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# Benzo (A) Pyrene Detoxification Potential of *Bacillus subtilis* BMT4i (MTCC 9447) Isolated From Srinagar, Garhwal, Uttarakhand, India

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**Abstracts:** Benzo(a)Pyrene (BaP), a pentacyclic high molecular weight polyaromatic hydrocarbon (HMWPAH) is a priority pollutant of extreme environmental concern due to its potent carcinogenic properties. *Bacillus subtilis* BMT4i (MTCC 9447) a well known BaP degrader may play a major role in the bioremediation of contaminated soils. Using multiple pathways, BMT4i efficiently degrades BaP into several metabolites, whose mutagenicity remains unknown. Present investigation aimed to analyze the BaP detoxification potential of BMT4i by evaluating the mutagenicity of BaP metabolites using the Ames test. For that, TA98 strain of *Salmonella typhimurium* (*his*D3052 mutant) was incubated with ethyl-acetate extracts of 7, 15 and 40 days grown cultures of BaP-BMT4i with or without S9 activation in the medium devoid of Histidine. The number of yellow revertant colonies representing mutation was counted and % detoxification potential was evaluated. The results showed that in the absence of S9 activation, a very small comparable number of revertant TA98 colonies were observed in negative control and test samples ($p>0.05$). After S9 activation a large number of revertant TA98 colonies (43±/48) appeared in negative control (BaP standard) which significantly reduced ($p< 0.001$) after 7 (23±3/48), 15 (12.4±2.7/48) and 40 days (8.3+1.9/48) demonstrating decrease in the mutagenicity of BaP by BMT4i. The percent BaP detoxification after 7 days was 45% which further increased to 70 and 80% after 15 and 40 days. The commendable in-vitro BaP detoxification by *Bacillus subtilis* BMT4i needs to be further tested through field trials in order to establish it as an efficient bio-weapon in bioremediation of HMW-PAHs contaminated sites.

**Keywords**: Ames test • *Bacillus subtilis* BMT4i • Benzo(a)Pyrene (BaP) degradation • BaP detoxification • mutagenicity

## Introduction

Benzo(a)Pyrene (BaP), a five ring high molecular weight polyaromatic hydrocarbon (HMWPAH) is a potent carcinogen with high recalcitrant nature. It is released into the environment primarily by anthropogenic activities including incomplete combustion of fossil fuel, coke oven emissions, coal combustion, conversion industries, aluminum smelters, vehicle exhausts, cigarette, cigar and marijuana smoking (Kanaly and Harayama 2010). The natural sources of BaP include forest fires, peat fires, volcanoes, burning of crude oil and shale oil (Juhasz and Naidu 2000). From contaminated soil and water, BaP enters into the food chain and is exposed to humans. In addition, ingestion of foods cooked over an open flame or smoked (Saito et al. 1978) and use of cosmetics which contain mineral oil (Grob et al. 1991) resulted in BaP exposure. In the liver, BaP is metabolized into its genotoxic form, which then interacts with nucleic acids and proteins, creating highly reactive macromolecular adducts. This leads to BaP-induced toxicity, mutagenesis, and carcinogenesis (IARC 2010, EPA 2017). Due to its abundance in the

environment and its potent mutagenic and carcinogenic effects in mammals, BaP is recently upgraded by International Agency for Research on Cancer (IARC) as a group 1 (Carcinogenic to humans) from its previous status as B2 (probable human carcinogen) compound (IARC 2018).

