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STRAIN IMPROVEMENT OF A POTENT BENZO-A-PYRENE (BAP) DEGRADER *Bacillus Subtilis* BMT4I (MTCC 9447)

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ABSTRACT

Benzo [a] pyrene (BaP), a pentacyclic polyaromatic hydrocarbon, is 1 of the 12 target compounds defined in the new US Environmental Protection Agency's strategy for controlling persistent, bioaccumulative, and toxic pollutants. We previously isolated a novel strain *Bacillus subtilis* BMT4i (MTCC) capable of utilizing BaP as sole source of carbon and energy and degrading BaP via an inducible chromosomally encoded pathway. The present study was done to improve the BaP degradation ability of *B. subtilis* BMT4i by means of inducing random mutations through treating BMT4i with physical mutagen (UV irradiation) or chemical mutagens such as ethyl methane sulfonate (EMS), 5-bromo uracil (5-BU) and acridine orange (AO). The observation showed that a UV mutant BMT4imuv2, amongst various other mutants exhibited highest BaP degradation up to 62% which was significantly superior in comparison to the control wild type BMT4i that showed 46% BaP degradation. The BMT4imuv2 was further characterized by time course experiment which showed almost 100% BaP degradation on completion of 28 days in contrary to 84.66% by control wild type BMT4i confirming a substantial improvement of the BaP degradation potential of BMT4i after treatment with physical mutagen UV (254 nm). The growth scenario of the mutant was found to be somewhat different. BMT4imuv2 showed a steep increase in the log values reaching to 16.60 after 2 days attaining maxima of 45.47 $\log_{10}$ CFU/ml after 7 days. Further, increase in incubation period led to decline in the cell number reaching 0.00 after 28 days. In view of the above, it could be concluded that

BMT4imuv2 is an improved version of BMT4i having a superior capability of BaP degradation, hence it could serve as a leading biological weapon to remediate BaP contaminated sites.

Key words: *Bacillus subtilis* BMT4i (MTCC 9447), Benzo-a-pyrene (BaP), Bioremediation, CFU/ml, Mutagen.

[I] INTRODUCTION

Benzo [a] pyrene (BaP), a high molecular weight polycyclic aromatic hydrocarbon (HMW-PAH) containing five benzene rings, is commonly found as pollutant in the air, water, and soil [1]. Due to its multiple ring structure and low aqueous solubility, BaP is thermodynamically stable and recalcitrant to microbial degradation [2, 3, 4]. BaP has been shown to possess potent carcinogenic, genotoxic and cytotoxic properties [5, 6, 7, 8]. The removal of BaP from contaminated soil 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 [9, 10]. Till today, only a few bacterial species, including *Mycobacterium* sp. [11, 12, 13], *Sphingomonas paucimobilis* [14], *Strenotrophomonas maltophilia* [15, 16] and *Mycobacterium vanbaalenii* Pyr-1; [17], are capable of degrading BaP co-metabolically. However, no study has yet demonstrated utilization of BaP as the sole source of carbon and energy [4, 18, 19].

In this context, we have reported previously for the first time, utilization of BaP as a sole source of carbon and energy by a novel strain *Bacillus subtilis* BMT4i (MTCC 9447) which is able to degrade more than 80% after 28 days growth via an inducible chromosomally encoded pathway [20, 21]. As an extension of our previous study, the present work was performed to improve the BaP degradation ability of BMT4i by inducing random mutations with physical mutagen eg. ultra-violet irradiation (UV 254 nm) or chemical mutagens including alkylating agent EMS, base analogue 5-BU and DNA intercalating fluorescent cationic dye AO. The improved mutant BMT4i strain could serve as a potent biological weapon in the development of an effective bioremediation protocol for the removal HMW-PAH including BaP from contaminated soil.

[II] MATERIAL AND METHODS

2.1. Chemicals and reagents BaP (99.9%) was purchased from Supelco, (Bellefonte, PA). Tryptone, peptone, beef extracts, bacto-agar, yeast extract, dextrose, EMS, 5-BU and AO were obtained from HiMedia Laboratories (Mumbai, India). General chemicals, including constituents of basal salt mineral medium (BSM) and solvents of analytical grade were purchased from Glaxo (Mumbai, India) and Merck (Mumbai, India).

