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16S rDNA GENE BASED IDENTIFICATION OF BACTERIA ISOLATED FROM SOIL AMENDED WITH *PARTHENIUM HYSTEROPHORUS* L.

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ABSTRACT

The present paper deals with the identification of bacteria through 16S rDNA based molecular technique. DNA isolated from pure culture of cropland soil and *Parthenium hysterophorus* amended was identified as *Methylobacterium* sp. HJM 27 (Gene bank accession number: HM243761.1) and *Bacillus* sp. BFF-3 (Gene bank accession number: EF031071.1) respectively. *Methylobacterium* sp. is a gram negative, rod shaped bacteria which forms pink pigmented and elevated bacterial colony. The *Bacillus* sp. is an aerobic, endospore forming and mobile gram positive rod shaped bacteria which form irregular and undulate bacterial colony. PCR amplification products of 1.5Kb in the form of amplicon bands were observed on agarose gel. Results of forward and reverse DNA sequencing of PCR amplicon with 8F and 1492R were 876 bp and 687 bp for *Methylobacterium* sp. HJM 27 and 839 bp and 989 bp for *Bacillus* sp. BFF-3. Comparison of DNA sequences of both isolates with nr database of NCBI gene bank database using BLAST showed 99 – 100% maximum identity and E - value equal to 0 for all closely related taxa. The phylogenetic study revealed their evolutionary status with other 10 homogenous close homologs.

INTRODUCTION

In a balanced soil, plants grow in an active and vibrant environment. Along with minerals and physical characteristics of soil, soil organisms also help to increase fertility and productivity of cropped land. Without the activities of soil organisms, organic materials would accumulate and litter the soil surface and there would be no food for plants. Soil biotas are thought to harbour a large part of the world's biodiversity that include organisms from microscopic microflora to macrofauna. The microbes are the important elements of the soil environment as they participate in the degradation of the organic matter and make the nutrients available to other soil organisms. More than 80% of the carbon which passes through the heterotrophic component of the ecosystem is released by microorganisms and the amount of nitrogen passing through the soil microbial population is more than twice that passes through the primary producer (Heal and Maclean, 1975). Soil microbes are also responsible for sequestration of green house gases especially methane. They oxidize atmospheric methane and are responsible for an estimated 5 – 10 % of its total removal from atmosphere (Cicerone and Oremland, 1988).

Although cultivation based biochemical techniques have been used for analysis of the specific groups of bacteria, several limitations are associated with such approaches. The introduction of high resolution molecular techniques has improved the analysis of diverse microbial population (Muyzer, 1999). The important advance has been the use of 16S rRNA as a molecular fingerprint to identify and classify organisms (Ohkuma and Kudo, 1996). A number of molecular genetics techniques, such as total DNA isolation and characterization, G + C composition, rRNA sequence, PCR amplification of rDNA, PCR amplification of functional genes are also being used to study microbial communities (Akkermans *et al.*, 1995).

16S rDNA gene has been the preferred gene target for describing the soil microbial diversity and for establishing phylogenetic relationship between unknown and uncultivated microorganisms. DGGE (Denaturing gradient gel electrophoresis) of 16S rDNA polymerase chain reaction products had also been used to analyze soil microbial community composition (Nakatsu *et al.*, 2000). The BLAST programmes and phylogenetic profiler provides tools for searching protein DNA databases for sequence similarities and to identify genes in a genome (Altschul *et al.*, 1990, 1997).

In this experiment, bacterial identification was done by isolating, amplifying and comparing 16S rDNA gene sequences with the nr database of NCBI genebank data base using BLAST search programme. The evolutionary distance matrix was also analyzed and on that basis phylogenetic tree was constructed.

MATERIALS AND METHODS

Experimental setup and soil bacteria culture

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Soil was collected from agro-ecosystem of Ranchi, Jharkhand and was kept in two separate plastic containers under moist condition. One container was considered as control (normal soil) and other was amended with 10% powdered *Parthenium hysterophorus*. Bacterial cultures were done from both soil sample by dilution plate method (Thornton, 1922; Thom and Raper, 1945). The isolation of bacteria from soil samples was initiated by taking 1g of soil from both groups and was diluted with 9 mL of sterilized deionized water till 10^{-7} dilution. 1 mL inoculums of the primary suspension was taken for bacteria culture in a petriplate (diameter = 100mm) containing Czapek Dox agar media (peptone - 10g/L, beef extract - 10g/L, agar - 15g/L NaCl- 5g/L, pH- 7.2) and were inoculated at 37°C for 48 h. Morphology of colonies so cultured was studied, gram staining and identification of irregular-undulate colony from *Parthenium* amended soil and circular pinkish colony from cropland soil was further done.