The removal of BaP from contaminated soil and water during remediation is essential in order to meet current "clean up" standards. Microbial degradation of BaP may play a major role in the decontamination of sediment and surface soils (Sims and Overcash 1983, Habe and Omori 2003). For aforementioned reasons, BaP degradation has been extensively studied in bacteria and fungi. Several metabolites have been identified which suggest execution of multiple degradation pathways in *Sphingomonas paucimobilis* EPA505, *Stenotrophomonas maltophilia* strain VUN10,003, *M. vanbaalenii* PYR-1, *Sphingomonas yanoikuyae* JAR02 and *Bacillus subtilis* BMT4i (Ye et al. 1996, Juhasz et al. 2000, Moody et al. 2004, Rentz et al. 2008, Lily et al. 2009, Lily et al. 2010, Bhatt et al. 2018). Regardless of the better understanding of BaP degradation pathways little is known about the toxicity and mutagenicity of the BaP metabolites which is essential requisite for better bioremediation strategies. Limited studies on cometabolic BaP degradation in *Sphingomonas paucimobilis* EPA505, *Stenotrophomonas maltophilia* strain VUN10, 003, *M. vanbaalenii* PYR-1 and *Sphingomonas yanoikuyae* JAR02 have shown reduction in the BaP mutagenicity after biodegradation reactions suggesting detoxification of BaP into less harmful metabolites (Ye et al. 1996, Juhasz et al. 2000, Moody et al. 2004, Rentz et al. 2008).

Extensive studies on BaP degradation in our laboratory have established *Bacillus subtilis* BMT4i (MTTC9747) to be an efficient degrader of BaP (Lily et al. 2009, Lily et al. 2010, Bhatt et al. 2018). BMT4i was able to degrade more than 80% of BaP in 28 days via chromosomally encoded pathways (Lily et al. 2009, Lily et al. 2010). In a recent study, using BaP as a sole source of carbon and energy, BMT4i degrades BaP into several metabolites by employing multiple pathways involving both dioxygenases and monooxygenases as initial attacking enzymes (Bhatt et al. 2018). The identified metabolites include benzo(a)pyrene-cis-7,8-dihydrodiol, benzo(a)pyrene-11,12-epoxide, 8-carboxy-7-hydroxy pyrene, 7,8,9,10-tetrahydrobenzo[pqr]tetraphene-7,8,9,10-tetraol, cis-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid, hydroxymethoxybenzo(a)pyrene, dimethoxybenzo(a)pyrene and chrysene-4 or 5-carboxylic acid. Among these metabolites, the ring cleavage products of BaP include cis-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid, chrysene-4 or 5-carboxylic acid and 8-carboxy-7-hydroxy pyrene. While their specific identities are known, the mutagenic potential of these metabolites remains uncertain. It is crucial to understand the BaP detoxification capabilities of BMT4i to assess its suitability as a candidate for remediating sites contaminated by automobile emissions. Therefore, this study aims to investigate the BaP detoxification potential of BMT4i by evaluating the mutagenic properties of its metabolites using the Ames test.

## Material and Methods

Analytical grade BaP (99.9% pure) was procured from Sigma-Aldrich Pvt. Ltd., USA. Solvents of HPLC-grade namely dimethylformamide, acetonitrile, methanol and formic acid were obtained from Merck Pvt. Ltd., Mumbai, India. Culture media including Nutrient Agar media and broth were purchased from Himedia, Mumbai, India. Basalt mineral media (BSM) constituents and solvents of analytical grade were sourced from GlaxoSmithKline Pvt. Ltd., Mumbai, and Merck Life Science Pvt. Ltd., Bengaluru, India.

**Preparation of BMT4i inoculum :** A single colony of BMT4i, grown on a BSM-BaP agar plate, was transferred into 10 ml of nutrient broth and incubated at 37°C with constant agitation until reaching an A600 of approximately 1.0 (approximately $1x10^8$ cells/mL). The cell culture was then centrifuged at 8000 rpm for 10 minutes and washed three times with BSM to remove residual nutrient broth. The BMT4i cell density was subsequently adjusted to $10^8$ cells/mL with BSM.

