J. Mountain Res. P-ISSN: 0974-3030, E-ISSN: 2582-5011 DOI: https://doi.org/10.51220/jmr.v19-i2.30
Vol. 19(2), (2024), 281-289

# Isolation and characterization of partial *nid*A gene homolog in *Bacillus subtilis* BMT4i

**Kamlesh Kumar Bhatt[1] • Madhuri Kaushish Lily[1] • Koushalya Dangwal[1]***

[1]*Department of Biotechnology, Modern Institute of Technology (MIT), Dhalwala, Rishikesh-249201, Uttarakhand, India*

*Corresponding Author Email id: kdangwal1@yahoo.co.in

**Received: 03.09.2024; Revised: 10.11.2024; Accepted: 2.12.2024**


**Abstract:** *Bacillus subtilis* BMT4i, a well-known degrader of Benzo(a)pyrene (BaP), was investigated for the presence of ring-hydroxylating dioxygenases (RHD). To achieve this, the homologous nidA gene of *Bacillus subtilis* BMT4i, associated with RHD, was amplified using nidA-specific primers. The amplified gene fragment was cloned into the pJET1.2 vector and subsequently sequenced by a commercial facility. The partial nidA gene sequence from BMT4i was then subjected to phylogenetic analysis. The study successfully cloned and sequenced the partial dioxygenase gene, revealing it to be 376 base pairs (bp) in length. Phylogenetic analysis indicated that the BMT4i-nidA gene is genetically the most distant from other *Mycobacterium* species sequences. However, the partial coding sequence (CDS) of the *Bacillus subtilis* BMT4i-nidA gene showed the closest homology to the partial nidA gene CDS from bacterium py116 (GenBank Accession no. HM049715.1).

**Key word:** Benzo (a) pyrene (BaP) degradation • High molecular weight- polyaromatic hydrocarbon (HMW-PAH) • nidA gene homolog • Ring hydroxylating dioxygenase (RHD) gene.

## Introduction

High-molecular-weight polyaromatic hydrocarbons (HMWPAHs) are composed of three or more aromatic rings arranged in linear, angular, or clustered structures and pose significant environmental concerns due to their potential toxicity to higher organisms (Patel et al., 2020). These compounds are notoriously difficult to degrade, given their chemically inert structure and hydrophobic nature, making them resistant to microbial degradation and persistent in the environment (Volkering et al. 1995, Harms and Bosma 1997). Their low bioavailability and bio-accessibility are further exacerbated by their slow diffusion kinetics and high octanol-water partition coefficient (Mackay and Callcott 1992, Lily et al. 2011).

Bacterial degradation of PAHs occurs via metabolic and co-metabolic pathways (Kanaly and Harayama 2010). In aerobic metabolism, PAHs are enzymatically converted into dihydroxy compounds through cis-dihydrodiols as intermediates. Multiple ring cleavage events (ortho, meta, or both) involving these dihydroxy compounds result in the formation of catechol, gentisate, protocatechuate, and phthalates, which are further metabolized into intermediates of the TCA cycle (Peng et al. 2008). Dioxygenase enzymes play a crucial role in bacterial PAH degradation by incorporating two oxygen atoms into adjacent carbon atoms of aromatic rings. These metalloenzymes, containing iron-sulfur centers at their active sites, are categorized into ring-hydroxylating dioxygenases (RHDs) and ring-cleaving dioxygenases (RCDs) (Kim et al. 2004, 2007). RHDs add two hydroxyl groups to adjacent carbons on a ring, while RCDs catalyze the cleavage of bonds between hydroxylated carbon atoms in aromatic compounds. RHDs utilize NADH/NADPH as electron donors to transfer electrons to the iron-sulfur center for the dioxygen activation reaction (Jakoncic et al.

2007, Wackett et al. 2002). The oxygenase (α3β3) component of RHDs contains a Rieske-type (2Fe-2S) cluster and a mononuclear iron center in each alpha subunit (Ferraro 2005, Vila et al. 2016).

