

Original Research Article

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

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 were isolated and identified from saline soil of North zone in India County, block Ashabutter khair forest in Punjab were investigated for their plant growth-promoting characters such as production of siderophores, 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 North zone in India County, block Ashabutter khair forest in Punjab. The use of halotolerant bacteria in saline soil (Indo-Gangetic plains) is interesting for future analysis and biotechnological development.

Keywords: Halophilic plant growth promoting bacteria, soil salinity, phenotype; 16SrRNA.

Introduction

Adaptation is a common characteristic of microorganisms in different extreme environments with possible applications in pharmaceutical, food, & bioenergy sectors. In the current investigation, a new test approach was proposed to demonstrate variability in extreme 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 acetophenoni*. The halotolerant organisms 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 (Roberts MF., 2005).

Halophilic and halotolerant bacteria are required in production of essential salty food e.g., pickling brines, Thai food sauces etc. (Margesin R *et al.*, 2001).

Decolorizing and utilization ability of azo dyes was reported in textile industry microbial isolates (Asad *et al.*, 2007). Ability of production of hydrolases, lipases, cellulases, proteases, amylases and biopolymers were found in halotolerant bacteria involved in composting process (Oliveira LC *et al.*, 2015). 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 (Kothari V *et al.*, 2015). *Corynebacterium xerosis* was reported as potential hydrocarbons degraders (petrol and diesel; Jyothi K *et al.*, 2012). Halophilic and halotolerant bacteria have different immunological properties and uses for the production of different enzymes (Shirazian P *et al.*, 2017) and also needed for maintaining the soil texture and nutrient recycling in a saline condition (Lavik G, *et al.*, 2009). The genus *Lysinibacillus* belongs to the family Bacillaceae of the phylum Firmicutes, and was proposed by Ahmed *et al.*, (2007). We studied the diversity of halotolerant and halophilic bacteria in saline soils in block Ashabutter khair forest in Punjab, North India.

Materials and methods

Bacterial strains

Two strains named L-PB424 and P-PB466 were isolated from salt-rich soil belong to North zone in India County, block Ashabutter khair forest in Punjab. I suggest adding a map of the location, within India and within Punjab State. 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.

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 solubilization index = total diameter (colony + halo zone) : colony diameter (Edi-Premono *et al.* 1996).

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 (NH_3) 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 NH_3 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. **OK, so the test is for AMMONIUM (NH_4^+) ion, not for AMMONIA (NH_3) specifically, so this should be properly mentioned. Also, add a citation.**

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. **Add a citation for this method.**

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 1500bp 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 was generated using RDP database and the phylogenetic tree was constructed using MEGA6. **Add a citation for this method.**

Results

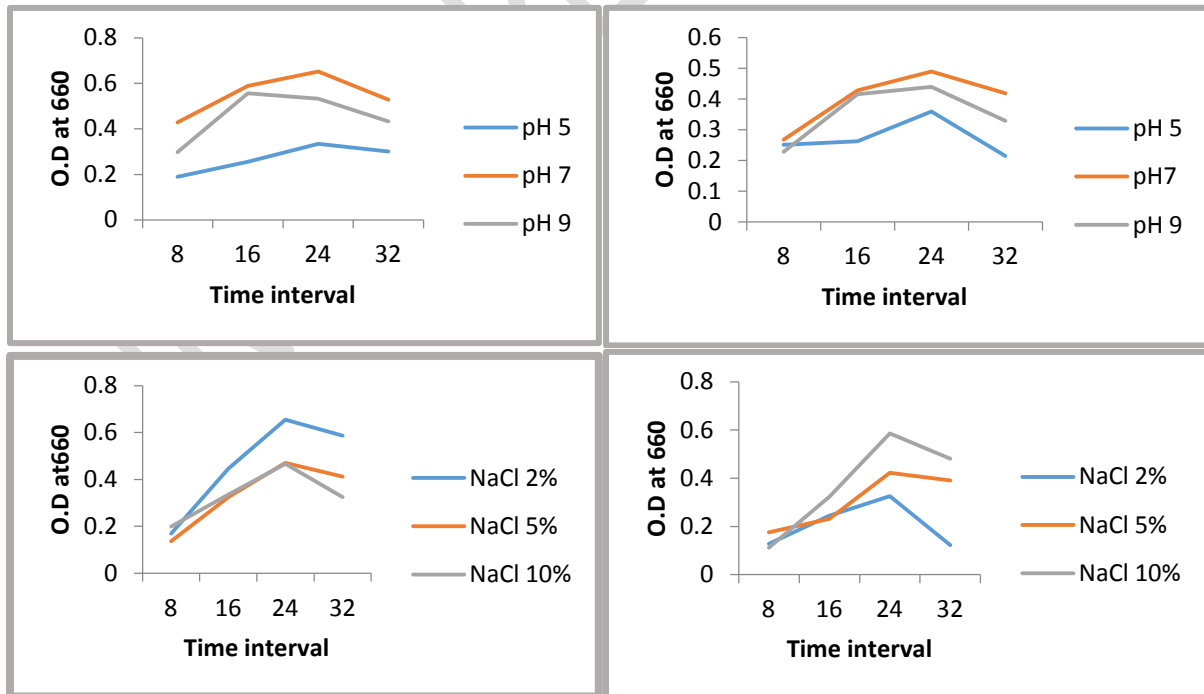
The recovered bacterial isolates were evaluated on the bases of morphology followed by biochemical, physiological and molecular identification (16SrRNA sequence). The morphological, physiological and biochemical characteristics of bacterial strains are given in **Table 1**. They have a very strict aerobic 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 P-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 Figure 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.