**Isolation of BaP metabolites :** All procedures were conducted under subdued yellow light to prevent the degradation of BaP by light exposure. The experiments were set up in triplicate, with each experiment consisting of a 50 mL BSM culture of *Bacillus subtilis* BMT4i (at $10^7$ cells/mL) supplemented with BaP (50 µg/mL) in amber bottles. These cultures were then incubated at 30°C with agitation at 120 rpm for 40 days. Additionally, a negative control was prepared using 50 mL of BSM containing 50 µg/mL BaP without the addition of BMT4i. At various time intervals (7, 15 and 40 days), 10 mL culture broth (from both sample and control) was withdrawn and processed for the recovery of the products by ethyl acetate extraction (Bhatt et al. 2018). For that, 10 mL ethyl acetate was added to all the cultures, vigorously vortexed and centrifuged for 5 min at 10,000 rpm. The upper ethyl acetate layers containing BaP metabolites were transferred in the fresh tubes. This step was repeated one more time and then 1 mL 0.1 N HCl was added to the aqueous layer to acidify it up to pH 4.0 to enhance the recovery of acidic metabolites and then ethyl acetate extraction was repeated twice. All the four ethyl acetate extracts for one culture were pooled and dried. The extracts from all three replicates for each time period were individually dried using a rotary evaporator (Perfit Pvt Ltd, Ambala, Punjab, India, Model No. 951) and subsequently reconstituted in 1.0 ml of dimethyl sulphoxide (DMSO). The concentration of BaP (obtained after incubation at different time intervals) was determined using the standard curve of BaP prepared by taking absorbance of BaP solutions (10-100 µg/mL BaP in DMSO) against concentration at 387 nm (Giger and Blumer 1974).

Detoxification potential of *Bacillus subtilis* BMT4i was checked using the protocol developed by Maron and Ames (1983). The BaP metabolite extracts were checked for mutagenicity involving bacterial strain namely TA98 of *Salmonella typhimurium.*

**Ames II™ Mutagenicity Assay (Maron & Ames 1983):** Ames test was performed using BioReliance Ames II™ mutagenicity kit supplied by Moltox Molecular Toxicology, Inc. Boone, North Carolina, USA. The test involved revival of mutant test strain (TA98) of *Salmonella typhimurium.* For that, the lyophilized disc of *Salmonella typhimurium* test strain TA98 was inoculated separately in the 10 mL growth medium (supplied along with kit) and incubated for 12 h in a shaker incubator at 37°C at 250 rpm.

The TA98 cell density was checked using UV-Vis Spectrophotometer (Systronics, India, model no. 119) by measuring absorbance at 600 nm. When absorbance reached to the $A_{600} \geq 2.0$, the culture was ready to be used for the Ames test.

For dosing, 24 well plates were labeled with culture type, test article name, and with or without S9 activation. S9 was the metabolic activation system which consisted of a microsomal fraction of rat liver homogenates having the ability to convert indirect mutagens to mutagens. Firstly, the mutagen exposure reaction mixture was prepared for the TA98 culture without S9 activation. 800 µL of overnight grown TA98 culture was added to 7.2 mL of exposure medium in a 10 ml test tube. Immediately, after mixing, 240 µL of TA98 culture-exposure medium was transferred into

each labeled well of 24 well plate. 10 µL each of negative control (DMSO solvent) and test samples (BaP standard and BMT4i-BaP extracts from 7 days, 15 days and 40 days) and 100 µL of positive control 2-nitrofluorene (2NF; 300 µg/ mL ) solution were added into the respective labeled wells. Then, plates were placed in an incubator shaker and incubated for 90 min at 37°C while shaking at 250 rpm. Same procedure was repeated with the S9 activation system. In a tube marked +S9 activation, 6.0 mL of exposure medium, 800 µL of TA98 culture and 1.2 mL of cold 30% S9 liver extracts were added sequentially and mixed thoroughly. Immediately afterwards, 240 µL of TA98 culture-S9-exposure medium was transferred into each of the labeled wells. After that, 10 µL each of negative control (DMSO solvent) and test samples (BaP standard and BMT4i-BaP extracts from 7 days, 15 days and 40 days) and 100 µL of positive control 2-aminoanthracene solution (100 µg/ mL), the indirect mutagen were added into the respective wells. Later on, the well plates were placed in an incubator shaker and incubated for 90 min at 37°C while shaking at 250 rpm.

