Secondary Structural Models (16S rRNA) of Polyhydroxyalkanoates Producing *Bacillus* Species Isolated from Different Rhizospheric Soil: Phylogenetics and Chemical Analysis

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Abstract: *Polyhydroxyalkanoates (PHAs) producing bacterial isolates are gaining more importance over the world due to the synthesis of a biodegradable polymer which is extremely desirable to substitute synthetic plastics. PHAs are produced by various microorganisms under certain stress conditions. In this study, sixteen bacterial isolates characterized previously by partial 16S rRNA gene sequencing (NCBI Accession No. KF626466 to KF626481) were again stained by Nile red after three years of preservation in order to confirm their ability to accumulate PHAs. Also, phylogenetic analysis carried out in the present investigation evidenced that the bacterial species belonging to genus Bacillus are the dominant flora of the rhizospheric region, with a potentiality of biodegradable polymer (PHAs) production. Again, RNA secondary structure prediction hypothesized that there is no direct correlation between RNA folding pattern stability with a rate of PHAs production among the selected isolates of genus Bacillus.*

Keywords: *Polyhydroxyalkanoates, Polymer, Rhizospheric, Bacillus, Phylogenetic tree.*

Introduction

PHAs productions have been reported among diverse bacteria. However, species of *Bacillus* have been widely studied [3, 7, 10, 12, 13] since the discovery of poly-*β*-hydroxybutyrate (PHB) in *Bacillus megaterium* [9]. Some species of *Bacillus* have been reported to produce Polyhydroxyalkanoates (PHAs) as much as 90% (w/w) of dry cells during nutrient-imbalanced conditions [11]. The genus has been widely used for a long time in industry and

academia for PHAs production, due to its absence of a lipopolysaccharides external layer, which makes the extraction much easier, its capability of growing in cheap raw materials and high growth rate in comparison to other bacteria [8]. Moreover, the isolates possess the ability to produce a variety of hydrolytic enzymes that can be exploited for cost-effective production of PHAs such as by utilization of agro-industrial and other waste materials [5].

Nowadays, 16S rRNA gene sequencing is the widely accepted modern molecular approach employed for identification of bacteria as the genotypic methods are more accurate than morpho-physiological or phenotypic identification [4]. Moreover, 16S rDNA is the significant point for molecular identification and taxonomical classification of bacteria due to the mosaic composition of phylogenetically conserved and variable regions within the gene [6]. The secondary structure of a 16S rRNA gene is more conserved than its counterpart DNA sequence; hence it is used in the phylogenetic analysis. The secondary-structure components such as helices, loops, bulges and separating single-stranded portions of RNA molecules are perfectly analyzed in this method [2]. Notably, it is an established fact that rRNA structure is highly conserved during the course of evolution, as most of the folding is functionally imperative despite primary sequence divergence. The prime objective of the present study was to characterize PHAs producing bacteria isolated from the rhizospheric soil region of various plants, using both morpho-physiological and 16S rRNA gene sequence analysis. Thus, the secondary rRNA structures of PHAs producing bacteria were predicted to assess the phylogenetic relationships as well as the genetic variations among the strains.

Materials and methods

Chemical and genetic analysis for screening of PHAs producing Bacillus species

In our preceding study, sixteen PHAs producing bacteria were isolated from the rhizospheric region of different plants such as *Saccharum officinarum*, *Ficus benghalensis*, *Bambusa vulgaris*, *Calotropis procera* and *Ipomoea batatas.* The morpho-physiological, molecular characterization and 16S rRNA gene sequencing of these bacteria were ascertained to be members of the genus *Bacillus* such as *Bacillus flexus* (MRK13), *Bacillus* sp. (S4-2013b), *Bacillus* sp. (P1-2013b), *Bacillus* sp. (S1-2013b), *Bacillus* sp. (S6-2013b), *Bacillus* sp. (O1), *Bacillus* sp. (P2-2013), *Bacillus* sp. (P3-2013), *Bacillus* sp. (P4-2013c), *Bacillus* sp. (B2-2013c), *Bacillus* sp. (B5-2013b), *Bacillus* sp. (C1-2013), *Bacillus* sp. (C3-2013), *Bacillus* sp. (O6), *Bacillus* sp. (S8), and *Bacillus thuringiensis* (RKD12), with GenBank accession numbers KF626466-KF626481 [10]. After three years of preservation, the accumulation of PHAs granule was observed by Sudan black and Nile red staining.