Table 1 Morphological and biochemical characteristics of isolated bacterial strains

S.NO	Bacterial strain	Colony Morphology				P-solubilization	NH ₃ Excretion	Carbohydrate fermentation	Gram Reaction	Bacterial species
		Cream	Irregular	Raised	Undulated					
1.	L-PB424	Cream	Irregular	Raised	Undulated	+	+	+	Gram positive(rod)	<i>Lysinibacillus acetophenoni</i>
2.	P-PB466	White	circular	Raised	Undulated	+	+	-	Gram negative(rod)	<i>Pseudomonas stutzeri</i>



(a)

(b)

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

PCR amplification of 16S rRNA gene:

Isolated DNA strains (L-PB424 and P-PB466) were investigated for 16SrRNA gene amplification. Its quality was evaluated on 1.0% agarose gel, a single band of high-molecular weight DNA has been observed and shown in Figure2. Fragment of 16SrRNA gene was amplified by 16SrRNA-F and 16SrRNA-R primers. A single discrete PCR amplicon band of 1500bp 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 16S rRNA gene was generated from forward and reverse sequence data using aligner software.

Phylogenetic analysis of strains:

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 using MEGA 10. **Maybe this paragraph should be moved to „Material and Methods”**

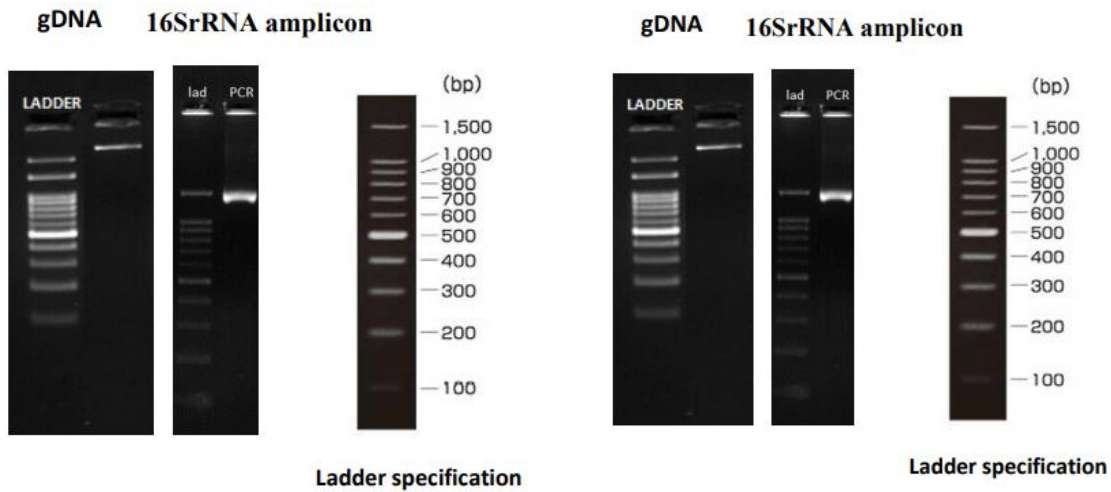
16SrRNA Gene sequence for L-PB424:

