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# Biochemical and molecular characterization of bacterial Endosymbionts associated with *Hishimonus phycitis* (Hemiptera: Cicadellidae), the insect vector of sesamum phyllody phytoplasma

**Mahalingappa Bandakkanavar, Ramappa HK, Nagaraju N, Prameela HA, Byregowda M and Sanjay MT**

**Abstract**
The present study, were carried out at GKVK, Bengaluru on diversity of culturable bacterial endosymbionts associated with *Hishimonus phycitis.* The leafhoppers (*H. phycitis*) were collected from sesamum crop at ZARS filed unit. The isolated seven endosymbiotic bacteria (Designated as EBHP-1 to 7). The study revealed that, all endosymbionts showed negative reaction to Indole and Voges-Proskauer test (EBHP-1 to 7). In citrate test, six endosymbionts showed positive reaction (EBHP-1, EBHP-2, EBHP-3, EBHP-4, EBHP-6 and EBHP-7) and EBHP-5, showed negative reaction. Further, in the methyl red test EBHP-2, EBHP-5, EBHP-6 and EBHP-7 showed positive and remaining three showed negative (EBHP-1, EBHP-3 and EBHP-4). Isolated DNA from the seven isolates of endosymbionts from their pure culture and subjected to PCR for amplification of gene encoding 16S rRNA using Fp1/Rp2 primers. DNA isolated from all samples yield the expected amplicon of 1.4 kb PCR product, which were sequenced. The phylogenetic analysis of the 16S rRNA gene and nucleotide identity indicated that EBHP-1, EBHP-3, EBHP-6 and EBHP-7 were closely clustered with *Pseudomonas* sp. and shared sequence similarity of 83-93(%). Similarly, EBHP 4 and EBHP 2 clustered with *Exiguobacterium* sp. and shared 64-93(%) sequence identity and EBHP-5 with *Achromobacter* sp. and 89-91(%) sequence similarity. These results suggest that, diverse endosymbiotic bacteria associated with the *Hishimonus phycitis*, which may have possible role in the transmission of sesamum phyllody.

**Keywords:** Sesamum phyllody (SP), *Hishimonus phycitis*, bacterial endosymbionts and 16S rRNA

## Introduction
Sesamum *(Sesamum indicum* L.) is one of the most important and ancient oilseed crop grown in India and many parts of the world. Sesamum seed is a rich source of edible oil. Sesamum phyllody disease is major constraint for sesamum production in India and causes yield loss up to 100 per cent, its caused by a phytoplasma, which is vectored by leafhopper species, *Orosius orientalis*, *Circulifer haematoceps*, *Neoaliturus haematoceps* and *Hishimonus phycitis* (Distant) (Naingroo, 2014; Nabi *et al*., 2013) [9, 10]. Among the leafhopper sps, *Hishimonus phycitis* is a major transmission of phyllody. Phytoplasma, is a cell wall-less bacteria which multiply in the plant phloem tissues and insect haemolymph (Bertaccini *et al*., 2014) [6]. Almost all insects are associated with heritable endosymbiotic bacteria. Endosymbiotic bacteria in insects exist in two forms, primary and secondary. Primary endosymbionts are vertically transmitted from mother to offspring and they provide their hosts with specific nutritional compounds that are important for their survival and development. Insect endosymbiotic microflora have played a key role in their ecological and evolutionary success (Douglas, 2011) [7]. Several roles have been characterized for bacterial endosymbionts associated with insects including nutritional ecology by providing essential nutrients that are limited or lacking in the diet or aid in digestion and detoxification of food and detoxification of toxic plant metabolites in the host's diet (Zhou and Zhang, 2013) [14]. Bacterial endosymbionts are commonly associated with insects (Beard *et al*., 2002) [3, 4]. This present study was aimed to shed light on diversity of culturable bacterial endosymbionts in leafhopper, *H. phycitis* and their biochemical characteristics.