2.2. Improvement of the BaP degradation ability of the BMT4i

Improvement of the BaP degradation ability of BMT4i was attempted by inducing random mutations as a result of treating BMT4i with physical mutagen eg. ultra-violet irradiation (UV 254 nm) or chemical mutagens including EMS, 5-BU and AO.

2.2.1. Treatment with physical mutagen: UV irradiation

All the experiments were set up in triplicates. For improving the BaP degradability of BMT4i by physical mutagen, the BSM-Dextrose (1%; BSMD) plate containing distinct colonies of

BMT4i was exposed to UV irradiation (254 nm) at the distance of 5 cm in a UV irradiation chamber (Vikrant Equipment India) for 15 min. UV treated 10 bigger colonies were inoculated into 5 ml BSMD broth and incubated for 24 h at 37^{o}C with constant stirring. After the incubation, 100 µl of each UV treated BMT4i culture were diluted up to 10^{-1}-10^{-10} and each dilution was spreaded onto the BSM-BaP agar plates and incubated at 37^{o}C for 48 h. The plate with distinct colonies (10^{-4}dilution) was selected for further investigation. Ten bigger colonies of all the UV treated BMT4i culture were selected from the BSM-BaP agar plate and grown in BSMD till the O.D. reaches one (approx.10^{8} cells/ml). The UV treated BMT4i cultures of each of the ten isolates were washed thrice with BSM and the cell numbers were adjusted to10^{7} cells/ml in BSM. Subsequently, 1 ml of each of the ten UV treated BMT4i culture was inoculated in 10 ml BSM-BaP broth and grown at 37^{o}C for 7 days. Thereafter, the viability of BMT4i in all the 10 cultures was determined by CFU count method and the BaP degradation efficiency of UV treated BMT4i isolates were determined by HPLC analysis of the BaP metabolites in methanolic extracts purified from cultures by ethyl acetate extraction as mentioned previously [20, 21].

2.2.2.Treatment with chemical mutagen

Similarly, for chemical mutagen treatment, the BMT4i (10^{8} cells) was inoculated in 10 ml BSMD broth in three separate flasks containing 40 µg/ml each of AO, EMS and 5-BU respectively and grown for 24 h at 37^{o}C. After the incubation, each mutagen treated BMT4i cultures were washed three times with BSM to remove trace of chemical mutagens and finally suspended in 1 ml BSM. 100 µl BSM cultures of each chemical mutagen treated cultures were diluted up to 10^{-1}-10^{-10} and spreaded onto the BSM-BaP agar plates and incubated at 37^{o}C for 48 h. The plate with distinct colonies (10^{-4}dilution) was selected for further investigation. Ten bigger sized colonies each of AO, EMS and 5-BU treated BMT4i culture were selected from BSM-BaP plate and grown in 10 ml BSMD broth till the O.D. reaches one (approx.10^{8} cells/ml). The 10 BMT4i isolate cultures from each of the AO, EMS and 5-BU treated BMT4i cultures were washed thrice with BSM and cell numbers were adjusted upto 10^{7} cells/ml. The 1.0 ml BSM suspension culture of each of the ten BMT4i isolates cultures was inoculated in 10 ml BSM-BaP broth separately and grown at 37^{o}C for 7 days. Afterwards, CFU/ml and efficiency of BaP degradation were determined for each chemical mutagen treated isolates of BMT4i.

2.2.3. Characterization of the BaP degradation ability of improved mutant BMT4i

Among the different mutant isolates of BMT4i, the one which showed significantly enhanced growth and BaP degradation was considered as improved mutant and was further characterized. The BaP degradation and growth kinetics of improved mutant were studied and compared with that of the wild type BMT4i as mentioned previously [20, 21].