After incubation, 2.8 mL of the indicator medium was added into each of the wells and mixed thoroughly by gentle pipetting up and down. 50 µL aliquots of the solution from each well of the 24-well plate were dispensed into 48 wells of the 98-well plate. Afterwards, the plates were covered carefully and placed into sealable plastic bags to prevent the evaporation. With and without S9 plates were placed into separate plastic bags and incubated at 37°C for 2 days. Later, the bags were removed from the incubator and the yellow colored positive wells (as the indicator of reversion mutants) and purple colored negative wells in each 48-well section were counted in front of the light source. % BaP detoxification by BMT4i was determined using following formula

$$\% \text{ BaP detoxification} = \frac{\text{Positive wells in negative control} - \text{Positive controls in test samples}}{\text{Positive wells in negative control}} \times 100$$

The % BaP detoxification was plotted against BaP-BMT4i extracts.

**Statistical analysis**

To rule out any discrepancies, all the experiments were performed in triplicates and each extract was analyzed at least three times for the Ames test. The results were expressed as the mean of three independent experiments (n = 3) with calculation of standard error (SE). The statistical analysis of the data was performed using Microsoft Excel and Prism 3 pad software (Microsoft, Redmond, WA, USA).

**Results and Discussion**

**Extraction and quantitation of BaP metabolites:** In order to analyze the BaP detoxification potentials of BMT4i, the residual BaP and metabolites resulted from degradation of BaP by BMT4i were extracted using ethyl acetate from the 7, 15, and 40 days test samples of BaP-BMT4i. The BaP was also extracted from the negative control (BaP without BMT4i) and test samples of 7, 15, and 40 days incubations. The concentrations of BaP (obtained after incubation at different time intervals) were determined using BaP as standard (10-100 µg/mL BaP in DMSO) (Giger and Blumer 1974) presented in Table 1. The observations evidently revealed significant loss of BaP in 7 (68%), 15 (78%) and 40 days (89%) BaP-BMT4i test samples as compared to that in respective negative controls. The observations of BaP degradation were consistent with earlier reports by Lily et al. (2009, 2010). Though residual BaP concentrations after degradation were significantly reduced in 7, 15 and 40 days BaP-BMT4i extracts, however the extracts contained several BaP metabolites as shown in the previous study by Bhatt et al. (2018) in our laboratory. The study identified a total eight compounds along with several unidentified

metabolites (Bhatt et al. 2018). The quantitation of individual metabolites was not feasible as their standard compounds are not commercially available and their absorbance maxima are not known.

**Table 1.** Concentration of BaP and BaP Metabolites in BMT4i-BaP extracts

| Time | BMT4i-BaP samples | Concentration (µg/mL) |
|---|---|---|
| 7 days | - BMT4i | 450 ± 11.9 |
| | + BMT4i | 140 ± 12.4 |
| 15 days | - BMT4i | 449 ± 15.9 |
| | + BMT4i | 101.28 ± 19.9 |
| 40 days | - BMT4i | 439 ± 22.9 |
| | + BMT4i | 47 ± 16.9 |

**BaP Detoxification Potential of BMT4i by Ames II™ Mutagenicity Assay:** In Ames II™ mutagenicity assay, frequency of induction of frameshift mutation in *his*D3052 mutant strain TA98 of *Salmonella typhimurium* by BMT4i-BaP extracts was estimated in triplicates. The assay is based on the facts that the *his*D3052 mutation in TA98 strain of *Salmonella typhimurium* makes it unable to grow in the *His*$^-$ medium and blue color of indicator medium remained unchanged. If the test compound is mutagenic then *his*D3052 mutation in TA98 is reverted back into wild types and cells will be able to grow in *His*$^-$ media that turns the blue color of the indicator medium to yellow (positive observation). If the color of the indicator medium remained blue it represented negative observation and the compound is considered as non mutagenic.