## Materials and Method

### Collection and isolation of endosymbiotic bacteria from the leafhopper (*H. phycitis* (distant)

Adults of *H. phycitis* leafhopper were collected from field of UAS, GKVK, Bengaluru using a aspirator and kept for starvation in *petridish* for 24 hours (Fig.1). Adults were surface sterilized with 70 per cent ethanol for 1 minute followed by 0.1 per cent sodium hypochloride for 1 minute then with sterile distilled water for 3 times and sterilized adults were crushed using 1 ml PBS solution. After crushing, suspension was added into eppendorf tube and it was centrifuged at 2000 rpm for 10 minutes. One ml of supernatant was taken and added in 9 ml water blank ($10^{-1}$ dilution). Again one ml aliquot taken from $10^{-1}$ dilution and added into another 9 ml water blank ($10^{-2}$ dilution).Then it was serially diluted up to $10^{-6}$ dilution. All media (NA, Mac Conkey Agar) were melted and poured in sterile petriplates. After solidification, 0.1ml aliqout was taken from required dilutions and added into the plates. The aliquot was spreaded using sterile spreader. All plates were incubated in incubator at 28 ºC for 1 day. After incubation, colonies (isolates) were selected from plates based on morphology (size, shape, colour) and made pure culture by streaking on media plates. All isolates were preserved in agar slants.


**Fig 1:** Insect vector of phytoplasma *Hishimonus phycitis* (Distant)

### Biochemical characterization of isolated endosymbiotic bacteria

The endosymbiotic bacteria were subjected to basic biochemical tests, including citrate utilization test and IMVIC reaction. Citrate utilization test in Simmons Citrate Agar, changes in colour as an indicator in the media, which is from green to blue, indicates positive for this test. IMVIC reactions consist of Indole production test in tryptone broth, after adding kovac's reagent, cherry red ring on the top layer of broth indicates the production of indole (positive). Methyl Red and Voges Proskauer tests in an MR-VP broth, for methyl red test, after adding methyl red, the production of red colour indicates the positive result and having ability to oxidize glucose. For Voges Proskauer, VP reagent 1 and 2 were added, and then pinkish red colour appeared which indicates the positive result. According to the method described by Benson (2002) [5].

### Molecular identification of bacterial endosymbionts DNA extraction and PCR amplification

The isolated bacteria were multiplied in Luria Bertani (LB) broth and bacterial genomic DNA was isolated by Sucrose buffer method. Kept isolated DNA at -20° C. The 16S rRNA gene was amplified from isolated genomic DNA of bacteria using forward primer and reverse primer. The full length bacterial 16S rRNA fragments were amplified by PCR from each representative isolate using primers, Fp1 forward primer (5'-AGAGTTTGATCCTGGCTCAG- 3') and Rp2 reverse primer (5'-ACGGCTACCTTGTTACGACTT- 3'). The PCR was carried out in a total volume of 30µl. The 16S rRNA fragment was amplified in PCR. The PCR program for the amplification of 16S rRNA gene consisted of initial denaturation at 94 °C for 4 minutes, 32 cycles of denaturation at 94 °C for 1 minute, annealing at 58 °C for 1 minute and extension at 72 °C for 1.30 minute with a final extension at 72 °C for 10 minutes. Amplification was performed in a DNA thermal cycler. The aliquot of each PCR amplified product was electrophoresed on a 1.0 per cent agarose gel in 1X TAE buffer at 70 V for 45 minutes, stained with ethidium bromide and the PCR products were visualized with a gel documentation unit.

### Phylogenetic analysis

The PCR products were sent for sequencing. DNA sequences corresponding to 16S rRNA gene, obtained from individual bacteria. Further DNA sequences were aligned using the clustlX software. The aligned DNA sequences were compared with the database of National Centre for Biotechnical Information (NCBI) for the identification of bacteria homology searches with 16S rRNA sequences in Gene Bank performed with Basic Local Alignment Search Tool (BLAST) program. Phylogenetic tree was developed using 16S rRNA sequence from the bacterial endosymbionts sample along with 12 sequences of bacterial endosymbionts strains downloaded from GenBank database (Table 1). A phylogenetic tree was constructed using the neighbour joining phylogenetic method using MEGA7 program with 1000 bootstrap replications. The nucleotide sequence identity matrixes for the bacterial endosymbionts were generated using Bio edit Sequence Alignment Editor (Vs. 5.0.9).