[III] RESULTS

3.1. Improvement of the BaP degradation ability of the BMT4i

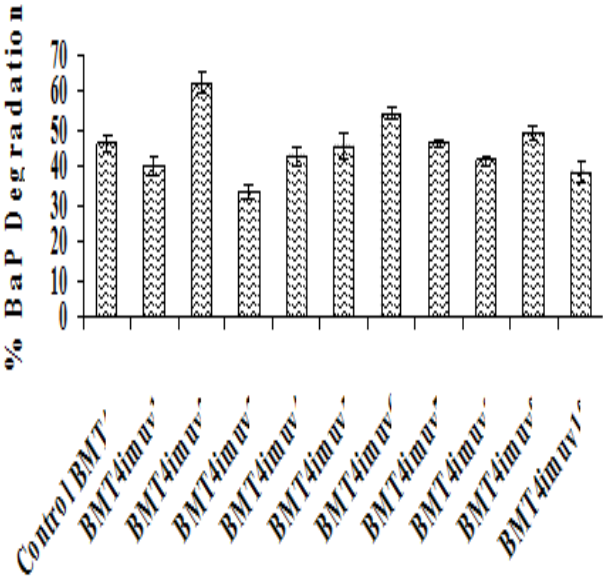
Improvement in the BaP degradation ability was attempted by inducing random mutations in the BMT4i by treating it with physical mutagen (UV_{254}) or chemical mutagens including EMS, 5-BU and AO.

3.1.1. Treatment with physical mutagen: UV irradiation

The attempt to improve the BaP degradation ability by random induced mutation with UV (254nm) resulted in the isolation of ten UV mutants of BMT4i designated as BMT4imuv1,

BMT4imuv2, BMT4imuv3, BMT4imuv4, BMT4imuv5, BMT4imuv6, BMT4imuv7, BMT4imuv8, BMT4imuv9, BMT4imuv10. All the above said mutants were checked for superior BaP degradation capabilities. Among the ten mutants, one mutant BMT4imuv2 exhibited highest BaP degradation up to 62% **[Figure-1]** followed by BMT4imuv6 (54%) which is better than that showed by control wild type BMT4i (46%).

Fig. 1: Comparison of BaP Degradation Efficiencies as Shown by Various UV Mutants of BMT4i in BSM-BaP After 7 Days.



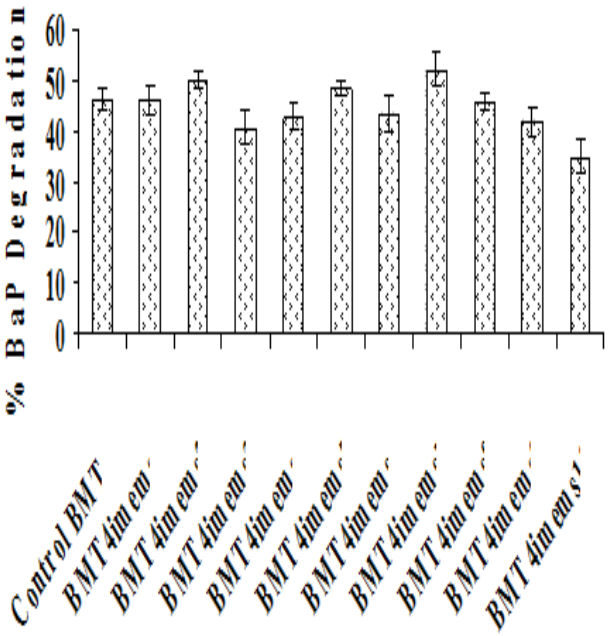
Most of the UV mutants including BMT4imuv4, BMT4imuv5, BMT4imuv7 BMT4imuv8 and BMT4imuv9 showed BaP degradation of approximately 42.92, 45.5, 46.2, 41.7 and 49% respectively **[Figure-1]** which is quite similar to that of the control wild type BMT4i (46%). However, two of the UV mutants designated BMT4imuv3 (33%) and BMT4imuv10 (38%) resulted in decline of BaP degradability.

3.1.2. Treatment with chemical mutagen

3.1.2.1. EMS

Ten EMS mutants of BMT4i (ranging from BMT4imems1 to BMT4imems10) were selected and were compared with the control BMT4i for their BaP degradation efficiency.