To assess the BaP detoxification potential of BMT4i, the biodegradation extracts of 7, 15 and 40 days which include residual undegraded BaP and metabolites were incubated with TA98 strain of *Salmonella typhimurium* with and without S9 activation extracts in the presence of indicator medium. After completion of incubation, the number of positive wells of revertant colonies with yellow color were counted (Fig. 2 and 3) and graphically presented (Fig. 3). The % BaP detoxification by BMT4i was determined as shown in Fig. 5.

The observations showed that in the absence of S9 activation, the majority of the TA98 cell colonies after exposure to BaP standard, BaP-BMT4i extracts of 7, 15 and 40 days remained blue (Fig.1 and 3). Only a very small fraction of the TA98 cell colonies were revertant turning yellow (2-3 out of 48) (Fig.1) which is comparable in BaP and test samples ($p>0.05$). This clearly demonstrated that BaP standard as well as BaP-BMT4i extracts (7, 15 and 40 day) does not cause frameshift mutation in TA98 strain indicating their non-mutagenic behavior in the absence of S9 activation (Fig.1 and 3). While in the positive control which are treated with a combination of direct acting mutagen (2-NF), all the wells turned yellow (positive) representing absolute mutagenicity (Fig.1 and 3).

In contrast to the above, a significant number of yellow TA98 colonies (43±2 out of 48) appeared after exposure to S9 activated BaP standard (Fig.2). The observation was consistent with the fact that BaP is a well known indirect acting mutagen (IARC 2018). S9 mediated metabolic activation of BaP causes production of mutagenic BaP metabolites which reverts *his*D3052 mutant of TA98 strain back into wild type enabling them to grow in His$^-$ medium and turning indicator from blue to yellow color.

In case of S9 activated BaP-BMT4i extracts, the number of yellow colored revertant TA98 colonies were found to be significantly reduced ($p< 0.001$) from 43/48 in BaP standard to 23±3/48, 12.4±2.7/48 and 8.3+1.9/48 in 7, 15 and 40 days of BaP-BMT4i incubation respectively (Fig.3)