**Table 1:** Sequences used in phylogenetic analysis of 16S rRNA of bacterial endosymbionts with different bacterial strains

| SI. No. | Bacterial strains | Accession number |
|---|---|---|
| 1 | *Pseudomonas* sp. strain P3 | MN400354.1 |
| 2 | Bacterium strain BS1064 | MK824252.1 |
| 3 | *Exiguobacterium alkaliphilum* strain 19 | MT225705.1 |
| 4 | *Exiguobacterium alkaliphilum* strain AS39 | MT214263.1 |
| 5 | *Pseudomonas mendocina* strain fsznc-01 | MK106366.1 |
| 6 | *Pseudomonas pseudoalcaligenes* strain81007 | FJ544320.1 |
| 7 | *Exiguobacterium aurantiacum* strain S11 | MK100772.1 |
| 8 | *Exiguobacterium acetylicum* strain S15 | MK100776.1 |
| 9 | *Achromobacter xylosoxidans* strain NA21 | JN585718.1 |
| 10 | *Achromobacter denitrificans* strain 1104 | KT832691.1 |
| 11 | Bacillus ATCC | NR074540.1 |
| 12 | *Xenorhabdus nematophila* strain DSM | NR042821.1 |

## Results and Discussion

### Isolation and biochemical characterization bacterial endosymbionts

Almost all insects have endosymbionts for their normal growth and development. Loss of these microorganisms often results in abnormal development and reduces survival of the insect host. Previously, many reports described the isolation of bacteria from leafhopper and other sap sucking insects. However, the present study is the first to identify bacteria associated with leafhopper, *H. phycitis*, employing biochemical characterization and 16S rRNA sequence analysis.

The present study results reviled that, the total seven endosymbiotic bacteria were successfully isolated from the *H. phycitis* and were assigned code numbers as endosymbiotic bacteria EBHP-1 to 7 for laboratory purposes (Plate 1a &1b). Similar results were found in previous work of Vijaykumar *et al.* (2019) [13] and Anand *et al.* (2009) [2] they were isolated five different bacteria from Diamondback moth (*Plutella*

*xylostella*) and eleven isolates from digestive tract of *Bombyx mori* respectively. Similar results were shown when five bacterial isolates (Rhizo 1, Rhizo 2, Rhizo 3, Rhizo 4, Rhizo 5) were isolated from stored tuber mealybug, *Rhizoecus amorphopalli*.

