MICROBIAL SCREENING AND EXPRESSION OF GENE INVOLVED IN CARBAZOLE DEGRADATION

SYNOPSIS

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By

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1. Introduction

Fossil fuels are the product of anaerobic decomposition of buried plants and animals under high pressure and temperature. Three major forms of fossil fuels are petroleum (oil), coal and natural gas. Oil plays a preponderant role in world's total energy consumption [1]. Majority of mass transportation is propelled by oil, mainly by diesel. Use of diesel fuel, rich in polycyclic and heterocyclic (mainly nitrogen and sulfur as heteroatom) aromatics, is directly correlated with environmental pollution. Its combustion leads to the emission of oxides of sulfur (SO_2) and nitrogen (NO, NO_2, N_2O), thus contributing to environmental problems like acid rain, particulate matter formation, greenhouse effects and destruction of the ozone layer [2]. Conventional hydrotreating technologies for refining diesel are energy and capital intensive, as they operate at very high temperature and pressure. Catalysts used in this process also known to have some critical influence on the composition of treated products. Moreover, the process also does not work when a minimum concentration of these contaminants is attained [3].

Biological processing requires relatively less severe reaction conditions and thus will be more economical. Biocatalysts operate at ambient temperature and pressure with high substrate selectivity and do not generate any undesirable by-products [4]. Carbazole (CAR) and its alkyl derivatives, are major representatives of nitrogen heterocyclic aromatic compounds present in diesel that are difficult to remove using conventional methods and thus chosen as model compounds for microbial degradation. Various microorganisms *viz. Arthrobacter*, *Burkholderia*, *Janthinobacterium*, *Klebsiella*, *Novosphingobium*, *Pseudomonas*, *Ralstonia*, *Sphingomonas* etc. have been reported to degrade CAR [5]. Biodegradation pathway and the genes involved in the degradation of CAR have been elucidated (Fig. 1). CAR is converted to anthranilic acid through enzymatic events catalyzed by carbazole-1,9a-dioxygenase (CARDO; encoded by *carAaAcAd*), 2'-aminobiphenyl-2,3-diol-1,2-dioxygenase (encoded by *carBaBb*) and 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoic acid hydrolase (encoded by *carC*). Due to complete degradation of CAR, microorganisms following this pathway will lead to the loss of fuel value and thus cannot be used in biorefining industry. Kilbane [6] proposed that in the absence of native microorganisms for selective removal of nitrogen from CAR, recombinant biocatalyst can be designed by combining the genes encoding for CARDO along with the genes encoding a suitable amidase.

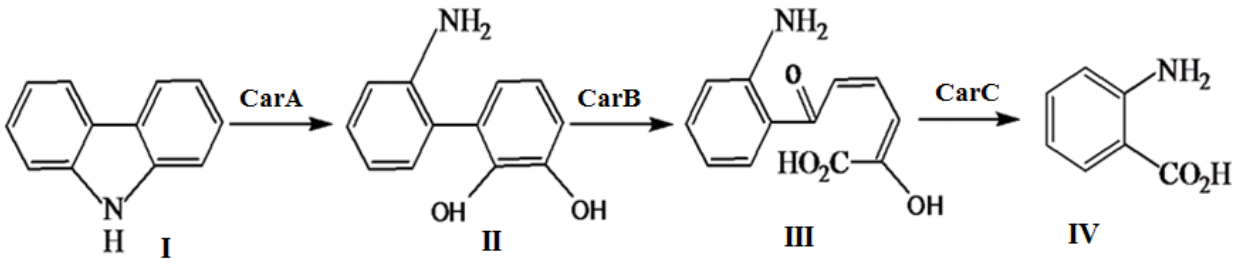

Fig 1: Degradation pathway of carbazole in *Pseudomonas* sp. strain CA10. CarA, carbazole-1,9a-dioxygenase; CarB, 2'-aminobiphenyl-2,3-diol-1,2-dioxygenase; CarC, 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoic acid hydrolase; I, Carbazole; II, 2'-aminobiphenyl-2,3-diol; III, 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoic acid (*meta*-cleavage compound); IV, Anthranilic acid.

The first step towards the design of such recombinant catalyst is the identification of CAR degrading microorganism and the cloning and expression of CARDO encoding gene and to check its suitability for petroleum refining industry.