Genomic analysis

DNA extraction and purification

For the total DNA isolation from the pure culture, bacterial cells were washed with TESS buffer [10 mM Tris/HCl, 1mM Na₂EDTA, 0.1 M NaCl and 0.1% Sarkosyl (N-lauroylsarcosine)] and resuspended in TE buffer (10 mM Tris/HCl, 1 mM Na₂EDTA). Cells were lysed with 50 mg lysozyme mL⁻¹ and 0.1% SDS. The subsequent phenol / chloroform extractions and ethanol precipitation were carried out as described by Sambrook *et al.* (1989). The quantity of the DNA was checked by running on 1.2% agarose gel. A single band of high molecular weight DNA has been observed. The extracted genomic DNA of both isolates were used as template DNA for amplification of the 16S rDNA gene.

Amplification of 16S rDNA gene by PCR

Amplification reaction was performed in 0.5 mL tubes containing 50 ng of the extracted DNA, 200 µM dNTPs, 1.5 mM MgCl₂, 1.25 U taq DNA polymerase (Sigma, USA) and 0.25 µM each of forward and reverse primers. PCR was performed under the following conditions: 45 S at 95°C and then 30 cycles of 15S at 94°C, 30 S at 53°C and 90 S at 72°C. Amplification products (PCR amplicon) of 1500 bp in the form of band were visualized on agarose gels (Fig. 1) and were used for further sequencing.

Sequence and sequence analysis

The PCR amplicon was purified to remove contaminants. Sequencing of 16S rDNA gene of both isolates were carried out with 8F and 1492R primers using BDT V3.1 cycle sequencing kit on ABI 373x1 genetic analyzer from both forward and reverse directions. The sequences obtained were compared with the nr database of NCBI genebank database using BLAST search program (<http://www.ncbi.nlm.nih.gov>) (Marchler - Bauer *et al.*, 2000; Pruitt *et al.*, 2005). The percentage of sequence matching were also analyzed and based on maximum identity score first ten sequences were selected and were aligned with those of closely related species using CLUSTAL W software (Thompson *et al.*, 1994).

Phylogenetic tree construction

The phylogenetic tree was constructed using Neighbour - Joining Tree MEGA - 4 bioinformatics software tool (Tamura

et al., 2007). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed.

RESULTS AND DISCUSSION

The bacterium isolated from control was identified as *Methylobacterium* sp. HJM 27 (Gene bank accession number: HM243761.1) based on nucleotide homology and phylogenetic analysis. It is a gram negative, rod shaped bacteria which forms pink pigmented and elevated bacterial colony. On the other hand, bacterium isolated from *Parthenium hysterophorus* amended soil was identified as *Bacillus* sp. BFF-3 (Gene bank accession number: EF031071.1). The genus *Methylobacterium* is a group of strictly aerobic, facultative or obligate, methylophilic bacteria that are able to grow on one - carbon compounds as sole carbon and energy sources (Green, 1992). They are often red to pink because of the presence of carotenoids (Trotsenko *et al.*, 2001). The *Bacillus* sp. is an aerobic, endospore forming and mobile gram positive rod shaped bacteria which form irregular and undulate bacterial colony.

Result of forward and reverse DNA sequencing of PCR amplicon were 876 bp and 687 bp for *Methylobacterium* sp. HJM 27 and 839 bp and 989 bp for *Bacillus* sp. BFF-3 when carried out with 8F and 1492R primer (Table 1- 4). Other close homologs of both isolates along with their accession number, based on BLAST programme have been shown in Table 5 and 6. The maximum identity of sequence was 99 - 100% and E - value is equal to 0 which depicts that all the 10 close homologs of both isolates are homogenous to identified strains. HM243761.1 and EF031071.1 shows maximum identity score of 100% and it implies that the sample analysed were *Methylobacterium* sp. HJM27 and *Bacillus* sp. BFF - 3.