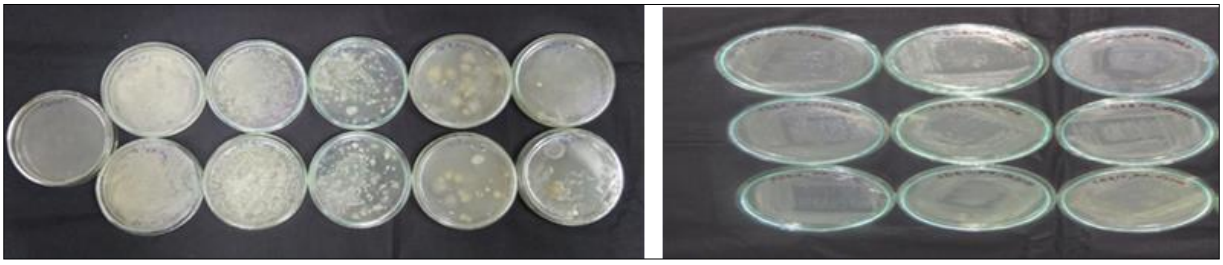
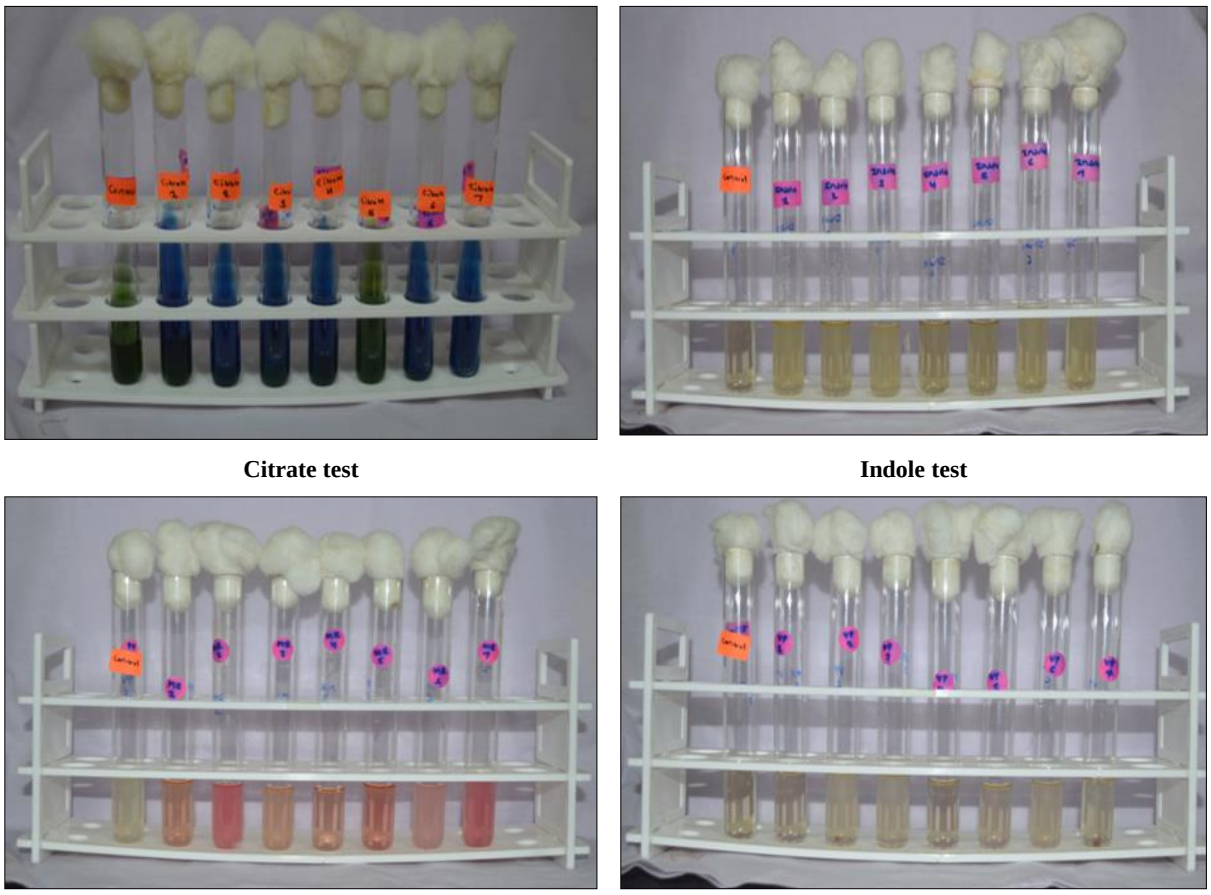


**Plate 1a:** Plates containing bacterial colonies isolated from *H. phycitis*

**Plate 1b:** Pure cultures of isolated endosymbiotic bacteria from *H. phycitis*

**Biochemical characterization bacterial endosymbionts**

All the bacterial endosymbionts were subjected to biochemical tests, after 48 hours. Observations were recorded and presented in Table 2 and Plate 2.



**Citrate test** **Indole test**

**Methyl red test** **Voges-proskauer test**

**Plate 2:** Biochemical characterization of endosymbiotic bacteria

**Table 2:** Biochemical characterization of bacterial endosymbionts

| Endosymbiotic bacteria | Indole test | Citrate test | Methyl red test | Voges-Proskauer test |
|---|---|---|---|---|
| EBHP1 | - | + | - | - |
| EBHP2 | - | + | + | - |
| EBHP3 | - | + | - | - |
| EBHP4 | - | + | - | - |
| EBHP5 | - | - | + | - |
| EBHP6 | - | + | + | - |
| EBHP7 | - | + | + | - |
| Control | - | - | - | - |

Note: - = Negative reaction, + = Positive reaction

**Indole production test**

We observed that the all endosymbiotic bacteria were developed cherry red colour in the tubes after adding kovac's reagent to each tubes (positive test). This indicated that all endosymbionts showed negative reaction to Indole production test. Ravikumar *et al*. (2014) [11] reported that, isolates Rhizo 1, Rhizo 2, Rhizo 3 were identified as *Bacillus* sp. as they showed negative for indole production.