Thus, the objectives were laid down as follows:

- Isolation, identification and characterization of bacterial strains with the ability to degrade CAR.
- Identification of genes responsible for CAR degradation.
- Cloning and expression of genes involved in the CAR degradation in *E. coli*.

2. Materials and methods

2.1. Chemicals

CAR (96% purity) and other PAHs (>98% purity) were purchased from Acros Organics (New Jersey, USA) and Sigma-Aldrich (St Louis, MO, USA), respectively. Organic solvents (HPLC grade) and other chemicals (analytical grade) were obtained from Qualigen (Mumbai, India), HiMedia Laboratories (Mumbai, India) and Merck (Darmstadt Hesse, Germany). Reagents used

in molecular studies were purchased from Sigma-Aldrich, Fermentas (Burlington, CDA), Stratagene (La Jolla, CA), Invitrogen (Carlsbad, CA) and New England Biolabs (Ipswich, MA).

2.2. Culture media

Basal salt medium (BSM), used for CAR and other PAHs degradation studies, composed of (per litre of solution) 2.44 g of KH_2PO_4; 5.57 g of Na_2HPO_4; 2 g of Na_2SO_4; 2 g of KCl; 0.2 g of $MgSO_4$; 0.001 g of $FeCl_3.6H_2O$; 0.02 g of $MnCl_2.4H_2O$ and 0.003 g of $CaCl_2.2H_2O$. During PAHs biodegradation studies, NH_4Cl (2 g/l) was added to the BSM as nitrogen source.

2.3. Enrichment, isolation and identification of carbazole degrading bacterial strains

For enrichment studies, PAHs and petroleum contaminated soil and liquid samples were collected from various Indian oil refineries (Jaipur, Gujarat, Mathura, Panipat), petrol pumps (Gujarat, Uttarakhand, Delhi and NCR), dye industries (Alpanil, Meghmani and Ami Pigments; Gujarat) and sewage treatment plants (Delhi and NCR). Enrichment experiments were performed in BSM containing 500 ppm of CAR. After several consecutive enrichments on CAR, pure bacterial strains were isolated using a spray plate technique. Phenotypic and genotypic characterizations of selected bacterial isolates were performed by biochemical tests and 16S rRNA sequencing, respectively.

2.4. Carbazole degradation study

Biodegradation studies by growing bacterial isolates were performed in BSM (pH 7.0) supplemented with CAR as sole carbon and nitrogen source. All experiments were carried out at 30°C and 180 rpm. For the time course of bacterial growth and CAR degradation analysis, culture samples were aliquoted at regular time intervals. Bacterial growth was estimated by recording optical density at 600 nm (OD_{600}) and viable cell count. Residual substrate concentration, after extraction in acidic condition, was analyzed using high performance liquid chromatography (HPLC). For the resting cell experiments bacterial cells, grown in BSM containing CAR, were harvested from the late exponential phase and resuspended in potassium phosphate buffer (pH 7.0) supplemented with CAR. Cultures were incubated at 30°C and 180 rpm. Samples were aliquoted at regular time intervals and analyzed for residual CAR concentration.

2.5. Substrate utilization

The isolated bacterial strain was inoculated in BSM containing 1% (v/v) of alkanes (*n*-hexane, *n*-heptane, *n*-octane, *n*-decane, *n*-undecane, *n*-dodecane and *n*-hexadecane) or 1 mM of PAHs (acenaphthene, acenaphthylene, anthracene, benzo[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, biphenyl, chrysene, dibenzothiophene, dibenzofuran, fluoranthene, 9H-fluorene, naphthalene, phenanthrene, pyrene and quinoline), separately as sole carbon source. Substrate utilization was assessed by bacterial growth and residual concentration analysis was performed using HPLC and gas chromatography (GC). Utilization of these compounds by resting cells was also performed.

2.6. Effect of abiotic environmental factors on carbazole degradation

Various abiotic environmental factors, affecting the bacterial cell growth and CAR degrading ability of isolated bacterial strain, were examined. These included temperature, pH, salinity, presence of additional carbon source (yeast extract) and initial concentration of CAR. Effect of various emulsifiers (SDS, Tween 80, Triton X-100 and CTAB) on CAR degradation was also determined.

2.7. Screening of biosurfactant production

Biosurfactant production was assayed by drop collapse assay, microplate analysis, emulsification index calculation and bacterial adhesion to hydrocarbon (BATH) assay.