All the 10 closely related homologs of identified bacteria were used for the construction of the phylogenetic dendrogram to know their evolutionary origin. The dendrogram showing the relation between *Methylobacterium* sp. HJM27 and their close homologs is shown in Fig. 2. The dendrogram showed that the sample - A is closely related with EU741076.1 with bootstrap value of 24 (at node). The monophyletic groups of EU741076.1, HM007540.1, AY468371.1 and EU741082.1 have close relation with HM243761.1 with 31% bootstrap value. AY468370.1, NR029082.1, AY251818.2, AY728074.1 and DQ490354.1 are the other distant members of ancestors of *Methylobacterium* sp. HJM27. Phylogenetic tree of sample - B (Fig. 3) shows that it is in close association with EF031071.1 which has bootstrap value of 99 (at node). This monophyletic group of sample and *Bacillus* sp. BFF-3 further show close relation with FJ644692.1 (*Bacillus cereus* strain MX3) and have bootstrap value 74. This entire group has bootstrap value 34 with HM003212.1 (*Bacillus cereus* strain WYLW1-7). Again the value for HM003208.1, GU085229.1 group, EU857430.1, GQ199727.1 group and FJ959367.1, FJ763650.1 monophyletic group is 18, 23 and 51 respectively. All these strains together have bootstrap value 64 and are also other descendants of ancestor of *Bacillus* sp. BFF-3 strain but they are distant members of sample - B and *Bacillus* sp. BFF-3 (EF031071.1) monophyletic group. Felsenstein (1985) proposed that bootstrap value of 95% or greater be considered

Table 1: Forward sequence: 876 bp of *Methylobacterium* sp. HJM27

```
CGTTGCTCGGAATCACTGGGCGTAAAGGGCGCGTAGCGGGCCGATTAAGTCGGGGGTGAAAGCCTGTGGCTCAACCACAGAATTG
CCTTCGATACTGGTTGGCTTGAGACCGGAAGAGGACAGCGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACC
AGTGGCGAAGGCGGCTGTCTGGTCCGGTTCTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAACGATGAATGCCAGCCGTTGGCCTGCTTGCAGGTCAGTGGCGCCGCTAACGCATTAAGCATTCCGCTGGGGAGTAC
GGTCGCAAGATTAATAACTCAAAGGAATTGACGGGGGCCCCGACAAAGCGGTGGAGCATGTGTTTAAATTCGAAGCAACGCGCAGAAC
CTTACCATCCCTTGACATGGCATGTACCTCGAGAGATCGGGATCCTCTTCGGAGGCGTGCACACAGGTGCTGCATGGCTGTCTGCA
GCTCGTGTGATGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCAGTCCTTAGTTGCCATCATTAGTTGGGGCACTTAGGGAG
ACTGCCGGTGATAAGCCGCGAGGAAGGTGTGGATGACGTCAAGTCTCATGGCCCTTACGGGATGGGCTACACACGTGCTACAATG
GCGGTGACAGTGGGACGCGAAACCGCGAGTTGAGCAAATCCCCAAAAGCCGTCTCAGTTCGGATTGCACTCTGCAACTCGGGTGC
ATGAAGGCGGAATCGTAGTAATCGTGGATCAGCACGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTACACCATG
GGAGTTGGTCT
```

Table 2: Reverse sequence: 687 bp of *Methylobacterium* sp. HJM27

```
GCGCCTCAGCGTCAGAACCGGACCAGACAGCCGCCTTCGCCACTGGTGTCTTGCGAATATCTACGAATTTACCTCTACACTCGCAG
TTCCGCTGTCTCTTCCGGTCTCAAGCCAACAGTATCGAAGGCAATTCTGTGGTTGAGCCACAGGCTTACACCCCGACTTAATCGGC
CGCCTACGCGCCCTTACGCCAGTGATTCCGAGCAAACGATAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGG
CTTATTTCCGGTACCTGATTCATGTCCTCCGCAAAAAGCTTTAACAACCTAAGGCCTTACACTACCGCGGATGGCTGGATCA
GGCTTGGCCCAATTGTCCAATATTCGCCACTGCTGCCTCCGTTAGGACTCTGGCCGTCTCAGTCCCAGTGTGGCTGATCCTCTC
TCAGACCAGTACTGATCGTCGCTTGGTAGGCCGTTACCCACCAACAAGCTAATCAGACGCGGGCCGATCCTTCGGCAGTAAACCT
TTCCCATAAGGGCGTATCCGGTATTAGCTCAAGTTTCCCTGAGTATTCCGAACCGAAGGGCACGTTCCACGTTACTACCCGCT
TGCCACTGACTTCCGAAGAAGCCCGTTCGACTTGCATGTGTTAAGCCTGCCGCCAGCGTTCCGCTCTGAG
```

