Lysinibacillus acetophenoni and Pseudomonas stutzeri with High Salt effect, Recovered from High Salinity Soil Area (Indo-Gangetic Plain of India)

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Authors’ contributions

This work was carried out in collaboration among all authors. Author PBK designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SS and SR managed the analyses of the study. Author VV managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJPSS/2021/v33i1430497

Received 28 March 2021
Accepted 02 June 2021
Published 17 June 2021

ABSTRACT

Soil salinity has affected many soil microbial communities as well as economic value of forest ecosystem for many years. The plant growth-promoting bacteria have developed several different mechanisms that have a positive influence on plant development and growth. Designated strain L-PB424 and P-PB466 was isolated and identified from saline soil of block Ashabutter khair forest in Punjab North zone in India, were investigated for their plant growth-promoting characters such as production of indole acetic acid, phosphate solubilization, Ammonium production and fermentation of polysaccharides. Comparative analysis of 16SrRNA gene sequences revealed that L-PB424 was closely related to Lysinibacillus manganicus DSM 26584 strain Mn1-7 (98.76%), on the other hand, strain P-PB466 was closely related to Pseudomonas songnenensis strain NEAUST5-5. This research paper is a study in evaluation and variety of possible halophilic/halotolerant bacterial strains in salt-affected soils of block Ashabutter khair forest in Punjab North zone in India. The use of Halophilic bacteria in saline soil is interesting for future analysis and biotechnological development.

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Keywords: Halophilic plant growth promoting bacteria; soil salinity; evolutionary tree; 16SrRNA.

1. INTRODUCTION

Acclimatization is an evolutionary process by which living creatures learn to adapt to new environments. Abiotic stressors, such as variations in soil and water salinity, temperature, pH, atmospheric humidity, air circulation, and radiation, affect lower to higher living species, yet they have evolved to cope with them [1]. In the current investigation, a new test approach was proposed to demonstrate erraticism in dangerous conditions with development and advancement of farming soil bacterial species in stepwise transformation in high salt (NaCl) conditions. Two different bacterial species were isolated from saline soil of Gangetic plain. These isolates, showed salt tolerance up to 10% NaCl concentration. Biochemical and molecular (16SrRNA sequencing) characterization revealed the strains to be Pseudomonas stutzeri and Lysinibacillus acetophenone. The halophilic bacteria keep an occasional degree of ionic fixations to include viable solutes, to regulate the diffusion level within the cytoplasm with respect to external environment. These balancing mechanisms of the internal environment and the properties of the cytoplasmic membrane facilitate them to acclimatize to changes within the saline condition as salt lakes, saline soils, and salinity mediated products [2].

Halophilic and halotolerant bacteria are required in production of essential salty food e.g., pickling brines, Thai food sauces etc. [3].

Decolorizing and utilization ability of azo dyes was reported in textile industry microbial isolates [4]. Ability of production of hydrolases, lipases, cellulases, proteases, amylases and biopolymers were found in halotolerant bacteria involved in composting process [5]. The halotolerant species (Bacillus atrophaeus, Halomonas shengliensis, Halomonas koreensis and Virgibacillus salarius) showed the ability to metabolize hydrocarbons and microorganisms like V. salarius and Brevibacillus sp. show potential towards bioremediation [6]. Corynebacterium xerosis was reported as potential hydrocarbons degraders [7]. Halophilic and halotolerant bacteria have different immunological properties and used for the production of different enzymes [8] and also needed for maintaining the soil texture and nutrient recycling in a saline condition [9]. The genus Lysinibacillus belongs to the family Bacillaceae of the phylum Firmicutes, and was proposed by Ahmed et al., [10]. We studied the diversity of halotolerant and halophilic bacteria in saline soils in block Ashabutter khair forest in Punjab, North India.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Two strains named L-PB424 and P-PB466 was isolated from saline soil belong to block Ashabutter khair forest in Punjab North zone in India. Nutrient agar medium and Nutrient broth were routinely used for culturing and maintenance of the recovered bacteria. The maintenance was performed at 4°C in the glycerol stock medium. Then investigated for plant growth promoting trait.

2.2 Indole Test

Some bacteria can produce indole from amino acid tryptophan using the enzyme typtophanase.

Production of indole is detected using Kovac's reagent. Indole reacts with the aldehyde in the reagent to give a red color. An alcoholic layer concentrates the red color as a ring at the top.