2.8. Identification of carbazole degrading gene

CAR degrading genes were identified by PCR amplification using primers designed from the consensus region of *car* gene sequence of well established CAR degraders. *Pseudomonas resinovorans* strain CA10 was used as a positive control for PCR amplification.

2.9. Cloning and expression of carbazole dioxygenase encoding gene

Overlap extension PCR was used to generate fusion construct of *carAa-carAcAd* [7]. Fusion product was ligated into pGEX-4T-3 vector and transformed into *E. coli* BL21. Expression of recombinant protein was then induced with 1 mM of IPTG (Isopropyl β-D-1-thiogalactopyranoside) at 25°C for 3 h. The resting cell activity, as mentioned above, was calculated for each and every aromatic and aliphatic compound listed above.

2.10. Analytical methods

Quantification of CAR and PAHs was performed by HPLC (Waters; Milford, MA) equipped with reverse-phase C8 column and photodiode array detector (PDA 2996; Waters). Acetonitrile: water (80:20 v/v) was used as a mobile phase with the flow rate of 0.5 ml/min. Gas chromatography, model GC-2010 Plus (Shimadzu Scientific Instruments; Kyoto, Japan), was used for the quantification of liquid aliphatic hydrocarbons (*n*-alkanes). Products formed during CAR degradation were identified by using gas chromatography-mass spectrometry (GC-MS) QP-2010 (Shimadzu, Japan) equipped with Rtx®-5Sil MS capillary column (Restek; Bellefonte, PA). Helium was used as the carrier gas with a constant flow rate of 1.21 ml/min. The column temperature program started from 80°C (2 min isothermal hold) followed by a gradual increase at a rate of 5°C/min till 280°C which was further heated to 320°C by a 10°C/min increment and held for 4 min. Detector and injector temperatures were 280°C and 270°C, respectively.

3. Results and discussions

3.1. Enrichment, isolation and identification of carbazole degrading microorganisms

Nineteen different soil and liquid samples, from PAHs or petroleum contaminated sites, were collected for enrichment studies. After enrichment in BSM containing 500 ppm of CAR, total 141 pure bacterial samples were isolated using spray plate method. Each bacterial isolates was then examined for its ability to degrade CAR in BSM supplemented with 500 ppm of CAR. Majority of isolates, 125 in numbers, showed less than 20% of CAR degradation, while 12 showed degradation between ranges 20-50%. There were only 4 isolates *viz.* A8 (62%), Alp6 (90%), Alp7 (91%) and GBS.5 (100%) that showed more than 50% of CAR degradation in 96 h. Due to their rapid growth in BSM and efficient CAR degradation, these four bacterial strains were selected for further studies.

Both phenotypic and genotypic studies were performed for the identification of these isolates. All the strains were aerobic, Gram negative rods and showed catalase production. Based on various biochemical analyses and by referring to Bergey's Manual of Determinative Bacteriology, strains GBS.5 and A8 was tentatively classified as belonging to the genus *Pseudomonas* and *Enterobacter*, respectively. Strains Alp6 and Alp7 were tentatively identified as *Acinetobacter spp*. 16S rRNA sequence of isolate Alp6 and Alp7 showed maximum sequence

similarity, 99.6% and 98.6%, respectively, with *Acinetobacter indicus* A648^T (GenBank Accession No. HM047743). These results were also supported by other phylogenetic trees constructed using minimum evolution, neighbor-joining and maximum likelihood methods. In case of 16S rRNA gene of strain GBS.5, 99.51% of sequence similarity was observed with *Pseudomonas oleovorans subsp. lubricantis* RS1^T (GenBank Accession No. DQ842018). The 16S rRNA sequences of isolates *Pseudomonas* sp. strain GBS.5, *Acinetobacter* sp. strain Alp6 and *Acinetobacter* sp. strain Alp7 are deposited under the GenBank accession numbers JX193073, JF828047 and JF828048, respectively.

Various PAHs degrading *Enterobacter* spp. [8] and *Acinetobacter* spp. [9] have been reported but there are no reports related to CAR degradation by these microorganisms. To the best of my knowledge *Acinetobacter* sp. strains Alp6 and Alp7 and *Enterobacter* sp. strain A8, isolated during this study, are the first reports for the CAR degrading bacteria belonging to respective genus.