RHD genes have been identified in various genera of both Gram-positive and Gram-negative bacteria. In Gram-positive bacteria, RHD genes include nid genes in *Mycobacteria* spp. and *Diaphorobacter* spp., pdo and dfn/fln genes in *Terrabacter* spp., and nar genes in *Rhodococcus* sp. strain NCIMB12038 (Abo-State and El-Kelani 2020). In Gram-negative bacteria, RHD genes include nah-like genes in *Pseudomonas* sp. strain G7 (Habe and Omori 2003), phd genes in *Comamonas testosterone* strain GZ39 (Martin et al. 2013), nag genes in *Ralstonia* sp. strain U2 (Muangchinda et al. 2014), and phn genes in *Burkholderia* sp. strain RP007 (Laurie and Lloyd-Jones 1999).

Given the essential role of RHD genes in HMWPAH biodegradation, numerous bacterial species capable of degrading PAHs are being studied for the presence of these genes. *Bacillus subtilis* BMT4i, a well-known PAH degrader isolated from the hilly region of Uttarakhand, India, can efficiently degrade BaP, using it as a sole source of carbon and energy through a chromosomally encoded pathway (Lily et al. 2009, 2010). HR-LC-MS-MS analysis of BaP metabolic intermediates produced by BMT4i indicated the presence of RHD as the initial ring-attacking enzyme (Bhatt et al. 2019). Therefore, the present study focused on isolating and characterizing the nidA gene, which encodes the catalytic subunit of the RHD enzyme, from *Bacillus subtilis* BMT4i.

## Material and Methods

### Amplification of Dioxygenase Gene (*nid*A)

The genomic DNA of BMT4i was extracted using the GeneiPure™ Bacterial DNA Purification Kit, following the manufacturer's instructions (Merck Pvt Ltd, India). PCR amplification of the large subunit of the dioxygenase gene (nidA) was conducted using the GeneiPure™ PCR Amplification Kit (Merck Pvt Ltd, India). The primers used for amplification were: forward primer 5′-ATGACCACCGAAACAACCGGAACAGC-3′ and reverse primer 5′-TCAAGCACGCCCGCCGAATGCGGGAG -3′. The 20 µl PCR reaction mixture comprised 1X PCR amplification buffer (GeneiPure™ PCR Amplification Kit), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 100 pmol each of forward and reverse primers, 100 ng of template genomic DNA, and 3 units of Taq DNA polymerase. The thermal cycler (Biometra, Germany, model Tpersonal) was programmed for the amplification of the nidA subunit of the dioxygenase gene under the following conditions: denaturation at 94°C for 30 seconds, annealing at 64°C for 1 minute, and extension at 72°C for 1 minute, repeated for 30 cycles. The amplified PCR products were purified using the HiPurA Purification Spin Kit, MB 505 (Himedia Pvt. Ltd, India), and separated on a 3.5% agarose gel. The amplified DNA fragments were then eluted from the gel and further purified using the HiPurA DNA Purification Spin Kit, MB 505 (Himedia Pvt. Ltd, India).

### Cloning and Sequencing of nidA Gene

The amplified DNA fragment was cloned into *E. coli* JM109 using the pJET1.2 cloning vector (Clone-JET PCR Cloning Kit, Thermo Fisher Scientific Inc.). The blunting reaction was initiated by adding 10 µL of 2X Reaction Buffer, 1 µL of the purified PCR product, and 1 µL of the DNA Blunting Enzyme provided by the manufacturer. The reaction volume was adjusted to 18 µL, vortexed, centrifuged for 3-5 seconds, and incubated at 70°C for 5 minutes, followed by immediate chilling on ice.

Next, the ligation reaction was prepared on ice by adding 1 µL of the pJET1.2 blunt cloning

vector (50 ng/µL) and 1 µL of T4 DNA ligase to the blunting reaction mixture. The mixture was vortexed, centrifuged briefly, and incubated at 22°C for 5 minutes. The ligation mixture was then stored at -20°C until further use.