**Voges-Proskauer test**

After adding V-P reagent I (napthol solution) and V-P reagent II (40% potassium hydroxide) to tubes including uninoculated

tube, we found that the ruby pink (red) colour developed in all endosymbiotic bacteria which indicated positive for this test. These results are in line with Anand *et al.* (2009) [2] obtained eleven isolates from digestive tract of *Bombyx mori* and labelled as isolate 1 to 11 and isolate 4, 7, 8, 9 and 11 shown positive for Voges- proskauer test.

**Citrate utilization test**

All endosymbiotic bacteria were given 48 hours incubation, observed that the endosymbiotic bacteria EBHP-1, EBHP-2, EBHP-3, EBHP-4. EBHP-6 and EBHP-7 tubes were changed the colour from green to blue indicated positive for this test whereas EBHP-5 showed negative reaction for this test which remains green in colour. Similar results were found with previous work of Ravikumar *et al*. (2014) [11] isolates Rhizo1, Rhizo 2 and Rhizo 3 were showed positive results for citrate utilization.

**Methyl-red test**

Methyl red indicator was added to each tubes including uninoculated tube and observed that endosymbiotic bacteria EBHP-2, EBHP-5, EBHP-6 and EBHP-7 were developed red colour throughout the tubes indicated positive for the test whereas, endosymbiotic bacteria EBHP-1, EBHP-3 and EBHP-6 were not found any red colour throughout the tubes indicated negative for the test. Similar results were found that, biochemical characters of isolate 2, 3, 4, 5, 6, and 10 shown positive result for methyl red test (Anand *et al.*, 2009) [2] and isolates PMB 1, PMB 2, PMB 12, PMB 14, PMB 18 and PMB 23 reacted positive to methyl red test (Abinaya *et al*., 2019) [1].

**Molecular identification of bacterial endosymbionts**

In total, seven endosymbiotic bacteria isolated from *Hishimonus phycitis* were identified and sequenced. The genomic DNA was isolated from the seven isolates of endosymbionts from their pure culture and subjected to PCR amplification of the 16S rRNA gene using universal primers. The thick DNA bands were visualized on agarose gel under gel documentation. All the isolated endosymbionts yield the expected amplicon of 1.4 kb size PCR product, which were sequenced, edited and compared with GenBank sequences using BLAST algorithms (Plate 3). Vijaykumar *et al*. (2019) [13] isolated bacterial genomic DNA and amplified using PCR with 16S rRNA primers (expected size 1000 bp). Amplified 4kb DNA fragments containing 16S-23S ribosomal DNA (rDNA) from the P-endosymbiont of 24 whiteflies from 22 different species of 2 whitefly subfamilies (Thao *et al.*, 2004).

The phylogenetic analysis and nucleotide identity revealed that four endosymbiotic bacteria (EBHP-1, EBHP-3, EBHP-6 and EBHP-7) closely clustered with *Pseudomonas* sp. and shared sequence similarity of 93-83 per cent. Similarly, two endosymbiotic bacteria (EBHP 4 and EBHP 2) clustered with *Exiguobacterium* sp. and shared 64-93 per cent sequence identity and one isolate (EBHP-5) with *Achromobacter* sp. and 89- 91 per cent sequence similarity (Table 3 and Fig.1). Singh *et al*. (2012) reported that phylogenetic analysis of 16S rDNA sequences of *Cardinium*, *Wolbachia*, *Rickettsia* and *Arsenophonus* showed that each of these bacteria form a separate cluster when compared to their respective counterparts from other parts of the world. Munson *et al* (1992) compared the 16s ribosomal RNA from the endosymbionts of three species of mealybugs and parsimony analysis of the sequences indicated that the mealybug endosymbionts are related and belong to the P-subdivision.