Phylogenetic analysis

Phylogenetic analysis of genes and proteins has served as a useful technique to study evolutionary relationships among different bacteria and virus population [1]. Nucleotide sequences of primarily identified PHAs producing *Bacillus* species were collected from the National Center for Biotechnology Information (NCBI) web server to perform a sequence analysis. Evolutionary divergence studies among 16S rRNA gene sequences of selected bacterial species were carried out with *Pseudomonas chlororaphis* DR2 (HG796168) as an out group. Multiple Sequence Alignments (MSAs) and construction of a phylogenetic tree among these sequences were performed using the Neighbor-Joining (NJ) algorithm [14] in MEGA 6.0 (Molecular Genetics Evolutionary Analysis) package [15]. The resultant tree topologies were evaluated by a bootstrap analysis of NJ data sets with 2000 resamplings.

RNA secondary structure prediction

16S rRNA of each bacterial sequence was predicted in the Mfold web server [16] to study and compare folding patterns among them. The minimum Gibb's free energy, ΔG, was computed by the mfold algorithm for each sequence, as the lowest Δ*G* maps to evolutionary stability of RNA structures for all sixteen PHAs producing bacterial sequences. During RNA structure prediction, the temperature was fixed to 37 °C and assumed to be constant within the range of temperatures that might occur *in vivo* or in the laboratory. RNA sequences were taken as linear; the ionic conditions were fixed at $[Na^{+}]$ = 1 M and $[Mg^{++}M]$ = 0. The predicted secondary structures were compared according to the number of stems, loops (exterior/interior), multiple loops, hairpin loops and bulges to study the conservation at the structural level.

Results and discussion

Chemical and phylogenetic analysis of PHAs producing Bacillus species

Gram staining and Nile red staining with phase contrast microscopic and UV-radiation photographs revealed that the bacteria were gram positive rod-shaped and *Bacilli*. The 16S rRNA gene sequences of *Bacillus* sp. (Table 1) were considered for phylogenetic tree construction whose percentage of PHAs accumulation was observed from Sudan black and Nile red staining (Table 2). A molecular phylogenetic approach was implicated to study the evolutionary relationships among sixteen *Bacillus* 16S rRNA sequences (KF626466 to KF626481). The resultant NJ based phylogenetic tree consisted of sixteen different taxa of *Bacillus* species such as *Bacillus* sp. (S8), *Bacillus* sp. (S1-2013b), *Bacillus* sp. (S4-2013b), *Bacillus* sp. (S6-2013b), *Bacillus* sp. (B2-2013C), *Bacillus* sp. (B5-2013b), *Bacillus thuringiensis* RKD12, *Bacillus flexus* MRK 13, *Bacillus* sp. O1, *Bacillus* sp. O6, *Bacillus* sp. (C1-2013), *Bacillus* sp. (C3-2013), *Bacillus* sp. (P1-2013b), *Bacillus* sp. P2, *Bacillus* sp. (P3-2013), *Bacillus* sp. (P4-2013), and *Pseudomonas chlororaphis* strain DR2 (HG796168) as an out group (Fig. 1).

The resultant tree was grouped into three different clusters, i.e. *Bacillus* sp. S8 and *Bacillus thuringiensis* RKD12 as one cluster and the rest fourteen *Bacillus* species as two other clusters, and the *Pseudomonas chlororaphis* strain DR2 (HG796168) was separated as an out group in the tree, indicating a distant evolutionary relation with the other sixteen isolates (Fig. 1). A strong evolutionary closeness with the support of a high bootstrap value was observed between *Bacillus* sp. (C3-2013), *Bacillus* sp. O6, and *Bacillus* sp. (P4-2013c); *Bacillus* sp. (S4-2013b) and *Bacillus* sp. P2; *Bacillus* sp. (B2-2013c), *Bacillus* sp. (S6-2013b), and *Bacillus* sp. (B5-2013b); *Bacillus flexus* MRK 13, *Bacillus* sp. (S1-2013b), and *Bacillus* sp. (P1-2013b); *Bacillus* sp. O1 and *Bacillus* sp. (P3-2013); *Bacillus* sp. S8 and *Bacillus thuringiensis* RKD 12.

Analysis of RNA folding patterns

The secondary structures of the ribosomal RNA of sixteen *Bacillus* species were predicted. The secondary structures with the lowest free energy were selected for a divergence analysis. The secondary structural features of the rRNA regions, as shown in Figs. 2-6, were analyzed based on conserved stems and loops, even in case of differences in the sequence length.