To prepare for cloning, *E. coli* JM109 competent cells were made according to Sambrook et al. (1999). A 5 µL aliquot of the ligation mixture was transferred to a sterile 1.5 mL microcentrifuge tube containing 50 µL of competent cells. After gentle mixing, the tube was kept on ice for 20 minutes. The cells were then heat-shocked by placing the tube in a 42°C water bath for 2 minutes without shaking. Immediately after that, the tube was returned to ice for 5 minutes before adding 500 µL of LB broth. The mixture was incubated at 37°C with shaking (~150 rpm) for 1 hour. Subsequently, 100 µL of the transformed culture was spread onto LB agar plates containing ampicillin (50 µg/mL) and incubated overnight (16–24 hours) at 37°C. The colonies that appeared on the LB-Amp plates were identified as recombinants.

The recombinant *E. coli* JM109, containing the pJET1.2 vector with the nidA gene fragment, was sent to Merck Millipore, Bengaluru, Karnataka, India, for sequencing. Sequence analysis was conducted using an automated ABI 3100 genetic analyzer, which employs fluorescent dye terminator cycle sequencing based on Sanger's method. The quality of pure and mixed bases was assessed using Sequencing Analysis Software v.5.1 with kb basecaller.

**Alignment and Phylogenetic Tree Analysis**

The partial sequence of the BMT4i-nidA gene obtained from Merck Millipore, Bengaluru, Karnataka, India, was used for phylogenetic analysis via the NCBI website (www.ncbi.nlm.nih.gov). A Nucleotide BLAST (Blastn) search was performed, and sequences with trimming and incomplete regions were manually removed from the 5' and 3' ends before conducting the similarity search on NCBI. The nidA and nidB sequences that showed alignment with the BMT4i-nidA query sequence were downloaded in FASTA format from the NCBI database.

The downloaded sequences, along with the input query sequence, were aligned using the "Muscle" multiple sequence alignment tool in MEGA 6 software, and a phylogenetic tree was generated (Kumar et al. 2016). Additionally, a phylogenetic tree was constructed using the maximum likelihood method with a bootstrap value of 5000. Evolutionary distances were estimated using the Kimura 2-parameter model (Kimura 1980).

## Result and Discussion

### Cloning of nidA Gene from BaP Degrading *Bacillus subtilis* BMT4i.

The BMT4i genomic DNA served as a template for amplifying the dioxygenase gene (Figure 1A). Since the nidA gene from *Mycobacterium* sp. strain PYR-1 is a highly conserved gene encoding the catalytic subunit of a novel RHD—and its homologs have been identified in numerous bacterial species with HMWPAH-degrading activities (Khan et al 2001, Kanaly and Harayama 2010)—the primer sequences used by Khan et al. (2001) for nidA were synthesized and employed to amplify the nidA gene in BMT4i. PCR amplification of the nidA homolog was performed under optimal conditions (Khan et al. 2001), and the amplified product was analyzed on a 3.5% agarose gel (Figure 1B). The results showed that the amplified fragment was approximately 350 base pairs (bp) in length, which is significantly smaller than the 1368 bp amplified product reported by Khan et al. (2001).

J. Mountain Res. P-ISSN: 0974-3030, E-ISSN: 2582-5011 DOI: https://doi.org/10.51220/jmr.v19-i2.30
Vol. 19(2), (2024), 281-289

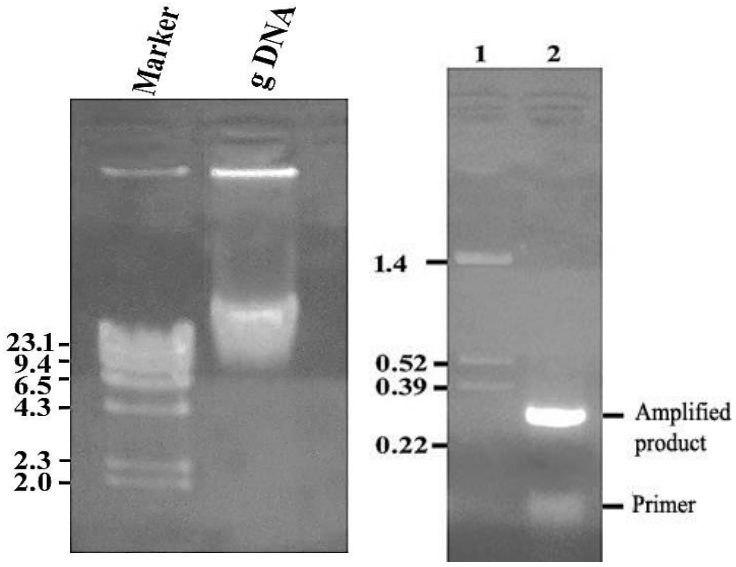


**Figure 1. A) BMT4i Genomic DNA; B) PCR Amplified nidA Partial Gene Fragment**

The purified 350 bp amplified DNA fragment was subsequently cloned into the pJET1.2 cloning vector. Recombinant colonies were then selected on LB-ampicillin plates (Figure 2).

