interval for 3 months.

Viability Assessment:

The viability of formulated *Pseudomonas* cells was assessed and compared to free-cell suspension (control, FC) to determine the effect of formulative materials. Viability assessment was performed every month for 6 months. During each assessment, 1.0 g of bentonite-based formulation was mixed with 15 ml of sterile distilled water and serially diluted until 10^4 dilution times. From each dilution factor, 0.05 ml aliquot was plated onto LB agar plates, incubated at room temperature (27 ± 2^0C) for 2 days and the number of colonies enumerated. This procedure was repeated by substituting bentonite formulations with 2.0 g of kaolin-based formulations and 0.50 ml of free-cell suspension. Formulations exposed to sunlight for 6 hours were enumerated in a similar manner.

Experimental Design and Analysis:

The experiments were conducted in a complete randomized design, with duplicates for each efficacy and viability assessments. Results obtained were analyzed with the SAS Program (Statistical Analysis System). The mean comparison for formulations exposed and non-exposed to sunlight was compared with Tukey's Studentized Range Test ($HSD_{0.05}$). The comparison of the effect of sunlight exposure for each formulation was compared with T-test ($P \geq 0.05$).

RESULTS AND DISCUSSION

Efficacy of Formulated* Pseudomonas *Cells in Degrading Toluene:

The degradation efficacies of formulated *Pseudomonas* cells in bentonite- and kaolin-based formulations were not significantly different from *Pseudomonas* free-cell suspension (FC), with mean degradation efficacy of 87.86%, 93.50%, and 93.26%, respectively (Figure 1), irrespective of the sunlight factor. This showed that generally, the presence of formulative materials such as clays, NFSM, sucrose and PABA, did not suppress the degradation of toluene, nor specifically enhanced the degradation activity when compared to free-cell suspension. Higher mean percentages of degradation were achieved by all formulation types exposed to sunlight, with a slightly higher percentage recorded by free-cell suspension, followed by kaolin- and bentonite-based formulations with 98.18%, 96.78% and 90.42%, respectively (Figure 1). The higher degradation activity observed in kaolin-based formulations were attributed to the physical nature of kaolin. Kaolin have been used as a catalyst in oil and gas refining process to aid in the catalytic cracking and breaking of heavier hydrocarbon molecules into lighter products (Rong and Xiao, 2002; Adamis, *et al*, 2005). It was also observed that formulations with NFSM and sucrose, applied singly (BS, BN, KN, KS) or in combinations (BSP, BNSP, KSP, KNSP), generally have a higher degradation activity compared to formulations without these enrichment materials (B, K) although the degradation efficacy is not significantly different.

Viability of* Pseudomonas *Cells in Various Formulations:

The highest viable cell recovery was from bentonite-based formulations, followed by kaolin-based formulations and free-cell suspension with cumulative mean viable colony count of 4.98 $\log_{10}$CFU ml^{-1}, 4.84 $\log_{10}$CFU ml^{-1} and 2.86 $\log_{10}$CFU ml^{-1}, respectively (Figure 2). This indicated that both the clay-based carrier materials due to their large surface areas for nutrient absorption and protection (Lunsdorf, *et al*., 2000) may have conferred some form of protection to the cells especially after exposure to sunlight. The bentonite clay was more suitable than kaolin in maintaining cell viability in both conditions as even with the presence of sunlight, the viable cell recovery was not significantly different from formulations without sunlight exposure (Table 1). This is attributed to bentonite having higher cation exchange capacity (Beveridge, 1988) and greater adsorption efficiency (Adamis, *et al*., 2005) compared to kaolin. This indicated the bentonite is useful to formulate cells for storage and field application purposes as the cell viability is not compromised. Contrary, kaolin is less suitable as in certain formulations, namely K, KN, KS and KNSP, cells were not protected from sunlight observed from the significantly lower cell viabilities after sunlight exposure (Table 1).