3.2. Biodegradation of carbazole by growing cells

Time course of CAR degradation and growth studies by strains GBS.5, Alp6, Alp7 and A8, performed in BSM containing 500ppm of CAR, revealed that strain GBS.5 was able to degrade 97% of CAR in 48 h, while strains Alp6, Alp7 and A8 showed 100%, 98% and 83% degradation, respectively in nine days. Rapid CAR degradation was observed during exponential growth phase of all strains. Control, *Pseudomonas resinovorans* strain CA10, showed only 54% of CAR degradation in nine days.

3.3. Resting cell experiment

Specific activity of CAR degradation by resting cells for all identified strains was calculated. By analyzing residual CAR concentration, from time wise aliquoted samples, specific activities of carbazole degradation for strains GBS.5, Alp6, and Alp7 were calculated to be 11.36, 7.96 and 5.82 µmol/min/g (dry cells)/l. Maximum specific activity of CAR degradation, reported till date, is 10.4 µmol/min/g (dry cells)/l for *Pseudomonas* sp. XLDN4-9 [10] compared to the 11.36 µmol/min/g (dry cells)/l for *Pseudomonas* sp. strain GBS.5.

Due to rapid CAR degradation, high specific activity of CAR degradation, *Pseudomonas* sp. strain GBS.5 was selected for further analysis.

3.4. Identification of products formed during carbazole degradation

To investigate the products formed during CAR degradation by the strain GBS.5, GC-MS analysis was carried out at different times of incubation. Peaks corresponding to long chain alkanes like *n*-tetradecane, *n*-pentadecane, *n*-hexadecane, *n*-heptadecane, eicosane and (9Z)-octadec-9-enoic acid were obtained. This MS spectrum correlates with the spectra of glycolipidic biosurfactants, produced by various microorganisms [11]. Biosurfactant production by strain GBS.5 was also confirmed by drop collapse, microplate, emulsification index (E_{24}) and BATH assays. The highest emulsification index of 53±1.52% was obtained after 48 h of incubation, which was stable for more than a month. Biosurfactant produced by the strain GBS.5 could be responsible for increasing bioavailability of CAR by solubilization and mobilization.

Pseudomonas spp. are reported to be involved in the degradation of various nitrogen containing heterocyclic aromatics [5, 12] and are also known to produce biosurfactants [13] but none of the *Pseudomonas* is reported to produce biosurfactant in the presence of CAR. This is the first report of any *Pseudomonas* sp. producing biosurfactant during CAR degradation.

3.5. Effect of abiotic environmental factors on carbazole degradation

Selected bacterial strain GBS.5 showed CAR degradation at a wide range of temperature (10 to 40°C), pH (6 to 9), salinity (5 to 35 g/l of NaCl) and CAR concentration (50 to 5000 ppm). CAR degradation ability was not influenced in the presence of additional carbon source (yeast extract). Rapid CAR degradation was observed in the presence of non ionic (Tween 80 and Triton X-100) and anionic (SDS) surfactant, while no degradation was seen in the presence of cationic surfactant (CTAB).

3.6. Substrate range analysis

3.6.1. Degradation of aromatic hydrocarbons by *Pseudomonas* sp. strain GBS.5

Ability of strain GBS.5 to utilize PAHs, present in petroleum products or listed as toxic environmental pollutants by Environmental Protection Agency, was tested. Apart from CAR, naphthalene and phenanthrene also supported the growth of strain GBS.5. In a separate experiment, all PAHs except biphenyl, dibenzothiophene and dibenzofuran were degraded by the resting cells (induced with CAR) of strain GBS.5. It was concluded from these findings that enzymatic system induced in the presence of CAR, has broad substrate range.

3.6.2. Degradation of aliphatic hydrocarbons *Pseudomonas* sp. strain GBS.5

Aliphatic hydrocarbons are the major component of diesel fuel which accounts for approximately 59% of total carbon content and hence the major contributor of fuel value. Microorganisms belonging to the genus *Pseudomonas* are widely reported for the degradation of alkanes [14]. Thus, *Pseudomonas* sp. strain GBS.5 was also screened for degradation of alkanes by its growing and resting cells.

Results of viable cell count revealed that all alkanes supported the bacterial cell growth. However, CAR induced resting cells did not show any reduction in concentration of alkanes, suggesting that enzymes induced by CAR were not able to metabolize alkanes.