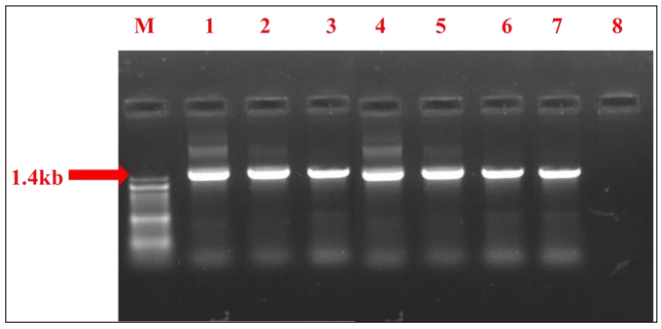



**Fig 4:** Plate 3: PCR amplification of bacterial 16S rRNA gene
Lane M: 1.5 kb ladder, Lane 1, 2, 3, 4, 5, 6 and 7: Bacterial DNA, Lane 8: Control

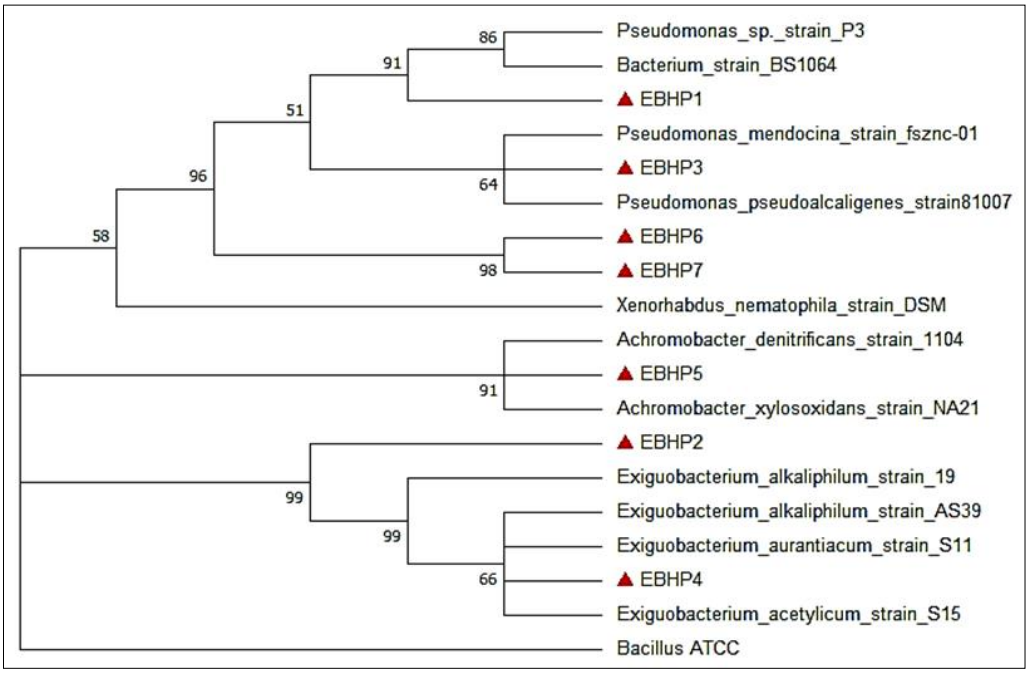



**Fig 5:** Phylogenetic tree generated by sequences from 16S rRNA gene of the bacterial endosymbionts

**Table 3:** Analysis of the sequence similarities among the 16S rRNA gene sequences from the bacterial endosymbionts and other bacterial isolates used in phylogenetic analysis