The incorporation of enrichment materials enhanced the cell viability by providing carbon source to support cell growth (Lee and Chang, 1993). Nevertheless, our results showed that the presence of NFSM and sucrose in bentonite- and kaolin-based formulations were not exceptional in enhancing cell viability, in both conditions with or without exposure to sunlight. In fact, their viable cell recovery was similar to viable cell count from formulations of bentonite (B) and kaolin (K) singly (Figure 2). In addition, the additive PABA (P) was observed to have a possible detrimental effect on the *Pseudomonas* cells, presumably due to its antimicrobial nature (Richards, *et al*., 1994). Formulations with PABA such as BP, BNP, BSP, BNSP, KP, KNP, KSP and KNSP, produced relatively low number of viable cells among all formulations (Figure 2).

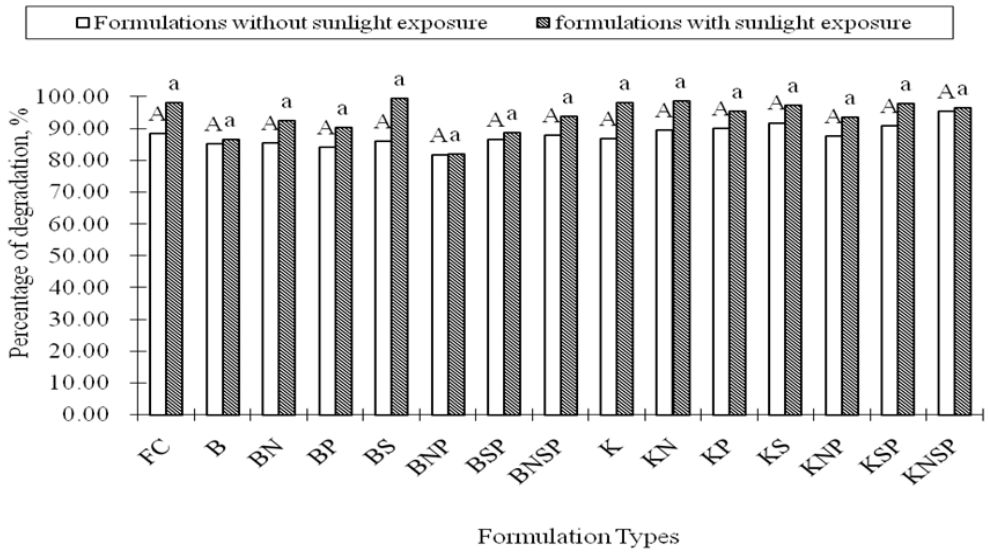

Fig. 1: Percentage of toluene degradation by *Pseudomonas* (UTAR EPA2) cells in various formulations with and without exposure to 6 h of sunlight. Means with the same letters and captions are not significantly different ($HSD_{(0.05)}$).