In group A (bacterial species isolated from the rhizosphere of *Saccharum officinarum*), among four *Bacillus* species, *Bacillus* sp. (S1-2013b) and *Bacillus* sp. (S8) shared common RNA folding (linear) but *Bacillus* sp. (S4-2013b) and *Bacillus* sp. (S6-2013b) showed the same number of branching with variable loops (Fig. 2).

Table 1. Molecular identification of bacterial isolates along with their corresponding accession number

Sl. No.	Bacterial code	Rhizospheric plant	Identified bacterial isolates	Accession number
1	B1	*Ficus benghalensis*	*Bacillus thuringiensis* RKD12	KF626481
2	B2	*Ficus benghalensis*	*Bacillus* sp. B2 (2013c)	KF626475
3	B3	*Ficus benghalensis*	*Bacillus flexus* MRK 13	KF626466
4	B5	*Ficus benghalensis*	*Bacillus* sp. B5 (2013b)	KF626476
5	C1	*Calotropis procera*	*Bacillus* sp. C1 (2013)	KF626477
6	C3	*Calotropis procera*	*Bacillus* sp. C3 (2013)	KF626478
7	O1	*Bambusa vulgaris*	*Bacillus* sp. O1	KF626471
8	O6	*Bambusa vulgaris*	*Bacillus* sp. O6	KF626479
9	S1	*Saccharum officinarum*	*Bacillus* sp. S1 (2013b)	KF626469
10	S4	*Saccharum officinarum*	*Bacillus* sp. S4 (2013b)	KF626467
11	S6	*Saccharum officinarum*	*Bacillus* sp. S6 (2013b)	KF626470
12	S8	*Saccharum officinarum*	*Bacillus* sp. S8	KF626480
13	P1	*Ipomoea batatas*	*Bacillus* sp. P1 (2013b)	KF626468
14	P2	*Ipomoea batatas*	*Bacillus* sp. P2 (2013)	KF626472
15	P3	*Ipomoea batatas*	*Bacillus* sp. P3 (2013)	KF626473
16	P4	*Ipomoea batatas*	*Bacillus* sp. P4 (2013c)	KF626474

Table 2. 16S rRNA gene of bacterial isolates from the rhizospheric soil regions of different plants, sorted in a descending order according to their structural folding stability (the lowest free energy) along with their PHAs production rate in percentage

Sl. No.	Identified bacterial isolates	Rhizospheric region	Free energy, (kcal/mol)	Rate of PHAs accumulation, %
1	*Bacillus* sp. C1 (2013)	Akund	-535.61	52.00
2	*Bacillus* sp. S6 (2013b)	Sugarcane	-527.63	58.00
3	*Bacillus* sp. P4 (2013c)	Sweet Patato	-522.49	61.00
4	*Bacillus flexus* MRK 13	Banyan	-518.44	70.00
5	*Bacillus* sp. B5 (2013b)	Banyan	-513.27	44.28
6	*Bacillus* sp. C3 (2013)	Akund	-500.43	39.00
7	*Bacillus thuringiensis* RKD12	Banyan	-498.66	78.94
8	*Bacillus* sp. S1 (2013b)	Sugarcane	-497.24	80.44
9	*Bacillus* sp. P1 (2013b)	Sweet Patato	-491.32	68.22
10	*Bacillus* sp. O1	Bamboo	-489.66	23.50
11	*Bacillus* sp. O6	Bamboo	-485.49	79.85
12	*Bacillus* sp. P2 (2013)	Sweet Patato	-473.12	37.00
13	*Bacillus* sp. S8	Sugarcane	-452.22	62.50
14	*Bacillus* sp. P3 (2013)	Sweet Patato	-442.97	45.54
15	*Bacillus* sp. S4 (2013b)	Sugarcane	-441.97	49.00
16	*Bacillus* sp. B2 (2013c)	Banyan	-440.93	36.10

Similarly, among the four isolates in group B (bacterial species isolated from the rhizosphere of *Ficus benghalensis*) the secondary structure of *Bacillus* sp. (B2-2013c) and *Bacillus flexus* MRK13 shared the same branching pattern whereas *Bacillus* sp. (B5-2013b) and *Bacillus* sp. RKD12 showed a common RNA folding pattern (Fig. 3).