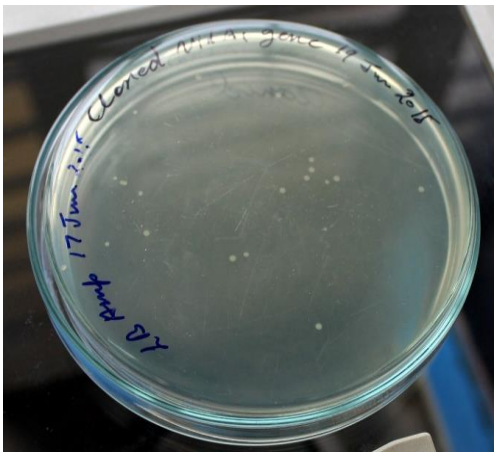

**Figure 2. Recombinant Colonies with Cloned BMT4i-nidA Partial Gene Fragment.**

**Sequencing of BMT4i-nidA Clone**

The *BMT4i*-nid*A* clone fragment was sequenced commercially by Merck Specialties Pvt Ltd, Bengaluru, Karnataka, India. The resulting electropherogram data (Figure 3) confirmed that the BMT4i-nid*A* clone was 376 bp in length (Figure 4).

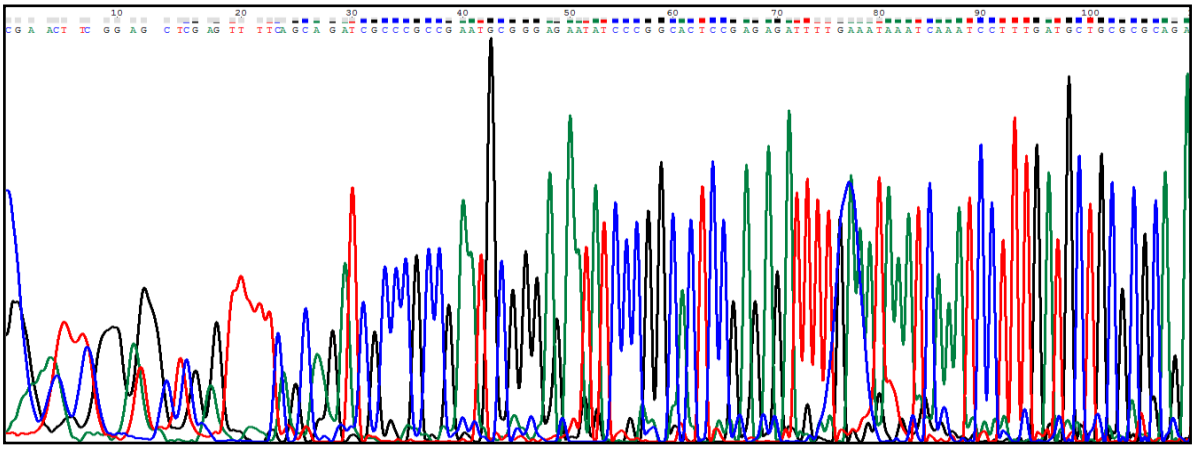

**Figure 3. Electropherogram Data of the BMT4i-nidA Clone**

**Phylogenetic Analysis of BMT4i-nidA Partial Gene Sequence**

The sequence of the BMT4i-nidA partial gene was submitted to the NCBI GenBank. This sequence was then used for phylogenetic analysis on the NCBI website (www.ncbi.nlm.nih.gov) using a Nucleotide BLAST (Blastn) search. The nidA and nidB sequences that aligned with the BMT4i-nidA query sequence were downloaded in FASTA format from NCBI. These sequences, along with the query sequence, were aligned using the "Muscle" multiple sequence alignment tool in MEGA 6 software. A phylogenetic tree was then constructed by estimating evolutionary distances with the Kimura 2-parameter model method (Tamura et al. 2007). The BLASTn search revealed that the BMT4i-nidA partial gene sequence shared homology with 22 nidA and nidB CDS sequences from *Mycobacterium* and other bacterial species.