Fig. 2: Comparison of BaP Degradation Efficiencies as Shown by Various EMS Mutants of BMT4i in BSM-BaP After 7 Days

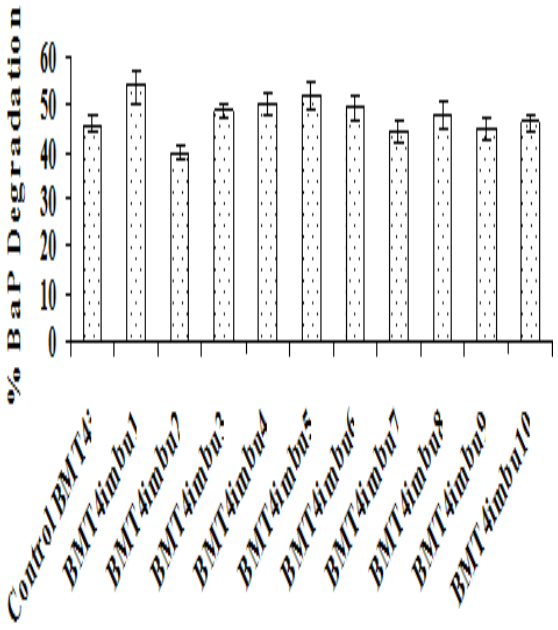


As shown in **Figure-2**, it could be clearly observed that none of the EMS mutants revealed any significant enhancement in BaP degradation in contrast to the wild type BMT4i (46%) except BMT4imems7 (52.23%). The BaP degradation efficiency of the EMS mutants namely BMT4imems1, BMT4imems2, BMT4imems3, BMT4imems4, BMT4imems5, BMT4imems6, BMT4imems7, BMT4imems8, BMT4imems9 and BMT4imems10 was found to be 46.30, 50.24, 40.7, 42.96, 48.34, 43.72, 52.23, 45.82, 41.68 and 34.99% respectively.

3.1.2.2. 5-BU

5-BU was employed for improving the BaP degradation efficiency of BMT4i. For that, ten 5-BU mutants were studied for their BaP degradation efficiencies. Out of the ten mutants, BMT4imbu1, BMT4imbu4 and BMT4imbu5 demonstrated the slight enhancement in % BaP degradation of 53.49, 50.19 and 51.66 respectively **[Figure-3]** as compared to that of 46% showed by control.

Fig. 3: Comparison of BaP Degradation Efficiencies as Shown by Various 5-BU Mutants of BMT4i in BSM-BaP After 7 Days.

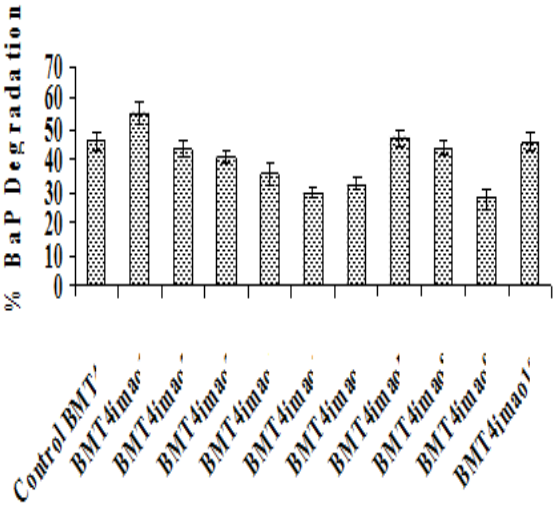


Rest of the mutants, BMT4imbu2 (40%), BMT4imbu3 (48.69%), BMT4imbu6 (49.28%), BMT4imbu7 (44.3%), BMT4imbu8 (47.82%), BMT4imbu9 (45%) and BMT4imbu10 (46.23%) showed comparable BaP degradation as that of the control BMT4i.

3.1.2.3. AO

Random mutations induced by AO did not result in any noteworthy augmentation in BaP degradation of BMT4i. Comparative assessment of BaP degradation potential of ten different AO mutants namely BMT4imao2, BMT4imao3, BMT4imao4, BMT4imao5, BMT4imao6, BMT4imao7, BMT4imao8, BMT4imao9 and BMT4imao10 revealed BaP degradation of 43.8, 41, 36, 30, 32.66, 47.2, 44.1, 27.7 and 45.9% respectively. The mutant BMT4imao1 showed better BaP degradation of 55.2% as compared to the control BMT4i **[Figure-4].** The data indicated noteworthy decline in BaP degradability of most of the AO mutants.