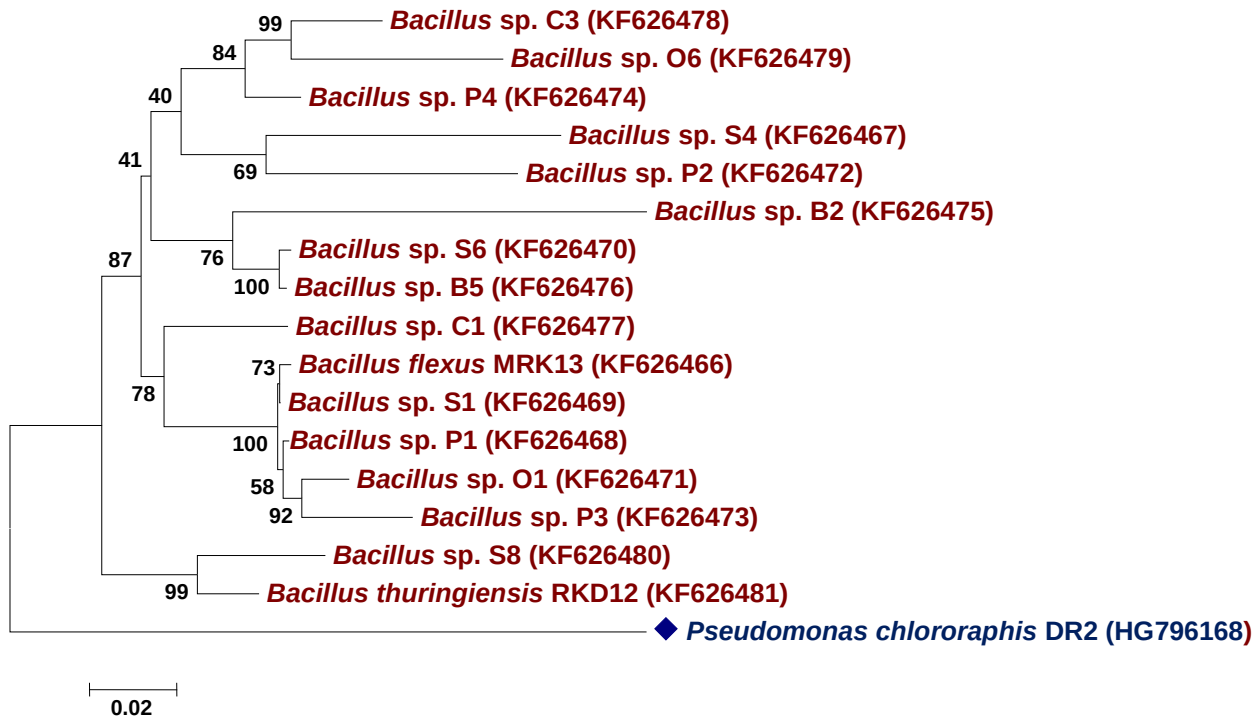

Fig. 1 Showing the phylogenetic relationship among the sixteen PHAs producing *Bacillus* species with an out group *Pseudomonas chlororaphis* DR2 [HG796168]