>nidAgene, *Bacillus subtilis* BMT4i (MTCC9447) 376bp.

GCTCGAGTTTTTCAGCAAGATCGCCCGCCGAATGCGGGAGAATATCCCGGCACTCCGAGAGATTTTGAAAT
AAATCAAATCCTTTGATGCTGCGCGCAGATCCGTTGGCAATGCCTTTTTCTTCATCGATACCGAGAACGATG
GCGGGCCTGTAATACCGCTCGACAAGCTTTGAAGCCACGATTCCGACGACACCGGGATTCCAGCCCGGCC
GCGCCGCGACGATCACCGGCCGGTCAAGCCCTTCCTCCTCGATCATCTGAACGGCTTCCTCTGTCATCTGAT
TGACGATTTTTTGGCGCTCCTTGTTCAAGGCGTTGATATCTTCGGCCAGCTGTTCCGGTTGTTTCGGTGGTC
ATATCTTTCTAGAAGTTCT

**Figure 4. Cloned BMT4i-nidA Partial Gene Sequence**

A phylogenetic tree was constructed using the maximum likelihood method with a bootstrap value of 5000, incorporating 23 nucleotide sequences: one from the partial coding sequence (CDS) of the *Bacillus subtilis* nidA gene and 22 nidA and nidB CDS sequences from *Mycobacterium* and other bacterial species

*subtilis* BMT4i-nidA gene was found to be genetically the most distant from the other sequences. Despite this, the *Bacillus subtilis* BMT4i-nidA gene partial CDS showed the closest homology to the nidA gene partial sequence CDS from bacterium py116 (GenBank Accession No. HM049715.1) (DeBruyn et al. 2012).

| Score | Expect | Identities | Gaps | Strand | Frame |
|---|---|---|---|---|---|
| 676 bits(366) | 0.0() | 373/376(99%) | 2/376(0%) | Plus/Minus | |

Features:

```
Query  12   GCTCGAG-TTTTCAGC-AGATCGCCCGCCGAATGCGGGAGAATATCCCGGCACTCCGAGA  69
           ||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  408  GCTCGAGTTTTTCAGCAAGATCGCCCGCCGAATGCGGGAGAATATCCCGGCACTCCGAGA  349

Query  70   GATTTTGAAATAAATCAAATCCTTTGATGCTGCGCGCAGATCCGTTGGCAATGCCTTTTT  129
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  348  GATTTTGAAATAAATCAAATCCTTTGATGCTGCGCGCAGATCCGTTGGCAATGCCTTTTT  289

Query  130  CTTCATCGATACCGAGAACGATGGCGGGCCTGTAATACCGCTCGACAAGCTTTGAAGCCA  189
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  288  CTTCATCGATACCGAGAACGATGGCGGGCCTGTAATACCGCTCGACAAGCTTTGAAGCCA  229

Query  190  CGATTCCGACGACACCGGGATTCCAGCCCGGCCGCGCCGCGACGATCACCGGCCGGTCAA  249
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  228  CGATTCCGACGACACCGGGATTCCAGCCCGGCCGCGCCGCGACGATCACCGGCCGGTCAA  169

Query  250  GCCCTTCCTCCTCGATCATCTGAACGGCTTCCTCTGTCATCTGATTGACGATTTTTTGGC  309
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  168  GCCCTTCCTCCTCGATCATCTGAACGGCTTCCTCTGTCATCTGATTGACGATTTTTTGGC  109

Query  310  GCTCCTTGTTCAAGGCGTTGATATCTTCGGCCAGCTGTTCCGGTTGTTTCGGTGGTCATA  369
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  108  GCTCCTTGTTCAAGGCGTTGATATCTTCGGCCAGCTGTTCCGGTTGTTTCGGTGGTCATA  49

Query  370  TCTTTCTAGAAGATCT  385
           |||||||||||| |||
Sbjct  48   TCTTTCTAGAAGTTCT  33
```