Fig. 4: Comparison of BaP Degradation Efficiencies as Shown by Various AO Mutants of BMT4i in BSM-BaP After 7 Days

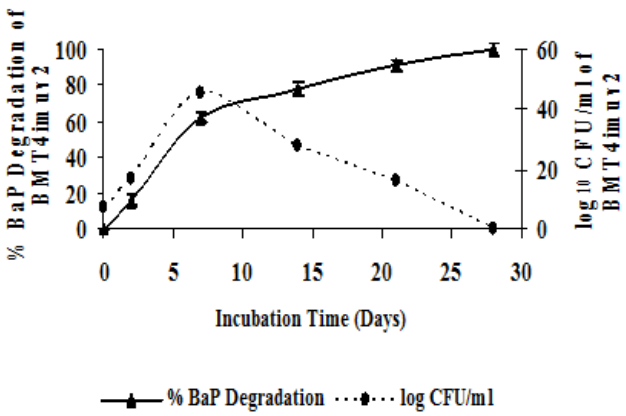


3.2. Characterization of the BaP degradation ability of BMT4imuv2

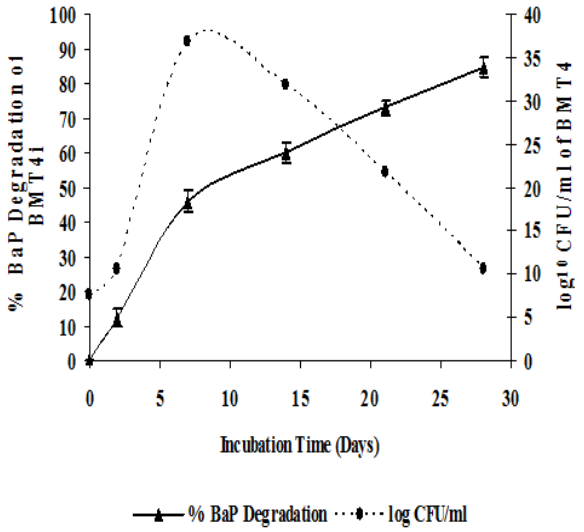
Among various BMT4i mutants, a UV mutant BMT4imuv2 showed noteworthy increment (1.5X) in the BaP degradation in comparison to the control. Therefore, it was tentatively considered as the improved version of BMT4i. In order to confirm and to exclude any possibility of doubt regarding the improvement, the degradation ability of BMT4imuv2 was further characterized by performing growth kinetics and BaP degradation kinetic experiments for 0, 7, 14, 21 and 28 days.

Fig. 5: Characterization of Growth and BaP Degradation Efficiency of Mutant BMT4imuv2 (A) and control wild type BMT4i (B) in BSM-BaP

A



B



At different time points of incubations, the log_{10} CFU/ml and assessment of % BaP degradation by HPLC analysis of respective cultures were performed. The data showed an exponential increase in the BaP degradation from 2nd day to 28th day **[Figure-5A]**. After 2nd day, BMT4imuv2 showed 16.31% BaP degradation which increased exponentially to 62.24%, 78.45% and 90.99% after 7, 14 and 21 days respectively achieving almost 100% on completion of 28 days. As far as the growth kinetic is concerned, the scenario was found to be somewhat different. As shown in **Figure – 5A**, starting with log_{10} CFU/ml of 7.30 on 0 day, there was a steep increase in the log values reaching 16.60 after 2 days attaining maxima of 45.47 log_{10} CFU/ml after 7 days. Further, increase in incubation time lead to decline in the log_{10} CFU/ml, achieving 28.00 and 16.39 log_{10} CFU/ml after 14 and 21 days respectively reaching 0.00 after 28 days. The BMT4imuv2 revealed almost 100% BaP degradation after 28 days in contrary to 84.66% by control wild type BMT4i **[Figure-5B]** confirming a substantial improvement of the BaP degradation potential of BMT4i after treatment with physical mutagen UV (254 nm). Therefore, it could be concluded that BMT4imuv2 is an improved version of BMT4i having a superior capability of BaP degradation.