After analyzing above results it was concluded that strain GBS.5, as whole, may be a good candidate for bioremediation of mixed PAHs contaminated sites, but not for petroleum refining. On applying in petroleum refining process *Pseudomonas* sp. strain GBS.5 would completely degrade CAR and other aliphatic hydrocarbons from diesel and hence would lead to loss of fuel value (carbon content). However, the ability of the strain GBS.5 to utilize aliphatic compounds by growing cells but not by resting cells suggested that microorganism has ability to use both aliphatic and aromatic hydrocarbons but *car* gene is not associated with the degradation of aliphatic compounds and thus will not affect the fuel value.

3.7. Identification of carbazole degrading genes

Genes responsible for CAR degradation were investigated for bacterial isolates, *Pseudomonas* sp. strain GBS.5, *Acinetobacter* sp. strain Alp6, *Acinetobacter* sp. strain Alp7 and *Enterobacter* sp. strain A8, by PCR. Various sets of primers, designed from conserved region of known *car* genes (*carAa*, *carAc*, *carBa*, *carBb* and *carC*) of well established CAR degraders, were used for gene amplification. As a result of PCR, only strain GBS.5 showed the amplification of all the genes similar to the positive control, *Pseudomonas resinovorans* strain CA10 [15, 16]. Amplicons were further cloned and sequenced. The nucleotide sequences of the *carAa*, *carBb*, *carC* and *carAc* genes are deposited under the GenBank accession numbers JX885589, JX885590, JX885591 and JX885592, respectively.

Comparative analysis of amino acid sequences encoded by respective *car* genes of *Pseudomonas* sp. strain GBS.5 with other CAR degraders revealed that terminal dioxygenase unit (CarAa) of

the enzyme CARDO, 2-aminobiphenyl-2,3-diol-1,2-dioxygenase catalytic subunit (CarBb) and meta-cleavage compound hydrolase (CarC) showed replacement of hydrophobic amino acid with hydrophilic and vice-versa. These amino acids are not present in the binding or catalytic sites of their respective proteins. Any effect of these changed amino acids is yet to be elucidated.

3.8. Cloning and expression of carbazole dioxygenase gene in *E. coli*

As Kilbane [6] suggested that, for selective removal of nitrogen from carbazole, recombinant biocatalyst should harbor gene encoding for carbazole dioxygenase and amidase. Gene *carAaAcAd* encoding for CARDO was cloned and expressed in *E. coli* strain BL21.

The enzyme CARDO catalyses the first step of CAR degradation pathway, converting CAR in to 2'-aminobiphenyl-2,3-diol. In *car* operon, genes *carAa* and *carAcAd* are separated by *carBaBbC* gene cluster. Fusion of *carAa* and *carAcAd* gene fragments was performed by using overlap extension PCR. Fused *carAaAcAd* gene fragment was then cloned in pGEX-4T-3 vector for expression studies. Recombinant pGEX-4T-3-*carAacd* vectors were transformed in *E. coli* strain BL21 cells. Expression of CARDO, via IPTG (1 mM) induction, was confirmed by SDS-PAGE.

3.9. Substrate range analysis of recombinant biocatalyst

IPTG induced recombinant cells were lysed by sonication and whole cell extracts were then examined for its substrate range analysis and specific activity of CAR degradation. Recombinant cells showed similar substrate range of PAHs as that of native GBS.5, but with increased activity, while no alkane degradation was observed by enzymatic action of CARDO. Specific activity of CAR degradation was calculated to be as 12.58 μmol/min/g (dry cells)/l.

After analyzing these findings it was concluded that a biocatalyst carrying CARDO encoding gene of *Pseudomonas* sp. strain GBS.5 would be suitable for constructing the recombinant catalyst for removing nitrogen form CAR.

4. Conclusions

Four bacterial strains *viz. Pseudomonas* sp. GBS.5, *Enterobacter* sp. A8, *Acinetobacter* sp. Alp6 and *Acinetobacter* Alp7, able to degrade CAR, were isolated. To the best of my knowledge this is the first report on CAR degradation by *Enterobacter* sp. and *Acinetobacter* spp. This study is also the first report for biosurfactant producing CAR degrading *Pseudomonas* sp.. None of the

reported *Pseudomonas* spp., till the compilation of this study, is known to produce biosurfactant during CAR degradation. A high specific activity against CAR (11.36 µmol/min/g (dry cells)/l) has projected the strain GBS.5 as a potential bioremediating strain for future.