**Figure 5. Blast Search to Retrieve the Cloned nidA Gene Sequence.**

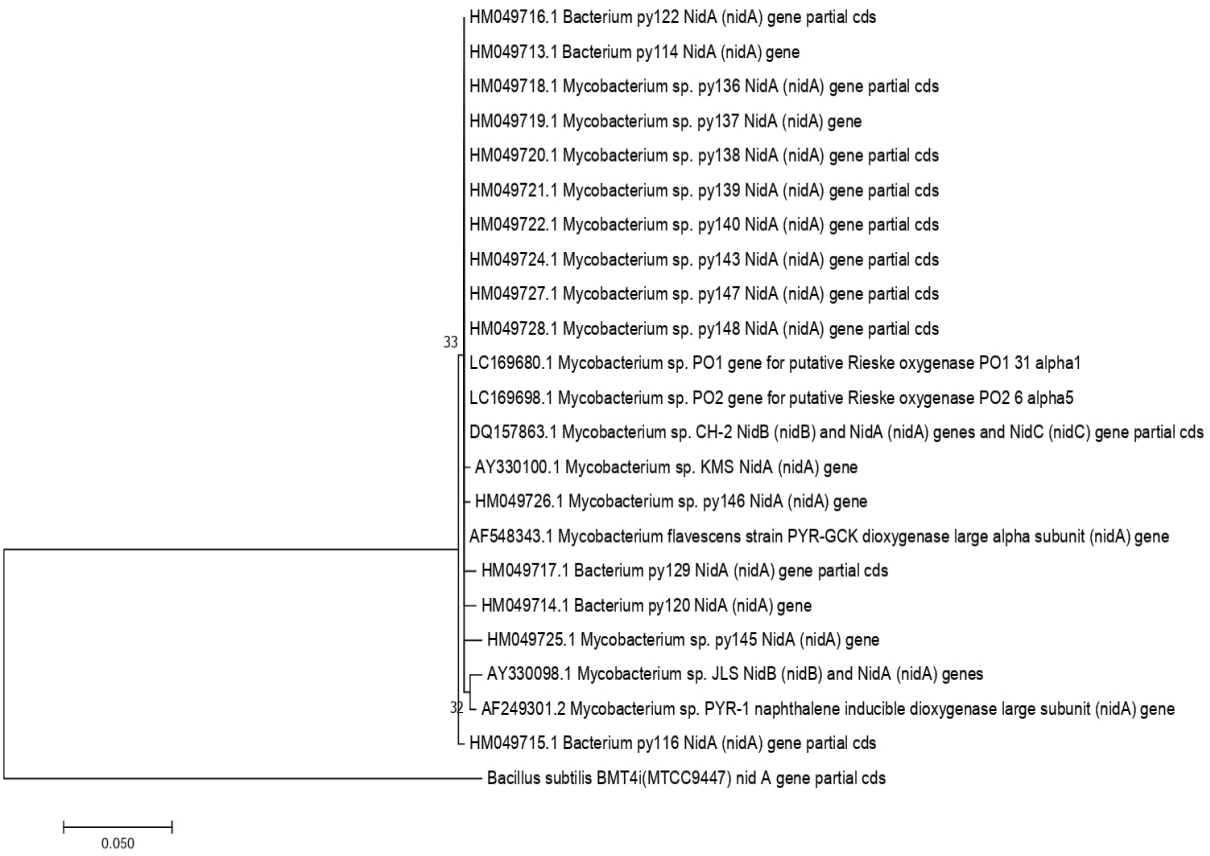


**Figure 6. Phylogenetic Tree of BMT4i-nidA Partial Gene Sequence Showing Homology with nidA and nidB Gene Sequences in Other Bacterial Strains**

To the best of our knowledge, this is the first report demonstrating the cloning of a nidA homolog from any Bacillus species. The presence of ring-hydroxylating dioxygenases (RHDs) in *Bacillus subtilis* BMT4i is consistent with findings from previous studies (Dacunha et al. 2006,). Yuliani et al. (2012) identified nidA and nidAc genes, which encode different components of a multicomponent RHD involved in pyrene degradation. Dacunha et al. (2006) reported the presence of two non-heme dioxygenases, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, in a *Bacillus* strain isolated from oil basins. Additionally, Stancu (2019) documented the presence of naphthalene dioxygenase (ndoM gene) in *Bacillus megaterium* IBB Po17.