Table 3: Forward sequence: 839 bp of *Bacillus* sp. BFF-3

```
GCGCTGCTAACTGCAGTCGAGCGAACGGATTGGAACCTTGGTTCTTGAATTCGCCGACAGGGGTGAATAACATGTGTGACCCCGC
CCATAACAGTGGTAACACTCCGGGAAACGAGGGCTAACACGAGAGAACCCTTTGAACCACGGGGTTCGGATTTGAAAGGGTGTCTCC
GGTGTATTATAGATGCCCCCGCTCACAAATCTTTTTGTGATGTAGCGGCTACCCAGGGCAATATGCATACCCACCTGAGGGG
GTGATCGGCCACACTGGGACAGACACAGGGCCACACCCCAAGGGGGCCGCTGTGGGGAATCTTCCACAGTGGAAAAATGTCA
GACAGAACCGCCCGCGAGTGTGAAAAGTGCTTTCAGGTCATAAATCTTTTTTAGGGAAAAACATGTGCTAGTTGAAGAACCCTGGC
ACCTTGGCGGTTCTAACCAAAAACGCCGCGGCTAACTACCTGCCAGCCGCCGCGTAATACGTAGGTGGCAAGTGTATCCAGAATTA
TGGGGCGTAGGGCGCGCGGAGGTGTTTTCTAATTATGATGTGAAACCCCGCGGCTCACCCGTGGAGGGTCAATTGAAAACCTGGGA
GACTTGAGTGCAGAAGAGGAAAAGTGAATTCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGGC
ACTTCTGGTCTGTAACACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACCGAT
GAGTGCTAAGTGTAGAGGGTTCCGCCCTTATGCTGAAGTTAACGCATTAAGCA
```

Table 4: Reverse sequence: 989 bp of *Bacillus* sp. BFF-3

```
CCCAATATGCGGCTGGCTCCAAAAGTTACCCACCCACTTCGGGTGTTACAAAACCTCTCGTGGTGTGACGGGCGGTGTGTACAAGGC
CCGGAAACGTATTACCCGCGCATGCTGATCCGCGATTACTAGCGATTCCAGTTCATGTAGGCGAGTTGCAGCCTACAATCCGAAC
TGAACCGTTTTATGAGATTAGCTCCACCTCGCGTCTTGCAGCTCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCAGGTCATAAG
GGGCATGATGATTTGACGTCATCCACCTTCTCCGGTTTGCACCGGCAGTCACCTTAGAGTGCCCAACTTAATGATGGCAACTAAG
ATCAAGGGTTGCGCTCGTTGCGGACTTAACCCAAACATCTCACGACAGGCTGACGACAACCATGCACCACCTGTACTCTGCTCCCG
AAGGAGAAGCCCTATCTCTAGGGTTTTAGAGGATGTCAAGACCTGGTAAGTTCTTCGCGTGTCTCGAATTAACCCACATGCTCCAC
CGCTTGTGCGGGCCCCGTCAACTCCTTTGAGTTTCAGCCTTCCGCGGCTACTCCCCAGGCGGAGTGTCTAATGCGTTAACTTCAGCAC
TAAAGGGCGGAAACCCCTAACACTTAGCACTACGTTTACGCGTGGACTACCAGGTATCTAATCCCTGTTTGTCTCCACGCTTTCCG
CGCCTCAGTGTGAGTTACAGACCAGAAAGTCCGCTTCCGCACTGGTGTCTCCATATCTCTACGCATTTACCCGCTACACATGGAATTC
ACTTTCCTTCTGCACTCAAGTCTCCAGTTTCCAATGACCCTCCAGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCCACTG
CGCGCGCTTACGCCAATAATTCCGGATACGCTTGCACCTACGTATTACCGCGGCTGTGGCACGTAGTAGCGTGGCTTTCTGCTA
GGTACGTCA
```