2.3 Phosphate Solubilization

Recovered strains were evaluated for the phosphate solubilization activity on Pikovskaya’s agar medium. The incubation temperature 28°C was provided for 5 days, after that the clear zone around the bacterial colonies were thought to be positive for the test. The capability of the phosphate solubilization capacity was described by the phosphorous solubilization index (Ed-Premono et al. 1996).

2.4 Ammonium Production Test

Bacteria, particularly those growing naturally in an environment exposed to urine may decompose urea by means of the enzyme urease. The occurrence of this enzyme can be tested by growing the organism in the presence of urea and testing for alkali (NH₃) production by means of a suitable pH indicator. An alternative method is to test for the production of ammonia from urea by means of Nessler's reagent and to detect NH₄⁺ production due to L-arginine breakdown. A positive reaction for presence of ammonia is a colour ranging from a pale yellow to a dark brown precipitate [11].
2.5 Carbohydrate Fermentation Test

The principle of carbohydrate fermentation states that the action of organism on a carbohydrate substrate results in acidification of the medium, detected by a pH indicator dye. Carbohydrate fermentation is the process bacteria use to produce energy. Most of the bacteria convert glucose to pyruvate during glycolysis; however, some organisms use alternate pathways. A fermentation medium consists of a basal medium containing a single carbohydrate (glucose, lactose, sucrose, mannitol etc.) for fermentation. However, the medium also contains various pH indicators. In addition to a pH indicator to detect the production of acid from fermentation, a Durham tube is placed in each tube to capture gas produced by metabolism. The carbohydrate fermentation patterns shown by different organisms are useful in differentiating among bacterial groups or species [12].

2.6 Molecular Identification

DNA was isolated from the culture with the help of HiMedia Bacterial DNA isolation kit. Its quality was evaluated on 1.0% agarose gel, a single band of high-molecular weight DNA has been observed. Fragment of 16SrRNA gene was amplified by 16SrRNA-F and 16SrRNA-R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16SrRNA-F and 16SrRNA-R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Consensus sequence of 16SrRNA gene was generated from forward and reverse sequence data using aligner software. The 16SrRNA gene sequence was used to carry out BLAST with the ‘nr’ database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix and phylogenetic tree was constructed was constructed using MEGA 10.

3. RESULTS

The recovered bacterial isolates were evaluated on the bases of morphology followed by biochemical, physiological and molecular identification (16SrRNA sequence). The morphological, physiological and biochemical characteristics of bacterial strains are given in Table 1. They have a very strict aerobic respiratory metabolism with oxygen but in some cases, nitrate has been used as an alternative that allows anaerobic growth.

In the study we have observed that the strain PB466 & L-PB424 were positive for P-solubilization and ammonium extraction while L-PB424 was found positive for carbohydrate fermentation and negative for PB-466.

The halophilic strain was tested for salinity tolerance. Both the strain showed growth in sodium chloride medium. One strain L-PB424 performed best at 10% NaCl concentration indicating they are highly salt tolerant. Another strain P-PB466 performed moderate growth at 2% and 5% thus, indicating they are moderately halophilic. Both strains show no growth at 5°C but show little growth 15°C and 50°C whereas both strains show highest growth at 32°C. At 32°C the highest growth rate was found (OD=0.98) after 24 hours of incubation to verify the effects of pH and temperature of medium on the growth rate of isolates, a number of experiments were carried out which were presented in Fig. 1 respectively. The optimal pH for the growth of the isolates was 7.0 while extreme pH was 5.0 which shows no growth and 9.0 which restricted the growth of strain P-PB466 but shows growth of strain L-PB424.

3.1 PCR Amplification of 16S rRNA Gene

Amplification of the 16SrRNA gene was examined in isolated DNA strains (L-PB424 and P-PB466). On a 1.0 percent agarose gel, it was found to contain a single band of high-molecular-weight DNA, as shown in Fig. 2. 16SrRNA-F and 16SrRNA-R primers were used to amplify a fragment of the 16SrRNA gene. When resolved on an agarose gel, a single distinct 1500bp PCR amplicon band was identified. To eliminate impurities, the PCR amplicon was purified. On an
ABI 3730xl Genetic Analyzer, forward and reverse DNA sequencing reactions of PCR amplicons were performed with 16SrRNA-F and 16SrRNA-R primers using BDT v3.1 Cycle sequencing kit. Using aligner software, a consensus sequence of the 16S rRNA gene was produced from forward and reverse sequencing data.