## Conclusion

The study successfully cloned a partial dioxygenase gene (nidA) and determined its sequence to be 376 bp in length. Phylogenetic analysis showed that the *Bacillus subtilis* BMT4i-nidA gene was the most genetically distant from other sequences. However, the partial coding sequence (CDS) of the *Bacillus subtilis* BMT4i-nidA gene exhibited the closest homology to the partial CDS of the nidA gene from bacterium py116 (GenBank Accession No. HM049715.1).

## Acknowledgment

We gratefully acknowledge the financial support from the Department of Science and Technology (DST), New Delhi, India (DST No: SR/SO/BB-0068/2012), which made this research possible.

## References

Abo-State MAM and El-Kelani TA (2020) Bacterial Biodegradation Pathways of Low and High Molecular Weight Polycyclic Aromatic Hydrocarbons (PAHs). Arch. Pet. Environ. Biotechnol. 5: 158. DOI: 10.29011/2574-7614.100058

Bhatt KK, Lily MK, Joshi G, Dangwal K (2018) Benzo(a)pyrene degradation pathway in *Bacillus subtilis* BMT4i (MTCC 9447). Turkish J Biochem.

Dacunha PT, Chernicharo CAL and Carvalho, J (2006) Detection of two non-heme dioxygenases in *Bacillus* strain isolate from oil basins. J. Ind. Microbiol. Biotechnol. 33(6): 439–447.

Das A, Das N, Rajkumari J, Pandey P and Pandey P (2024) Exploring the bioremediation potential of *Bacillus* spp. for sustainable mitigation of hydrocarbon contaminants. Environ. Sustain. https://doi.org/10.1007/s42398-024-00309-9

DeBruyn, JM, O'Connor SM and Unkefer CJ (2012) Characterization of a dioxygenase gene cluster involved in the degradation of polycyclic aromatic hydrocarbons (PAHs) in Mycobacterium sp. PYR-1." Appl. Environ. Microbiol. 78(24): 8495–8503.

Ferraro DJ, Gakhar L and Ramaswamy S (2005) Rieske business: Structure-function of Rieske non-heme oxygenases. Biochem. Biophys. Res. Commun. 338: 175–190

Habe H and Omori T (2003) Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. Biotechnol. Biochem. 67(2): 225–243

Harms H and Bosma T (1997) Mass transfer limitation of microbial growth and pollutant degradation. J. Ind. Microbiol. Biotechnol. 18: 97–104

Jakoncic J, Jouanneau Y, Meyer C and Stojanoff V (2007) The catalytic pocket of the ring-hydroxylating dioxygenase from *Sphingomonas* CHY-1. Biochem. Biophys. Res. Commun. 352: 861–866

Kanaly RA and Harayama S (2010) Advances in the field of high molecular weight polycyclic aromatic hydrocarbon biodegradation by bacteria. Microb. Biotechnol. 3:136–164

Khan ST, McKinnon JC and Bouwer EJ (2001) Phylogenetic Analysis of the Ring-Hydroxylating Dioxygenases from *Mycobacterium* sp. Strain PYR-1 and Related Bacteria. Appl. Environ. Microbiol. 67(8): 3600–3608.

Kim SJ, Jones RC, Cha CJ, Kweon O, Edmondson RD and Cerniglia CE (2004) Identification of proteins induced by polycyclic aromatic hydrocarbon in *Mycobacterium vanbaalenii* PYR-1 using two-dimensional polyacrylamide gel electrophoresis and de novo sequencing methods. Proteomics 4: 3899–3908

Kim SJ, Kweon O, Freeman JP, Jones RC, Adjei MD, Jhoo JW, Edmondson RD and Cerniglia CE (2006) Molecular cloning and expression of genes encoding a novel dioxygenase involved in low- and high-molecular weight polycyclic aromatic hydrocarbon degradation in *Mycobacterium vanbaalenii* PYR-1. Appl. Environ. Microbiol. 72: 1045–1054