Table 5: Close homologs of *Methylobacterium* sp. HJM27

Accession	Description	Max. score	Total score	Query coverage	E value	Max. ident.
HM243761.1	<i>Methylobacterium</i> sp. HJM27	2488	2488	100%	0.0	100%
EU741082.1	<i>Methylobacterium</i> sp. 13635J	2488	2488	100%	0.0	99%
AY728074.1	Uncultured alpha proteobacterium clone S1-10-CL6	2483	2483	100%	0.0	99%
HM007540.1	Uncultured bacterium clone A23	2483	2483	100%	0.0	99%
NR029082.1	<i>Methylobacterium populi</i> BJ001	2477	2477	100%	0.0	99%
AY251818.2	<i>Methylobacterium</i> sp. BJ001	2477	2477	100%	0.0	99%
EU741076.1	<i>Methylobacterium</i> sp. 13632G	2477	2477	100%	0.0	99%
AY468371.1	<i>Methylobacterium</i> sp. 1sub	2471	2471	100%	0.0	99%
AY468370.1	<i>Methylobacterium</i> sp. MM4	2471	2471	100%	0.0	99%
DQ490354.1	Methylobacteriaceae bacterium KVD-1982-01	2466	2466	100%	0.0	99%

Max. score = Maximum score; E value = Expected value; Max. ident. = Maximum identification

statistically significant and indicate support for a clade; alternative nodes can be rejected if they occur in less than 5% of the bootstrap estimates. Hillis and Bull (1993) also stated

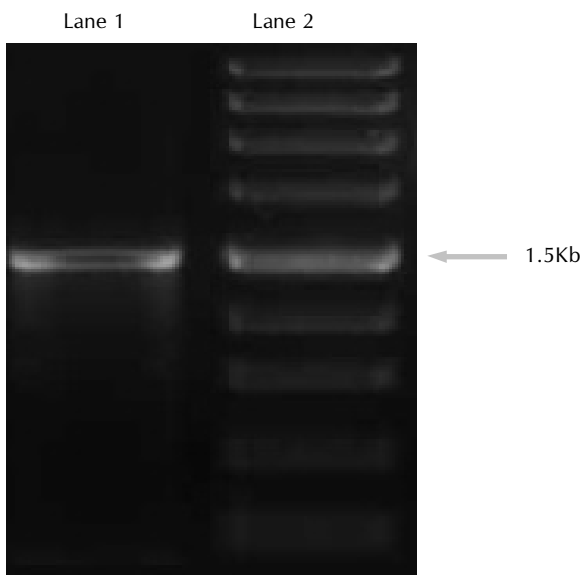
that bootstrap values of 50% or more may be overestimates of accuracy.

In the dendrogram of bacteria shown, 11 strains are present

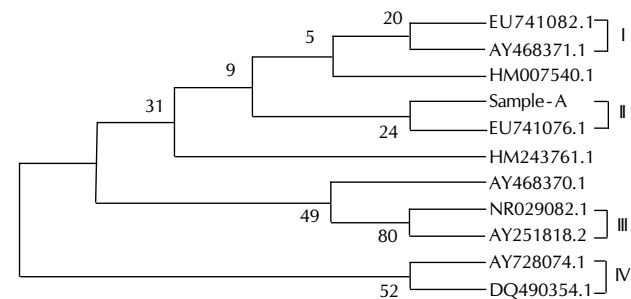
Table 6: Close homologs of *Bacillus* sp. BFF-3

Accession	Description	Max.score	Total score	Query coverage	E value	Max. ident.
EF031071.1	<i>Bacillus</i> sp. BFF-3	1827	1827	100%	0.0	100%
HM003212.1	<i>Bacillus cereus</i> strain WY1W1-7	1784	1784	99%	0.0	99%
FJ644692.1	<i>Bacillus cereus</i> strain MX3	1784	1784	99%	0.0	99%
FJ263046.1	<i>Bacillus cereus</i> strain ZD19	1784	1784	99%	0.0	99%
HM003208.1	<i>Bacillus cereus</i> strain WY1W1-1	1783	1783	99%	0.0	99%
FJ959367.1	<i>Bacillus subtilis</i> strain 0-2	1783	1783	99%	0.0	99%
GU085229.1	<i>Bacillus</i> sp. BD-31	1783	1783	99%	0.0	99%
FJ763650.1	<i>Bacillus cereus</i> strain S72	1783	1783	99%	0.0	99%
EU857430.1	<i>Bacillus cereus</i> strain B1	1783	1783	99%	0.0	99%
GQ199727.1	<i>Bacillus</i> sp. 210_25	1783	1783	99%	0.0	99%