[IV] DISCUSSION

In general, strain improvement is considered as one of the major process involved in the achievement of higher titers of industrial metabolites [22]. Conventionally, strain improvement has been achieved through mutation, selection, or genetic recombination [23]. Among these, the mutation induction using physical and chemical mutagens is the commonly employed technique for strain improvement. The most widely used mutagens are nitrosoguanidine, methylmethane sulfonate, EMS, N-methyl-N-nitro-N-nitrosoguanidine, 5-BU, AO and UV [24, 25, 26, 27, 28, 29, 30, 31]. In view of the above, the improvement of the BaP degradation ability of *B. subtilis* BMT4i was attempted by inducing random mutations as a result of treating BMT4i with physical mutagen UV or chemical mutagens including EMS, 5-BU and AO. Although numerous mutants of BMT4i were obtained after mutagen treatments but only the UV irradiation resulted in an improved BMT4i mutant having better BaP degradation ability than the wild type BMT4i. The UV mutant BMT4imuv2 showed an improved BaP degradation up to 62 % (1.5X of wild type BMT4i). BMT4imuv2 exhibited 100% BaP degradation as compared to 84% by wild type BMT4i after 28 days. Furthermore, BMT4imuv2 also showed an improved enhancement in log_{10} CFU/ml (38 fold) as compared to that was shown by the control (29 fold) after 7 days. To our best of knowledge, the present study is the first report demonstrating the improvement of PAHs degradation ability of any bacterial strain.

[V] CONCLUSION

It could be concluded that BMT4imuv2 is an improved version of BMT4i having a superior capability of BaP degradation, hence it could

serve as a leading biological weapon to remediate BaP contaminated sites.

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REFERENCES

[1] Environmental Protection Agency [1997] Public health goal for Benzo [a] Pyrene in drinking water. Office of Environmental Health Hazard Assessment, Berkeley, CA, 1‑42.

[2] Heitkamp MA, Cerniglia CE. [1987] The effects of chemical structure and exposure on the microbial degradation of polycyclic aromatic hydrocarbons in freshwater and estuarine ecosystems. *Environ Toxicol Chem* 6:535‑546.

[3] Shuttleworth KL, Cerniglia CE. [1995] Environmental aspects of PAH biodegradation. *Appl Biochem Biotechnol* 54:291‑302.

[4] Kanaly RA, Harayama S. [2000] Biodegradation of high molecular weight polycyclic aromatic hydrocarbons by bacteria. *J Bacteriol* 182(8):2059‑2067.

[5] Miller RM, Singer GM, Rosen JD, Bartha R. [1988] Photolysis primes biodegradation of Benzo[a] pyrene. *Appl Environ Microbiol* 1724‑1730.

[6] Kalf DF, Crommentuijn T. [1997] Environment quality objectives for 10 polycyclic aromatic hydrocarbons. *Ecotoxicol Environ Safe* 36:89‑97.

[7] NTP-National Toxicological Program [2002] Tenth Report on Carcinogens. Report of the NTP on Carcinogens. National Academy Press, Washington.

[8] Hsu GW, Huang X, Luneva NP, Geacintov NE, Beese LS. [2005] Structure of a high fidelity DNA polymerase bound to a Benzo[a] pyrene adducts that blocks replication. *J Biol Chem* 280(50):3764‑3770.

[9] Sims RC, Overcash MR. [1983] Fate of polynuclear aromatic compounds (PNAs) in soil-plant system. *Residue Rev* 88:1‑68.

[10] Habe H, Omori T [2003] Genetic of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci Biotechnol Biochem* 67:225‑243.

[11] Heitkamp MA, Cerniglia CE. [1989] Polycyclic aromatic hydrocarbon degradation by a *Mycobacterium* sp. In microcosms containing sediment and water from a pristine ecosystem. *Appl Environ Microbiol* 55:1968‑1973.

[12] Schneider J, Grosser R, Jayasimhulu K, Xu W, Warshawsky D. [1996] Degradation of pyrene, Benzo[a]anthracene and Benzo[a]pyrene by Mycobacterium sp. strain RJG II-135, isolated from a former coal gasification site. *Appl Environ Microbiol* 62:13‑19.