Apart from CAR degradation studies, enzyme CARDO from the strain GBS.5 was also cloned and expressed in *E. coli*. Specific activity of CAR degradation was calculated to be as 12.58 µmol/min/g (dry cells)/l. Substrate range analysis of recombinant biocatalyst revealed its ability to degrade a wide range of PAHs present in diesel but not aliphatic hydrocarbons, indicating its suitability as a candidate for biorefining industry.

5. Future perspectives

It has been shown in this study that CAR dioxygenase has the ability to degrade wide range of PAHs but does not degrade the aliphatic hydrocarbons. Thus, to design a biocatalyst suitable for biorefining industry, future work will focus on:

- Designing a recombinant biocatalyst with the ability of denitrogenation and dearomatization.
- Designing a biocatalyst for cleaner fuel production by expressing *car* gene in sulfur utilizing host.
- Analyzing and exploiting bioremediation potential of other isolated strains.

References

[1] World energy outlook 2012, *International Energy Agency*., pp. 1-700, Nov. 2012.

[2] J.S. Gaffney, N.A. Marley, "The impacts of combustion emission on air quality and climate-from coal to biofuels and beyond," *Atmos. Environ.*, vol. 43, pp. 23-36, 2009.

[3] C. Song, X. Ma, "New design approaches to ultra-clean diesel fuels by deep desulfurization and deep dearomatization," *Appl. Catal. B: Environ.*, vol. 41, pp. 207-238, 2003.

[4] S.L. Borgne, R. Quintero, "Biotechnological processes for the refining of petroleum," *Fuel Process. Technol.*, vol. 81, pp. 155-169, 2003.

[5] A.L. Larentis, H.C.C. Sampaio, C.C. Carneiro, O.B. Martins, T.L.M. Alves, "Evaluation of growth, carbazole biodegradation and anthranilic acid production by *Pseudomonas stutzeri*," *Braz. J. Chem. Eng.*, vol. 28, pp. 37-44, 2011.

[6] J.J. Kilbane II, "Microbial biocatalysts developments to upgrade fossil fuels," *curr. opin. Biotechnol.*, vol. 17 , pp. 305-314, 2006.

[7] K.L. Heckman, L.R. Pease, "PCR-mediated overlap extension can create specific nucleotide mutations or generate chimeric gene products," *Nat Protoc.*, vol. 2, pp. 924-932, 2007.

[8] L.F. Bautista, R. Sanz, M.C. Molina, N. Gonzalez, D. Sanchez, "Effect of different non-ionic surfactants on the biodegradation of PAHs by diverse aerobic bacteria," *Inter. Biodeter. Biodegr.*, vol. 63, pp. 913-922, 2009.

[9] S.H. Yu, L. Ke, Y.S. Wong, N.F. Tam, "Degradation of polycyclic aromatic hydrocarbons by a bacterial consortium enriched from mangrove sediments," *Environ. Int.*, vol. 31, pp. 149-154, 2005.

[10] L. Li, Q. Li, F. Li, Q. Shi, B. Yu, F. Liu, P. Xu, "Degradation of carbazole and its derivatives by a *Pseudomonas* sp.," *Appl. Microbiol. Biotechnol.*, vol. 73, pp. 941-948, 2006.

[11] V. Patel, S. Cheturvedula, D. Madamwar, "Phenanthrene degradation by *Pseudoxanthomonas* sp. DMVP2 isolated from hydrocarbon contaminated sediment of Amlakhadi canal, Gujarat, India," *J. Hazard. Mater.*, vol. 201-202, pp. 43-51, 2012.

[12] T. Stobdan, A. Sinha, R.P. Singh, D.K. Adhikari, "Degradation of pyridine and 4-methylpyridine by Gordonia terrea IIPN1," *Biodegradation*., vol. 19, pp. 481-487, 2008.

[13] P.K.S.M. Rahman, E. Gakpe, "Production, characterization and applications of biosurfactants-review," *Biotechnology*, vol. 7, pp. 360-370, 2008.

[14] F. Mehboob, H. Junca, G. Schraa, A.J.M. Stams, "Growth of *Pseudomonas chloritidismutans* AW-1^T on *n*-alkanes with chlorate as electron acceptor," *Appl. Microbiol. Biotechnol*, vol. 83, pp. 739-747, 2009.

[15] S. Sato, N. Ouchiyama, T. Kimura, H. Nojiri, H. Yamane, T. Omori, "Cloning of genes involved in carbazole degradation of *Pseudomonas* sp. strain CA10: nucleotide sequence of genes and characterization of metacleavage enzyme and hydrolase," *J Bacteriol*, vol. 179, pp. 4841-4849, 1997.