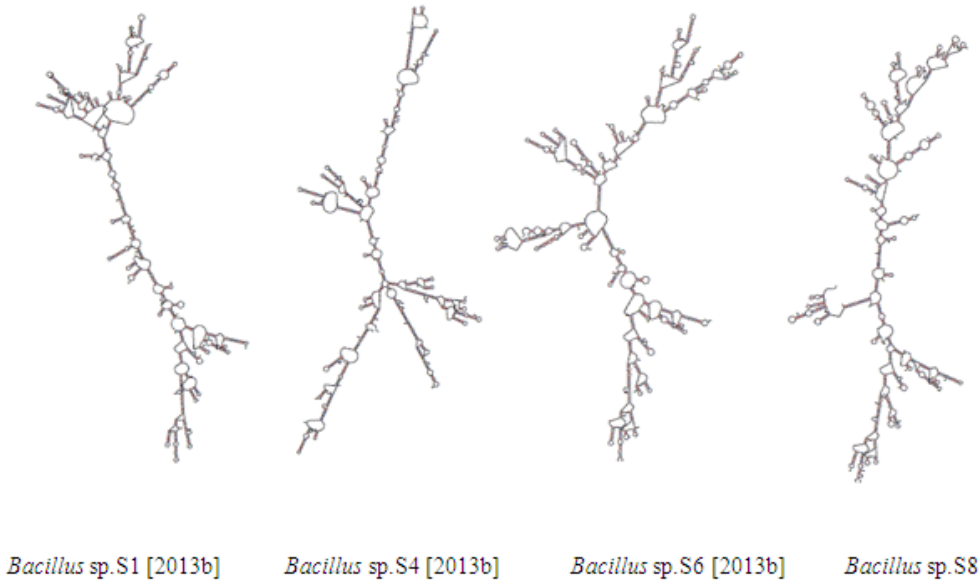

Fig. 2 Secondary structure of 16S rRNA of bacteria isolated from the rhizospheric soil region of Sugarcane (*Saccharum officinarum*, Group A)

Likewise, in group C (bacterial species isolated from the rhizosphere of *Bambusa vulgaris*) *Bacillus* sp. (O1) and *Bacillus* sp. (O6) shared the same RNA folding pattern but differed in terms of numbers of the loops (Fig. 4).

However, in group D (bacterial species isolated from rhizosphere of *Calotropis procera*) the RNA secondary structure of *Bacillus* sp. (C1-2013) and *Bacillus* sp. (C3-2013) differed in terms of pattern of branching and loops but shared a similar folding pattern (Fig. 5). In case of

group E (bacterial species isolated from rhizosphere of *Ipomoea batatas*), out of four isolates, *Bacillus* sp. (P2-2013), *Bacillus* sp. (P3-2013) and *Bacillus* sp. (P4-2013) shared a common RNA folding pattern, but the folding pattern of *Bacillus* sp. (P1-2013b) differed from the other three isolates (Fig. 6).

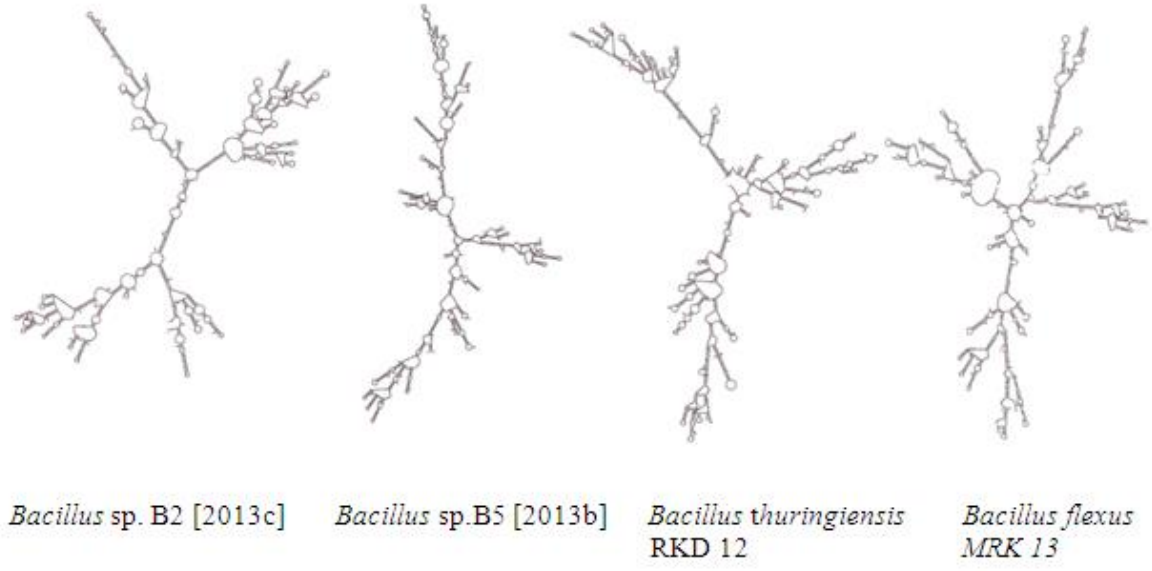

Fig. 3 Secondary structure of 16S rRNA of bacteria isolated from the rhizospeheric soil region of Banyan (*Ficus benghalensis*, Group B)