Kimura M (1980) A Simple Method for Estimating Evolutionary Rates of Base Substitutions through Comparative Studies of Nucleotide Sequences. J. Mol. Evol. 16(2): 111–120. doi:10.1007/BF01731584

Kumar S, Stecher G, Tamura K (2016) "MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33(7): 1870–1874. doi:10.1093/molbev/msw054

Laurie AD and Lloyd-Jones G (1999) The phn genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. J. Bacteriol. 181(2): 531–540

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

Lily MK, Bahuguna A, Dangwal K and Garg V (2010) Optimization of an inducible chromosomally encoded benzo(a)pyrene (BaP) degradation pathway in *Bacillus subtilis* BMT4i (MTCC 9447). Ann. Microbiol. 60: 51–58

Lily MK, Bahuguna A, Dangwal K and Garg V (2011) Biodegradation of Benzo-a-pyrene (BaP) by *Bacillus subtilis* BMT4i (MTCC 9447): Isolation, Identification of BaP Degrading Bacteria & Characterization of BaP Degradation Activity. LAP LAMBERT Academic Publishing GmbH & Co. KG, Dudweiler Landstr. 9966123 Saarbrücken, Germany. ISBN 978-3-8443-2455-6

Lu X, Zhang Y, Wang X and Li Y (2011). "The bioremediation potential of indigenous microorganisms isolated from contaminated soils and sediments." J. Hazard. Mater. 185, 687–695.

Mackay D and Callcott D (1998) Partitioning and physical and chemical properties of PAHs. In The Handbook of Environmental Chemistry Vol. 3 Part I PAHs and Related Compounds, edited by A. H. Neilson. Springer-Verlag Berlin Heidelberg

Martin F, Malagnoux F, Violet F, Jakoncic J and Jouanneau Y (2013) Diversity and catalytic potential of PAH-specific ring-hydroxylating dioxygenases from a hydrocarbon-contaminated soil. Appl. Microbiol. Biotechnol. 97(11): 5125–5135

Muangchinda C, Chavanich S, Viyakarn K, Watanabe K, Imura S, Vangnai AS and Pinyakong O (2014) Abundance and diversity of functional genes involved in the degradation of aromatic hydrocarbons

in Antarctic soils and sediments around Syowa Station. Environ. Sci. Pollut. Res. 22(6): 4725–4735

Patel AB, Shaikh S, Jain KR, Desai C and Madamwar D (2020) Polycyclic Aromatic Hydrocarbons: Sources, Toxicity, and Remediation Approaches. Front. Microbiol. 11:562813.

Peng X, Liang Y, Yang S and Zhang T (2008) "Enhanced degradation of polycyclic aromatic hydrocarbons by combined use of microbial consortium and surfactant." J. Environ. Sci. 20, 212–217.

Sambrook J, Fritsch EF and Maniatis T (1999) Molecular Cloning: A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Stancu M C (2019) Identification and characterization of naphthalene dioxygenase from *Bacillus megaterium* IBB Po17. J. Appl. Microbiol. 127(5): 1335–1348.

Tamura K, Dudley J, Nei M and Kumar S (2007) Mega4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24(8): 1596–1599.

Vila MA, Umpiérrez D, Seoane G, Rodríguez S, Carrera I and Veiga, N (2016) Computational insights into the oxidation of mono- and 1,4 disubstituted arenes by the Toluene Dioxygenase enzymatic complex. J. Mol. Catal. B Enzym. 133: S410–S419

Volkering F, Breure AM, Van Andel JG and Rulkens WH (1995) Influence of nonionic surfactants on bioavailability and biodegradation of polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 61: 1699–1705

Wackett LP (2002) Mechanism and applications of Rieske non-heme iron dioxygenases. Enzym. Microb. Technol. 31: 577–587

Yuliani S, Riwanto S and Purwanto S (2012) Identification and characterization of genes involved in the degradation of polycyclic aromatic hydrocarbons (PAHs) by *Mycobacterium* sp. strain PYR-1. Int. J. Environ. Sci. Technol. 9(3): 395–406.