![Graph](image1.png)

**Fig. 1** Different pH and NaCl concentration for growth of bacterial isolates: (a) L-PB424 and (b) P-PB466

**16SrRNA Gene sequence for L-PB424:**

```
TTGGGATAAATCGTGAGACGGGCTATACCGGATAATTACATAGCTCCTTGATATTTTGAA
AGATGTCTTGGCTGTATCGAGTGGCGCACCGCGATTACATAGTTGGTATAGGTACACGGCC
TACCAAGGGAGATGGTGAGCGCACTTGAGAAGTTAGGCGACACGGACTGGGAACACGGCC
CAGACTCTACCGGAGGACAGATGAGGAACTTTCCACAAATTGGAGAAAGAGTCCTGATAC
GCCGCTGTAGTGAAAGAGTGTTCGG
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**16SrRNA Gene sequence for P-PB466:**

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TTGGGATAAATCGTGAGACGGGCTATACCGGATAATTACATAGCTCCTTGATATTTTGAA
AGATGTCTTGGCTGTATCGAGTGGCGCACCGCGATTACATAGTTGGTATAGGTACACGGCC
TACCAAGGGAGATGGTGAGCGCACTTGAGAAGTTAGGCGACACGGACTGGGAACACGGCC
CAGACTCTACCGGAGGACAGATGAGGAACTTTCCACAAATTGGAGAAAGAGTCCTGATAC
GCCGCTGTAGTGAAAGAGTGTTCGG
```
16SrRNA Gene sequence for P-PB466:

GCGGCAGGCTAACACATGCAAGTGAGTGGAGCTTGCTCCATGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTATACCGCATACGTCCTACGGGAGAAAGTGGGGGAATCTTCGGACCTACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTTGTGAAGAAGGTCTTCGGATGTAAAGCACTTTAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCCGCTAACTCCGCAAGCCGCGTAATACGAAGGGTGCAAGCGTTAATCGAATTACTGGGCGTAAAGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTATGGCAGAGGGTGGTGGAATTTCCTGTG

Fig. 2. 16SrRNA gene (1500bp) amplification of bacterial strain (a) L-PB424 (b) P-PB466

3.2 BLAST Result of L-PB424 & P-PB466

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. Fig. 4 depicts the tree with the highest log likelihood (-2380.84). The initial tree(s) for the heuristic search were automatically generated by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances calculated using the Maximum Composite Likelihood (MCL) technique, and then picking the topology with the best log likelihood value. The branch lengths are measured in the number of substitutions per site, and the tree is depicted to scale. There were 11 nucleotide sequences in this study. 1st+2nd+3rd+Noncoding codon locations were included. The total number of locations in the final dataset was 1440. MEGAX was used to perform evolutionary analysis [14].
### Table 1. Morphological and biochemical characteristics of isolated bacterial strains

| S.NO | Bacterial strain | Colony morphology | IAA | P- solubilization | NH$_3$ excretion | Carbohydrate fermentation | Gram reaction | Bacterial species |
|------|------------------|-------------------|-----|-------------------|------------------|--------------------------|---------------|------------------|
| 1.   | L-PB424          | Cream irregular   | Raised Undulated | +    | +                | +                        | Gram positive(rod) | Lysinibacillus acetophenoni |
| 2.   | P-PB466          | White circular    | Raised Undulated | +    | +                | -                        | Gram negative(rod) | Pseudomonas stutzeri |
Table 2. BLAST result of L-PB424

| Description                                      | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession    |
|--------------------------------------------------|-----------|-------------|-------------|---------|------------|--------------|
| Lysinibacillus manganicus DSM 26584 strain Mn1-7 | 2569      | 2569        | 99%         | 0       | 98.76%     | NR_118533.1  |
| Lysinibacillus acetophenoni strain JC23          | 2549      | 2549        | 98%         | 0       | 98.81%     | NR_135864.1  |
| Lysinibacillus massiliensis 4400831 = CIP 108448 = CCUG 49529 | 2429      | 2429        | 96%         | 0       | 97.80%     | NR_043092.1  |
| Lysinibacillus chungkukjangi strain 2RL3-2       | 2412      | 2412        | 98%         | 0       | 97.00%     | NR_109669.1  |
| Lysinibacillus endophyticus strain C9            | 2401      | 2401        | 98%         | 0       | 96.75%     | NR_146821.1  |
| Lysinibacillus halotolerans strain LAM612        | 2350      | 2350        | 96%         | 0       | 96.94%     | NR_134073.1  |
| Lysinibacillus alkaliphilus strain OMN17         | 2344      | 2344        | 96%         | 0       | 96.67%     | NR_136779.1  |
| Ureibacillus defluvi strain DX-1                 | 2342      | 2342        | 97%         | 0       | 96.30%     | NR_133885.1  |
| Lysinibacillus odysseyi 34hs-1 = NBRC 100172 strain 34hs1 | 2335      | 2335        | 97%         | 0       | 96.28%     | NR_025258.1  |
| Lysinibacillus telephonicus strain S5H2222      | 2333      | 2333        | 96%         | 0       | 96.47%     | NR_157637.1  |