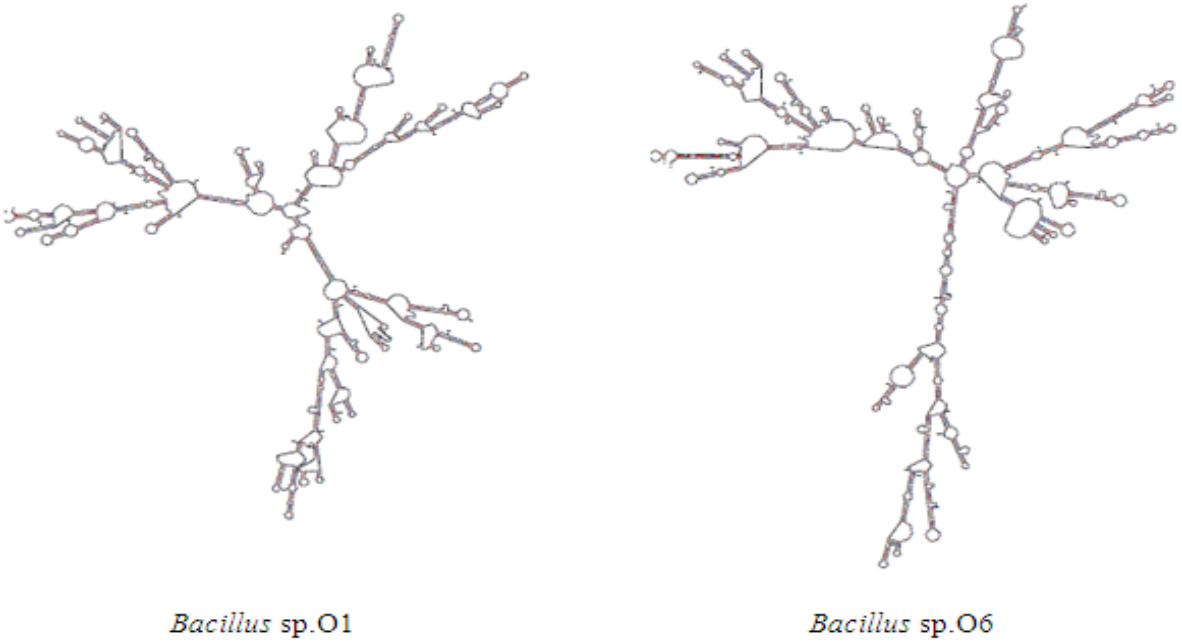

Fig. 4 Secondary structure of 16S rRNA of bacteria isolated from the rhizospeheric soil region of Bamboo (*Bambusa vulgaris*, Group C)

The only difference in the topology of their secondary structure is due to differences in the nucleotide sequences. These secondary structure predictions indicate that the domains base pair to form a core region central to several stem features, implying that the conservedness is more important for the proper rRNA folding pattern. The topology of the secondary structure

based only on the predicted RNA secondary structure of the *Bacillus* sp. resolved most relationships among the species studied.

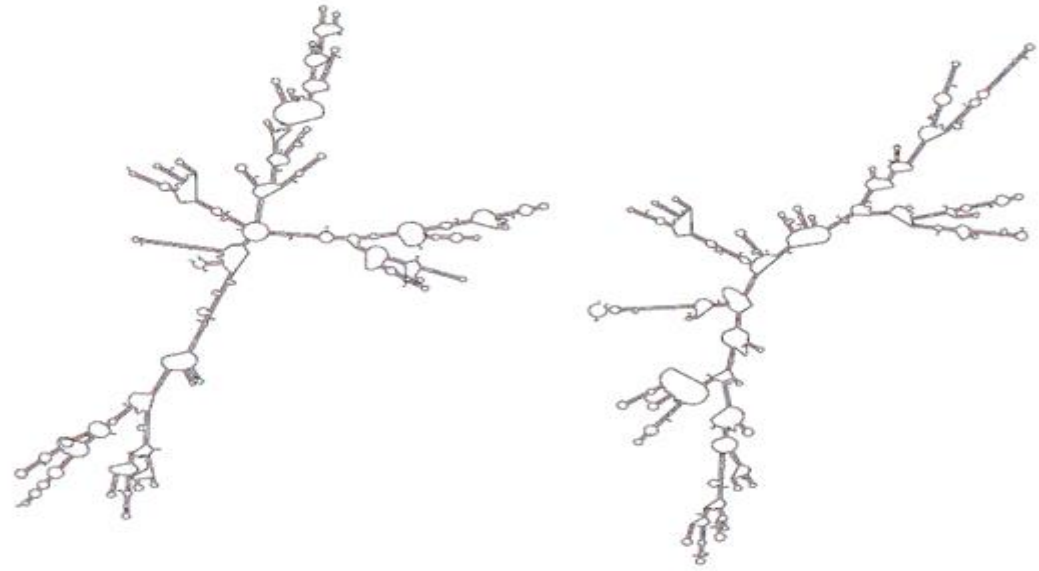

Fig. 5 Secondary structure of 16S rRNA of bacteria isolated from the rhizospeheric soil region of Akund (*Calotropis procera*, Group D)