[16] S. Sato, J. Nam, T. Kasuga, H. Nojiri, H. Yamane, T. Omori, "Identification and characterization of genes encoding carbazole 1,9a-dioxygenase in *Pseudomonas* sp. strain CA10," *J. Bacteriol*, vol. 179, pp. 4850-4858, 1997.

Author's publications

International Journals

[1] **G.B. Singh**, A. Srivastava, A. Saigal, S. Aggarwal, S. Bisht, S. Gupta, S. Srivastava, N. Gupta, "Biodegradation of carbazole and dibenzothiophene by bacteria isolated from petroleum contaminated sites," *Bioremed. J.*, vol. 15, pp. 189-195, 2011.

[2] **G.B. Singh**, S. Gupta, S. Srivastava, N. Gupta, "Biodegradation of carbazole by newly isolated *Acinetobacter* spp.," *Bull. Environ. Contam. Toxicol.*, vol. 87, pp. 522-526, Nov. 2011.

[3] **G.B. Singh**, S. Srivastava, S. Gupta, N. Gupta, "Evaluation of carbazole degradation by *Enterobacter* sp. isolated from hydrocarbon contaminated soil," *Recent Res. Sci. Technol*., vol. 3, pp. 44-48, 2011.

[4] **G.B. Singh**, S. Gupta, N. Gupta, "Carbazole degradation and biosurfactant production by newly isolated *Pseudomonas* sp. strain GBS.5," *Int Biodeter Biodegr*, Accepted, 2013.

[5] **G.B. Singh**, K. Mehta, S. Gupta, N. Gupta, "Expression and characterization of carbazole dioxygenase from *Pseudomonas* sp. GBS.5," *Biotech Lett*, Communicated, 2013.

Conferences

[1] **G.B. Singh**, S. Gupta, S.K. Sharma, N. Gupta, "Screening and isolation of Microorganisms involved in refining fossil fuels," in *International conference on climate change and sustainable management of natural resources*, Madhya Pradesh, India, 2009, p. 70.

[2] **G.B. Singh**, S. Gupta, S.K. Sharma, N. Gupta, "Screening of microorganisms from soil utilizing dibenzothiophene and Carbazole," in *National conference on potentials of biotechnology and microbiology*, Ghaziabad, India, 2009, p. 23.

[3] **G.B. Singh**, S. Srivastava, N. Gupta, "Biodegradation of carbazole by a promising gram-negative bacterium," in *International conference on environmental science and engineering*, Singapore, 2010, pp. 783-786.

GenBank Submissions

[1] **G.B. Singh**, S. Srivastava, S. Gupta, N. Gupta, "*Acinetobacter* sp. enrichment culture clone Alp6 16S ribosomal RNA gene, partial sequence," GenBank Accession No. JF828047, 2011.

[2] **G.B. Singh**, S. Srivastava, S. Gupta, N. Gupta, "*Acinetobacter* sp. enrichment culture clone Alp7 16S ribosomal RNA gene, partial sequence," GenBank Accession No. JF828048, 2011.

[3] **G.B. Singh**, S. Srivastava, S. Gupta, N. Gupta, "*Pseudomonas* sp. enrichment culture clone GBS.5 16S ribosomal RNA gene, partial sequence," GenBank Accession No. JX193073, 2012.

[4] **G.B. Singh**, S. Gupta, S. Srivastava, N. Gupta, "*Pseudomonas* sp. GBS.5 carbazole terminal oxygenase component (*carAa*) gene, partial cds," GenBank Accession No. JX885589, 2012.

[5] **G.B. Singh**, S. Gupta, S. Srivastava, N. Gupta, "*Pseudomonas* sp. GBS.5 2-aminobiphenyl-2,3-diol 1,2-dioxygenase catalytic subunit (*carBb*) gene, partial cds," GenBank Accession No. JX885590, 2012.

[6] **G.B. Singh**, S. Gupta, S. Srivastava, N. Gupta, "*Pseudomonas* sp. GBS.5 meta-cleavage compound hydrolase (*carC*) gene, complete cds," GenBank Accession No. JX885591, 2012.

[7] **G.B. Singh**, S. Gupta, S. Srivastava, N. Gupta, "*Pseudomonas* sp. GBS.5 carbazole ferredoxin component (*carAc*) gene, partial cds," GenBank Accession No. JX885592, 2012.