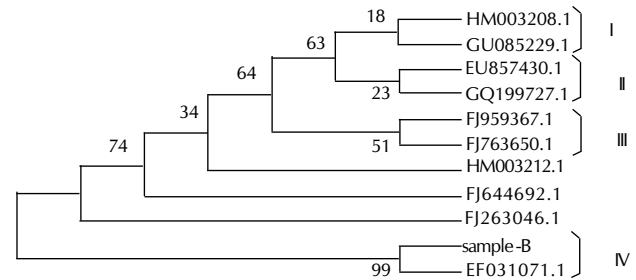
Max. score = Maximum score; E value = Expected value; Max. ident. = Maximum identification

**Figure 1: Gel image of 16S rDNA amplicon**

Lane 1: 16S rDNA amplicon band; Lane 2: DNA marker

**Figure 2: Phylogenetic tree showing position of sample - A (*Methylobacterium* sp. HJM27)**

of which 8 strains are distributed in 4 distinct groups (I, II, III and IV) and three strains are remained ungrouped (Figs. 2 and 3). All the grouped and ungrouped strains of the *Methylobacterium* sp. HJM27 monophyletic group are members of *Methylobacterium* sp. except two which are Uncultured alpha proteobacterium clone S1-10-CL6 and Uncultured bacterium clone A23. In Aken et al. (2004) experiment, the closest relatives to *Methylobacterium* sp. strain BJ001 are *Methylobacterium thiocyanatum*, *M. extorquens*, *M. zatmanii* and *M. rhodesianum*, with sequence similarities

**Figure 3: Phylogenetic tree showing position of sample - B (*Bacillus* sp. BFF-3)**

of 99.3, 99.1, 98.6 and 98.5%, respectively. *Methylobacterium* sp. is well known for its ability to degrade chloromethane or dichloromethane as the sole carbon and energy source (Vannelli et al., 1998, 1999; Studer, 2001; McDonald et al., 2002). Jing and Huyop (2006) showed that this bacterium also has the ability to utilize the herbicide 2, 2 - dichloropropionate (DALAPON) as a sole carbon source. The genus *Methylobacterium* belongs to the $\alpha - 2$ subclass of the class proteobacteria. It has been shown that these bacteria produce phytohormones (Trotsenko et al., 2001; Koenig et al., 2002; Omer et al., 2004) which stimulate seed germination and growth of certain plants (Lee et al., 2006; Kutschera, 2007). These growth promoting *Methylobacteria* form symbiotic interaction with their host organisms that were characterized in detail (Kutschera, 2007; Schauer and Kutschera, 2008). In addition *Methylotrophic* bacteria are also involved in the consumption of methane that is released from anaerobic environments and whose green-house effect is 20 times more elevated than CO_2 (Aken et al., 2002).

All the ungrouped strains of *Bacillus* sp. BFF-3 monophyletic group are members of the *Bacillus cereus* (Bc) group and 50% of the grouped members are also of Bc group. However, all the strains are members of genus *Bacillus* and family Bacillaceae. *Bacillus cereus* is an opportunistic pathogen causing food poisoning manifested by diarrhoeal or emetic syndromes (Ivanova et al., 2003). It is closely related to the animal and human pathogen *Bacillus anthracis* and *Bacillus thuringiensis*. Xu and Cote (2003) stated that *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides* and *Bacillus thuringiensis* belongs to the same group from 40 Bacillaceae studied species. The phenotypic and genotypic similarities between all four species have been well documented (Logan and Berkeley, 1984; Claus and Berkeley, 1986; Ash et al., 1991). Recently Helgason et al. (2000) proposed to regroup *B. anthracis*, *B.*

cereus and *B. thuringiensis* in a single species on the basis of genetic evidence. Since the genus *Bacillus* was first described (Cohn, 1872), the number of *Bacillus* species has fluctuated widely. Rossler *et al.* (1991) grouped nine *Bacillus* species into four clusters. In particular, 16S rRNA gene sequence analysis by Ash *et al.* (1991) revealed five phylogenetically distinct clusters of species and three ungrouped species from 51 *Bacillus* sp. Studied. Many *Bacillus* species that belonged to these phylogenetic groups have been reclassified as members of novel genera or have been transferred to other genera (Shida *et al.*, 1997; Jeon *et al.*, 2005). Despite the reduction in the number of species in the genus *Bacillus*, the genus is considered as one of the largest genera and additional *Bacillus* species from diverse habitats have been described recently (Heyrman *et al.*, 2005; Shivaji *et al.*, 2006; Ko *et al.*, 2006).

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