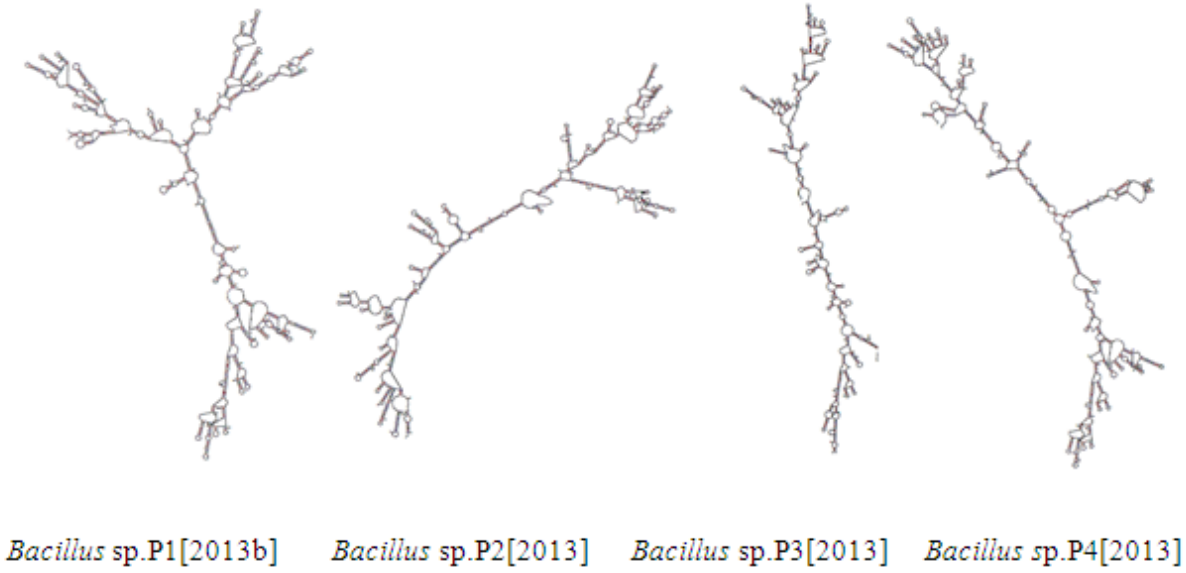

Fig. 6 Secondary structure of 16S rRNA of bacteria isolated from the rhizospeheric soil region of Sweet potato (*Ipomoea batatas*, Group E)

Conclusion

In silico molecular phylogenetic analysis and study of rRNA folding patterns along with phenotypic characterization of 16S rRNA genes have served together as a more useful method for identification of bacteria than when these techniques are used alone. Characterization of bacteria isolated from the plant rhizosphere soil regions shows the dominance of gram-positive bacteria (*Bacillus* species) exhibiting a high production of PHAs, which is evidenced from Nile Red staining even after three years of preservation of the selected isolates. The evolutionary relationship has been studied through a molecular phylogenetic

approach, which revealed a strong closeness among PHAs producing *Bacillus* isolates of rhizospheric soil. The secondary structure of a 16S rRNA is more conserved than its counterpart DNA sequence. Thus, in the present investigation, genetic variability has been observed at RNA secondary structures, indicating evolution apparently acting at structural levels of RNA. It has also been observed that the r-RNA folding pattern in *Bacillus* sp. C1 (2013) is the most stable energetically (i.e. -535.61 kcal/mol) compared to other *Bacillus* species with a capability to produce PHAs of 52% (i.e. more than 50%) which was isolated from the rhizosphere region of Akund. On the contrary, the r-RNA folding energy of *Bacillus* sp. S1 (2013b) isolated from the rhizosphere region of Sugarcane is -497.24 kcal/mol, with a capability to produce PHAs of 80.44%. Hence, here it is hypothesized that there is no direct correlation between the RNA folding pattern stability and the rate of PHAs production among the *Bacillus* species isolated from different plants rhizosphere soil regions. Functional characterization of genes involved in PHAs production can be studied. However, 16S ribosomal divergence has been carried in order to understand its variability.

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