| Organism | EBHP1 | EBHP2 | EBHP3 | EBHP4 | EBHP5 | EBHP6 | EBHP7 | B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | B10 | B11 | B12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EBHP1 | ID | | | | | | | | | | | | | | | | | | |
| EBHP2 | 0.780 | ID | | | | | | | | | | | | | | | | | |
| EBHP3 | 0.868 | 0.755 | ID | | | | | | | | | | | | | | | | |
| EBHP4 | 0.794 | 0.841 | 0.765 | ID | | | | | | | | | | | | | | | |
| EBHP5 | 0.823 | 0.898 | 0.842 | 0.753 | ID | | | | | | | | | | | | | | |
| EBHP6 | 0.846 | 0.789 | 0.834 | 0.801 | 0.769 | ID | | | | | | | | | | | | | |
| EBHP7 | 0.867 | 0.910 | 0.846 | 0.765 | 0.798 | 0.721 | ID | | | | | | | | | | | | |
| B1 | 0.935 | 0.670 | 0.906 | 0.681 | 0.582 | 0.840 | 0.853 | ID | | | | | | | | | | | |
| B2 | 0.934 | 0.754 | 0.905 | 0.681 | 0.582 | 0.841 | 0.853 | 0.999 | ID | | | | | | | | | | |
| B3 | 0.682 | 0.842 | 0.660 | 0.935 | 0.515 | 0.641 | 0.652 | 0.692 | 0.692 | ID | | | | | | | | | |
| B4 | 0.680 | 0.698 | 0.658 | 0.938 | 0.514 | 0.640 | 0.650 | 0.690 | 0.690 | 0.997 | ID | | | | | | | | |
| B5 | 0.913 | 0.655 | 0.905 | 0.675 | 0.580 | 0.836 | 0.849 | 0.967 | 0.968 | 0.690 | 0.688 | ID | | | | | | | |
| B6 | 0.878 | 0.765 | 0.922 | 0.677 | 0.598 | 0.837 | 0.844 | 0.922 | 0.923 | 0.673 | 0.671 | 0.923 | ID | | | | | | |
| B7 | 0.680 | 0.765 | 0.657 | 0.939 | 0.514 | 0.640 | 0.650 | 0.689 | 0.689 | 0.996 | 0.999 | 0.687 | 0.671 | ID | | | | | |
| B8 | 0.680 | 0.643 | 0.657 | 0.938 | 0.514 | 0.640 | 0.650 | 0.689 | 0.689 | 0.997 | 0.998 | 0.687 | 0.671 | 0.997 | ID | | | | |
| B9 | 0.752 | 0.674 | 0.743 | 0.664 | 0.910 | 0.720 | 0.742 | 0.771 | 0.771 | 0.697 | 0.697 | 0.770 | 0.754 | 0.697 | 0.698 | ID | | | |
| B10 | 0.751 | 0.625 | 0.742 | 0.664 | 0.890 | 0.719 | 0.740 | 0.769 | 0.769 | 0.697 | 0.697 | 0.768 | 0.753 | 0.697 | 0.698 | 0.990 | ID | | |
| B11 | 0.696 | 0.592 | 0.685 | 0.816 | 0.525 | 0.656 | 0.670 | 0.707 | 0.708 | 0.865 | 0.864 | 0.714 | 0.695 | 0.864 | 0.863 | 0.703 | 0.703 | ID | |
| B12 | 0.762 | 0.652 | 0.752 | 0.681 | 0.562 | 0.729 | 0.752 | 0.786 | 0.786 | 0.714 | 0.714 | 0.783 | 0.764 | 0.713 | 0.713 | 0.778 | 0.781 | 0.712 | ID |

Note: B1 = *Pseudomonas* sp. strain P3, B2 = Bacterium strain BS1064; B3 = *Exiguobacterium alkaliphilum* strain 19; B4 = *Exiguobacterium alkaliphilum* strain AS39; B5 = *Pseudomonas mendocina* strain fsznc-01; B6 = *Pseudomonas pseudoalcaligenes* strain 81007; B7 = *Exiguobacterium aurantiacum* strain S11; B8 = *Exiguobacterium acetylicum* strain S15; B9 = *Achromobacter xylosoxidans* strain NA21; B10 = *Achromobacter denitrificans* strain 1104; B11 = *Bacillus* ATCC; B12 = *Xenorhabdus nematophila* strain DSM

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