[13] Grosser RJ, Warshawsky D, Vestal JR. [1991] Indigenous and enhanced mineralization of pyrene, benzo[a] pyrene and carbazole in soils. *Appl Environ Microbiol* 57:3462‑3469.

[14] Ye D, Siddiqi MA, Maccubbin AE, Kumar S, Sikka HC. [1996] Degradation of polynuclear aromatic hydrocarbons by *Sphingomonas paucimobilis*. *Environ Sci Technol* 30:136‑142.

[15] Juhasz AL, Naidu R. [2000] Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. *Int Biodeterior Biodegrad* 45:57‑88.

[16] Juhasz AL, Stanley GA, Britz ML. [2002] Metabolite repression inhibits degradation of benzo[a]pyrene and dibenz[a, h]anthracene by *Stenotrophomonas maltophilia* VUN 10, 003. *J Ind Microbiol Biotechnol* 28:88‑96.

[17] Moody JD, Fu PP, Freeman JP, Cerniglia CE. [2004] Degradation of benzo[a] pyrene by *Mycobacterium vanbaalenii* PYR-1. *Appl Environ Microbiol* 70:13‑19.

[18] Peng RH, Xiong AS, Xue Y, Fu XY, Gao F, Zhao W, Tian YS, Yao Q. [2008] Microbial biodegradation of polyaromatic hydrocarbons. *FEMS Microbiol Rev* 32:927‑955.

[19] Seo JS, Keum YS, Li QX. [2009] Bacterial degradation of aromatic compounds. *Int J Environ Res Public Health* 6:278‑309.

[20] Lily MK, Bahuguna A, Dangwal K, Garg V. [2009] Degradation of Benzo[a]pyrene by a novel strain *Bacillus subtilis* BMT4i (MTCC 9447). *Braz J Microbiol* 40 (4): 884-892.

[21] Lily MK, Bahuguna A, Dangwal K, Garg V. [2010] Optimization of an Inducible Chromosomally Encoded Benzo[a]pyrene

(BaP) Degradation Pathway in *Bacillus subtilis* BMT4i (MTCC 9447). *Ann Microbiol* 60(1): 51-58.

[22] Podojil M, Blumauerova M, Culik K, Vanek Z. [1984] The tetracyclines: properties, biosynthesis and fermentation. In: Vandamme EJ, editor. Biotechnology of industrial antibiotics. New York: Marcel Dekker; 259-279.

[23] Parekh S, Vinci VA, Strobel RJ. [2000] Improvement of microbial strains and fermentation processes. *Appl Microbiol Biotechnol* 54: 287–301.

[24] Baltz RH. [1999] Encyclopedia of bioprocess technology: fermentation, biocatalysis and separation. New York, Wiley.

[25] Sidhu GS, Sharma P, Chakrabarti T, Gupta JK. [1997] Strain improvement for the production of a thermostable α-amylase. *Enz Microbial Technol* 21(7): 525-530.

[26] Haq I, Javed S, Ashraf H. [2002] Production of amyloglucosidase by UV irradiated strain of *Aspergillus niger*. *Biotechnol* 1(1): 34-39.

[27] Gohel V, Megha C, Vyas P, Chhatpar HS. [2004] Strain improvement of chitinolytic enzyme producing isolate *Pantoea dispersa* for enhancing its biocontrol potential against fungal plant pathogens. *Ann Microbiol* 54(4): 503-515.

[28] Abdelghani TTA, Kunamneni A, Ellaiah P. [2005] Isolation and mutagenesis of Streptokinase producing bacteria. *Am J Immunol* 1(4): 125-129.

[29] Rao RS, Jyothi CP, Prakasham RS, Rao CS, Sarma PN, Rao LV. [2006] Strain Improvement of *Candida tropicalis* for the production of xylitol: biochemical and physiological characterization of wild-type and mutant strain CT-OMV5. *J Microbiol* 44(1): 113-120.

[30] Adsul MG, Bastawde KB, Varma AJ, Gokhale DV. [2007] Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production. *Biores Technol* 98(7): 1467-1473.

[31] Veerapagu M, Jeya KR, Ponmurugan K. [2008] Mutational effect of *Penicillium chrysogenum* on antibiotic production. *Advanced Biotech* 16-19.