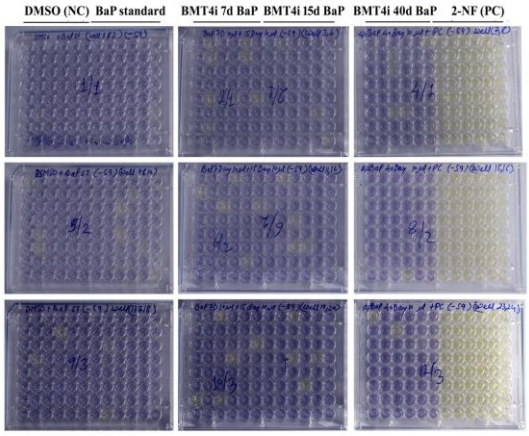


**Fig 1.** Ames test without S-9 Activation Extracts, PC-Positive Control and NC- Negative Control

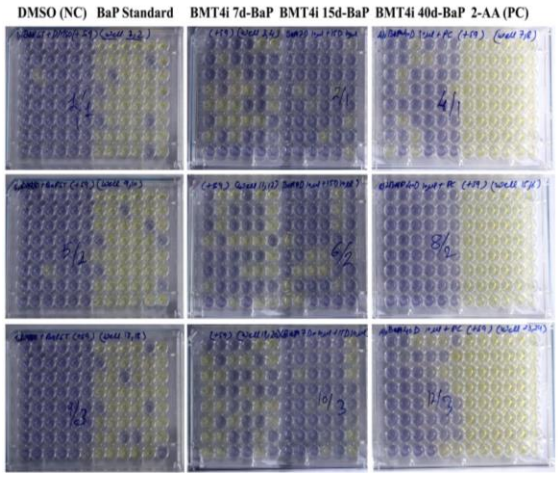


**Fig 2:** Ames test with S-9 activated extracts, PC-Positive Control and NC- Negative Control

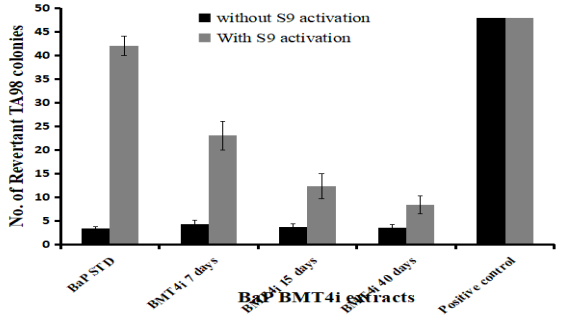


**Figure 3.** BaP-BMT4i extracts induced revertant TA98 colonies with or without S9 activation

indicating decrease in the mutagenicity of BaP after exposure to BMT4i. When mutagenicity (number of revertant colonies) was plotted against BaP concentrations in negative control, 7, 15, 40 day BaP-BMT4i extracts test samples a BaP dose response linear curve was obtained (Fig.4) which clearly demonstrated decrease in the number of revertant colonies as BaP concentration was decreased (Fig. 4).

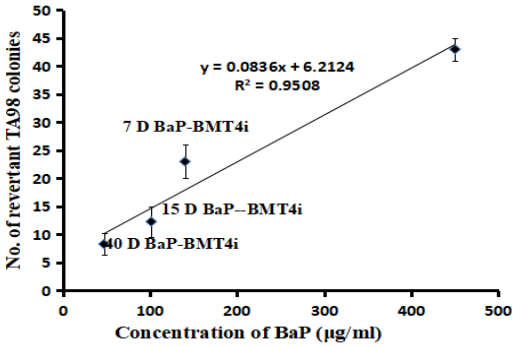


**Figure 4.** Correlation between no. of revertant TA98 colonies and BaP concentrations in negative control, 7, 15, 40 day BaP-BMT4i extracts test samples

Considering S9 activated BaP standard as hundred percent mutagenic and toxic, % BaP detoxification of BMT4i was calculated and plotted against BaP incubation periods (Fig.5). The graph showed that 45% of the total BaP was detoxified after 7 days of BaP-BMT4i incubation. The % detoxification was further increased up to 70 and 80% after 15 day and 40 days incubation (Fig.5).