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TTGGGATAACTCCGTGAAACCGGGGCTATACCGGATAATACAATCTAGCTCCTGCTA
ATGTTGAAAGATGGTTTCGGCTATCGCTATGGATGGGCCCGCGGCATTACTAGTT
GGTAGGGTAACGGCCTACCAAGGGACGATGCGTAGCCGACCTGAGAGGGTGAGGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCGG
ATCGTAAAACCTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGTACCTTG
ACGGTACCTTATTAGAAAGCCACGGCTAACTAGTGCCAGCAGCCGCGGTAATACGT
GGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCCGGTCCTTTA
AGTCTGATGTGAAAGCCCTCGGCTCAACCGAGGAGGGTCATTGGAAACTGGAGGAC
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TTGGAGGAACACCAGTGCGAAGGCGACTTTCTGGTCTGTAAGTACGCTGAGGCG
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CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA
TGAGTGCTAAGTGTTAGGGGGTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGC
ACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTGGAAGCAACGCGAAGAACCTTACCAG
GTCTTGACATCCCGCTGACCGCTATGGAGACATAGCCTTCCCTTCGGGGACAGCGGT
GACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCCG
CAACGAGCGCAACCCTTGTTCTTAGTTGCCATCATTTAGTTGGGCACTCTAAGGAGA
CTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTA
TGACCTGGGCTACACACGTGCTACAATGGACGGTACAAACGGTTCGCGAAGTCGCGA
GACGAAGCCAATCCGATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCT
ACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTT
CCGGGCCTTGACACACCGCCCGTCACACCACGAGAGTTTGTAAACCCCGAAGTCG
GTGGGGTAACCCTTACGGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGA
AGTCGTACAAGGGGGTTAACCGAAAA

16SrRNA Gene sequence for P-PB466:

GCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAGTGGAGCTTGCTCCATGATTC
AGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTT
TCGAAAGGAACGCTAATACCGCATACTCCTACGGGAGAAAGTGGGGGATCTTCGG
ACCTCACGCTATCAGATGAGCCTAGGTTCGGATTAGCTAGTTGGTGAGGTAAAGGCTC
ACCAAGGCGACGATCCGTAACCTGGTCTGAGAGGATGATCAGTCACACTGGAAGTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGA
AAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT
TAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTTGACGTTACCAACAGAA
TAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGTAATACGAAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAA
GCCCCGGGCTCAACCTGGGAACTGCATCCAAAACCTGGCGAGCTAGAGTATGGCAGA
GGGTGGTGGAAATTTCTGTGTAGCGGTGAAATGCGTAGAGGAAGGAACACCAGTGG
CGAAGGCGACCACCTGGGCTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGTCGACTAGCCGTTGGG
ATCCTTGAGATCTTAGTGGCGCAGCTAACGCATTAAGTCGACCGCCTGGGGAGTACG
GCCGCAAGGTTAAAACCTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTGGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAAC
TTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCCGTAACGAGCGCAACCCTTGTC
TTAGTTACCAGCACGTTAAGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGG
AGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACG
TGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATA
AAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCG
CTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGACACACC
GCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAATAGCTAGTCTAACCTTCGGGGG
GACGGTTACCACGGAGTGATTC



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

BLAST Result of L-PB424 & P-PB466:

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 defluvii 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

Table 2 BLAST result of L-PB424

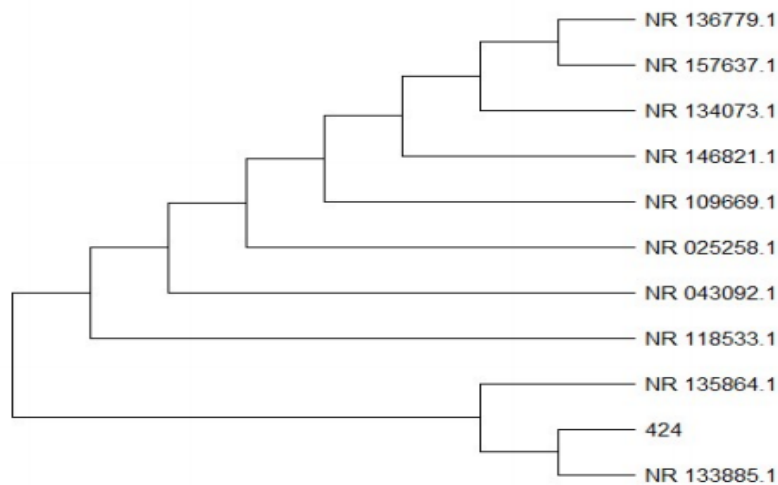


Figure 3 Evolutionary relationship of taxa with reference to L-PB424

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 Figure 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 (Kumar *S. et al.*, 2018)