Fig. 3 Evolutionary relationship of taxa with reference to L-PB424

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-3419.39) is shown in Fig. 3. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum
Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the variety of substitution per website this analysis is concerned for the 11 sequences and sequence clusters with respect to position and there is 1464 sequence position within the final dataset and MEGAX was used to analyze the evolutionary relationship between the sequences [14].

Table 3. BLAST result of P-PB466

| Description                                      | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession   |
|--------------------------------------------------|-----------|-------------|-------------|---------|------------|-------------|
| Pseudomonas stutzeri ATCC 17588 = LMG 11199      | 2647      | 2647        | 100%        | 0       | 99.93%     | NR_041715.1 |
| Pseudomonas stutzeri strain CCLUG 11256          | 2647      | 2647        | 100%        | 0       | 99.93%     | NR_118798.1 |
| Pseudomonas stutzeri strain NBRC 14165            | 2643      | 2643        | 100%        | 0       | 99.86%     | NR_113652.1 |
| Pseudomonas stutzeri ATCC 17588 = LMG 11199      | 2641      | 2641        | 100%        | 0       | 99.86%     | NR_103034.2 |
| Pseudomonas stutzeri strain VKM B-975             | 2636      | 2636        | 100%        | 0       | 99.79%     | NR_116489.1 |
| Pseudomonas songnenensis strain NEAU-STS-5        | 2556      | 2556        | 100%        | 0       | 98.82%     | NR_148295.1 |
| Pseudomonas chloridismutans strain AW-1           | 2516      | 2516        | 100%        | 0       | 98.26%     | NR_115115.1 |
| Pseudomonas kunmingensis strain HL22-2            | 2497      | 2497        | 100%        | 0       | 98.05%     | NR_133828.1 |
| Pseudomonas guariconensis strain PCAVU11          | 2488      | 2488        | 100%        | 0       | 97.92%     | NR_135703.1 |
| Pseudomonas knackmussii B13                       | 2486      | 2486        | 99%         | 0       | 98.04%     | NR_117756.1 |

Fig. 4. Evolutionary relationship of taxa with reference to P-PB466
4. DISCUSSION

The evolutionary tree of L-PB424 showed that the strain shows close resemblance with *Lysinibacillus manganicus* 98.76%, *Lysinibacillus massiliensis* 97.80% and *Lysinibacillus chungkujiangi* 97.00% with this strain. It indicates that strain L-PB424 belongs to genus Bacillus. Hence, it is designated as *Lysinibacillus acetophenoni*. While the evolutionary tree of strain L-PB424 showed its close resemblance with *Lysinibacillus manganicus* with maximum 98% homology on the other hand P-PB466 showed that the strain shows close resemblance with *Pseudomonas stutzeri* 99.93%, *Pseudomonas songnenensis* 98.82% and *Pseudomonas chloritidismutans* 98.26% with this strain. It indicates that strain P-PB466 belongs to genus Pseudomonas. Hence, it is designated as *Pseudomonas stutzeri*. While the evolutionary tree of strain P-PB466 showed its close resemblance with *Pseudomonas stutzeri* with maximum 99% homology. The submission of 16SrRNA sequence to the NCBI GenBank and find the accession number of both sequences are MW362768 and MW44489. These two strains are halotolerant and possess adaptive mechanism to tolerate hypersaline condition.

5. CONCLUSION

It is evident from the current sample that the sampling area contains a diverse range of bacterial species. In tryptophan broth, they react to IAA development in a significant way. IAA answer is good across the board in all isolates. Apart from that, isolates have demonstrated a significant role in phosphate solubilization, carbon source which have high impact on growth of the plant. Generation of IAA and Phosphorous Solubilizing Index has the highest response. Finally, it is clear that bacteria retrieved from saline were not only halotolerant, but also had traits that promoted plant growth. These bacteria can be used as biofertilizers to combat abiotic stress and are a blessing for sustainable agriculture in stressful situations.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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