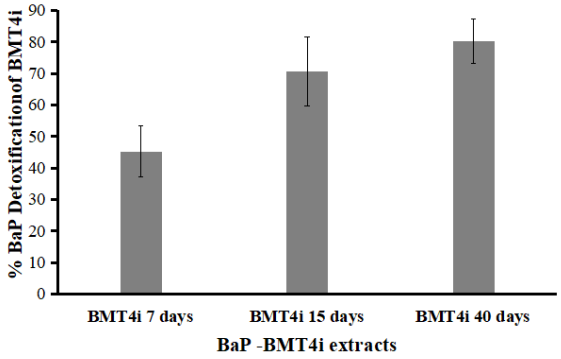


**Figure 5.** % BaP detoxification after 7, 15 and 40 days of BaP-BMT4i incubation

In spite of excellent BaP detoxification activity, a significant mutagenicity in decreasing order was also observed in 7, 15 and 40 days BMT4i-BaP extracts. This indicated two possibilities: first, the observed mutagenicity is due to presence of residual BaP as shown in Table 1. As a result of biodegradation, BaP concentration was in decreasing order in 7, 15 and 40 days BaP-BMT extracts which is highly correlated ($R^2$ = 0.951) with the decreasing order of mutagenicity in the same samples (Fig. 4). Second, it is also possible that some of the metabolites are indirect mutagens that might get activated by S9 and contribute to the total number of revertant yellow colonies. Metabolites identified in BMT4i-BaP extracts after 15 days included BaP-cis-7,8-dihydrodiol, 7,8,9,10-tetrahydrobenzo[pqr]tetraphene-7,8,9,10-tetraol, BaP-11,12-epoxide, 7-hydroxy-8-carbaldehyde pyrene, dimethoxy-BaP, hydroxymethoxy-BaP, as well as ring cleavage products like cis-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid and chrysene-4 or 5-carboxylic acid. Out of aforementioned metabolites, BaP-11, 12-epoxide has been reported to be a weak pulmonary carcinogenic activity in newborn mice (Wislocki et al. 1978) although; the same metabolite is reported to be non-tumorigenic in C3H/Cumf mice (Kouri et al. 1980). The BaP-cis-7, 8-dihydrodiol and chrysene- 4- or 5-carboxylic acid have been suggested to be nontumorigenic (Moody et al. 2004). Moreover, 10-oxabenzo[*def*]chrysen-9-one and 7-hydroxy-8-carbaldehyde pyrene cannot form a bay region diol epoxide and is therefore unlikely to be carcinogenic (Schneider et al. 1996, Moody et al. 2004). The mutagenicity of the rest of the metabolites identified in 7 and 15 days BMT4i-BaP extracts have not yet been analyzed by any of the previous studies.

Our observations are in consonance with the previous studies demonstrating detoxification potentials of few bacterial strains *Stenotrophomonas paucimobilis* EPA 505, *Stenotrophomonas maltophilia* strain VUN10,003, *Mycobacterium vanbaalenii* PYR-1, and *Sphingomonas yanoikuyae* JAR02 (Ye et al. 1996, Juhasz et al. 2000). Ye et al. (1996) indicated that biodegradation of BaP by *Sphingomonas paucimobilis* EPA 505 reduces the mutagenicity associated with the BaP. Mutagenicity of BaP biodegraded metabolites was tested in *Salmonella typhimurium* strain TA98 with and without metabolic activation. However, the reduction in mutagenicity has not been quantified. Juhasz et al. (2000) evaluated the ability of *Stenotrophomonas maltophilia* strainVUN 10,003 to degrade and detoxify high molecular weight polycyclic aromatic hydrocarbons (PAHs) like pyrene, fluoranthene, BaA, BaP, dibenz[a,h]anthracene and coronene in basal liquid medium. Mutagenicity assays (AmesTest) demonstrated a decrease in the mutagenic potential of dichloromethane culture extracts from all cultures containing single PAH over the incubation period, corresponding to the decrease in the concentration of the PAH. When liver microsome extract (S9) was present, a dose-dependent mutagenic effect was observed in *Salmonella typhimurium* strain TA100 at concentrations exceeding 25 µg of PAH per plate. However, at the highest concentrations, toxic effects were noted. Conversely, no dose-response was observed in the absence of mammalian microsomal activation. Mutagenicity assays performed on extracts from control cultures containing PAHs, inoculated with $HgCl_2$-killed cells, demonstrated little change in mutagenicity over the incubation period. However, inoculation of the PAH-containing medium with strainVUN 10,003 resulted in a smaller decrease in the number of revertant colonies in *S. maltophilia* strainVUN 10,003-BaP extracts (18%). The BaP detoxification potential of *S. maltophilia*

strainVUN 10,003 (18%) is much lesser than the BaP detoxification potential of *Bacillus subtilis* BMT4i (80 %) in the present study

To the best of our knowledge, the present study is the first one to systematically analyze the bacterial BaP detoxification potential. The commendable in-vitro BaP detoxification potential of *Bacillus subtilis* BMT4i needs to be further tested through field trials in BaP contaminated sites in order to establish it as an efficient bio-weapon in decontamination and bioremediation of HMW-PAHs contaminated sites.

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