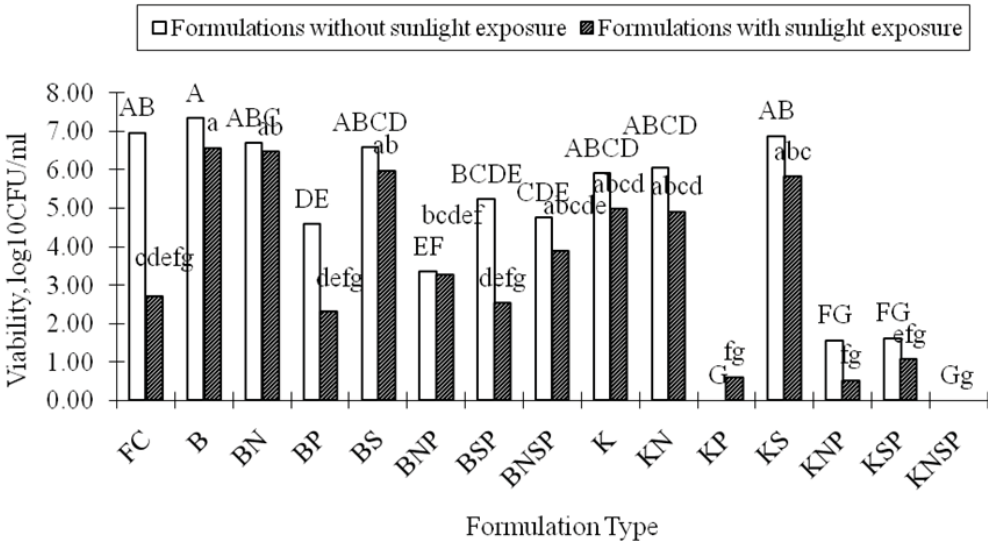

Fig. 2: Mean viable cell count of *Pseudomonas* (UTAR EPA2) cells in various formulations with and without exposure to 6 h of sunlight. Means with the same letters and same captions are not significantly different ($HSD_{(0.05)}$).

When compared with their association to toluene degradation efficacy, we found that high viability does not necessary lead to subsequent effective degradation efficacy. Viability for bentonite-based formulations was the highest, but their degradation efficacy was lower than kaolin-based formulations and free-cell suspension. In our study, we observed that kaolin-based formulations, which have significantly lower viable cell count (2.86 $\log_{10}$CFU ml^{-1}) compared to bentonite-based formulations (4.98 $\log_{10}$CFU ml^{-1}), have shown relatively higher degradation activity, especially after sunlight exposure with 96.78% compared to 90.42%, respectively. Even free-cell suspension with only 2.70 $\log_{10}$CFU ml^{-1}, after sunlight exposure, was able to achieve 98.18% degradation activity. Similarly, formulations without sunlight exposure produced a similar trend, where kaolin-

Table 1: Results of T-test comparison on the viability of formulations exposed and non-exposed to sunlight within a single formulation

T-test	t Value	Pr > |t|
FC	3.83	0.01
B	0.37	0.08
BN	0.68	0.52
BP	2.48	0.05
BS	1.99	0.09
BNP	0.80	0.45
BSP	2.39	0.05
BNSP	1.10	0.31
K	2.85	0.03
KN	3.26	0.02
KP	-1.00	0.36
KS	3.66	0.01
KNP	0.97	0.37
KSP	0.55	0.60
KNSP	0.00	0.00

Note: FC: free-cell suspension, B: bentonite clay, K: kaolin clay; N: non-fat skim milk, S: sucrose and P: PABA.

based formulations with only 3.15 $\log_{10}$CFU ml^{-1} recorded 90.22% degradation activity compared to bentonite-based formulations with 85.29% degradation activity from 5.51 $\log_{10}$CFU ml^{-1}. In both cases, free-cell suspension (FC) has good degradation activity with 88.34% and 98.18%, although the number of viable cells was significantly reduced. In addition, no viable cells were recovered from KNSP, yet the degradation efficacy was relatively high. This observation again reaffirmed that that the physical aspects of the formulative materials may have an influence on the degradation activity as it is associated with the surface contact areas which influence degradation activity. As at this moment, the binding and adsorption of clay-based materials with toluene and their role in toluene bioremediation is not well understood, hence we propose further investigations to establish this. Based on the results from efficacy and viability assessments, bentonite clay applied singly (B) and kaolin-based formulation with sucrose (KS) were the two formulations with the most potential for further development.

To conclude, formulated *Pseudomonas* UTAR EPA2 cells can degrade toluene effectively in conditions with and without exposure to 6 h sunlight. Bioformulation of the cells are highly recommended as cells can be protected during storage and when applied to the environment. Among the three enrichment and additive materials tested, we discovered that enrichment materials were effective in enhancing cell viability especially sucrose and NFSM, but were not exceptionally beneficial in the degradation process. Contrary, the additive PABA was observed to have no beneficial role in the bioformulation as it negatively impacted the cell viability and degradation efficacy, despite its UV protectant claims. Formulation using bentonite clay (B) singly was the most suitable formulation as highest viability and good efficacy results were obtained, even after exposure to sunlight. We also suggest more studies be done to look into the physico-chemicals aspects of the formulations and their influence on toluene degradation.

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