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 CCUG 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_103934.2
Pseudomonas stutzeri strain VKM B-975	2636	2636	100%	0	99.79%	NR_116489.1
Pseudomonas songnenensis strain NEAU-ST5-5	2556	2556	100%	0	98.82%	NR_148295.1
Pseudomonas chloritidismutans 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

Table 3 BLAST result of P-PB466

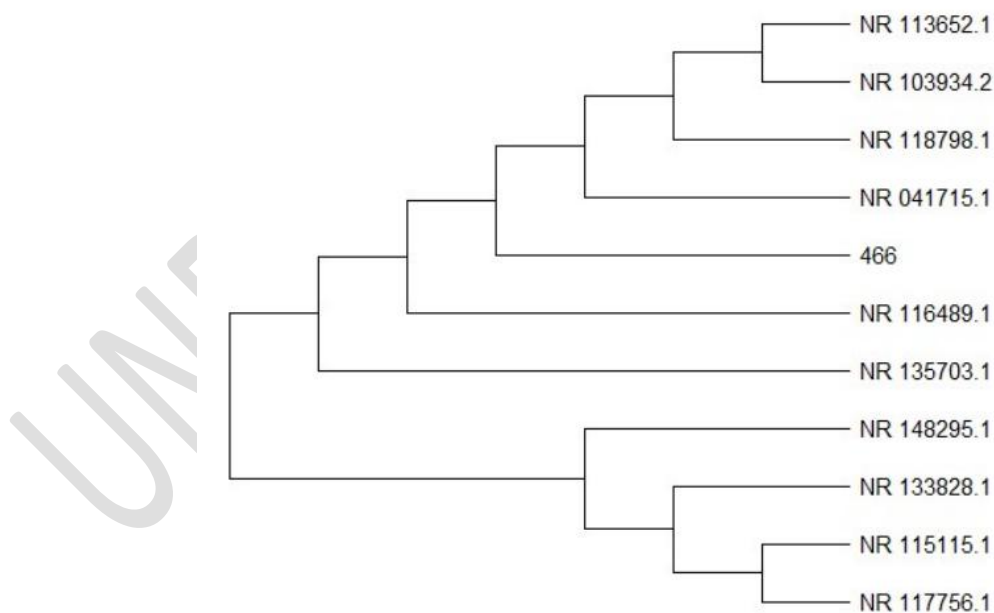


Figure 4 Evolutionary relationship of taxa with reference to P-PB466

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-2380.84) is shown in Figure 4. 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 number of substitutions per site. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1440 positions in the final dataset. Evolutionary analyses were conducted in MEGAX (Kumar S. *et al.*, 2018). This paragraph mostly repeats the previous. It would be easier to understand if you just mention that 1464 positions were found for one strain and 1440 for the other.

The evolutionary tree of L-PB424 showed that the strain shows close resemblance with *Lysinibacillus manganicus* 98.76%, *Lysinibacillus massiliensis* 97.80% and *Lysinibacillus chungkukjangi* 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.

Discussion:

The aim of our current inspection was to isolate plant growth-promoting *Bacillus* and *Pseudomonas* isolates and test them for physiological stress tolerance, such as pH and NaCl concentrations. Bacterial isolates were obtained from saline-rich soil in the block Ashabutter khair forest in Punjab, North India, in order to carry out this research. These PGP bacterial strains are beneficial to plant growth and defend plants from diseases and abiotic stress through a range of mechanisms. Those that form strong associations with plants, such as endophytes, may be more effective in promoting plant growth and increasing resistance to osmotic stress. Since Gram-positive bacteria develop endospores, which have the ability to survive in harsh environmental conditions, the P-PB466 strain showed more resistance to high salinity (10 percent NaCl) than the P-PB 424 strain at pH 9. Our findings matched those of Damodarachari K., *et al* (2018), who tested bacteria for high temperatures (50°C), salinity (7%) and drought (-1.2MPa), finding that *Pseudomonas* isolates were less resistant than *Bacillus* isolates. Youssef *et al.* found that bacterial strains derived from salty soil grew at a comparable rate (2003). As L-PB424 was grown on nutrient broth with NaCl (2%, 5%, and 10%) and pH (5.0, 7.0, and 9.0) for two days at an 8-hour period, the maximum growth curve was obtained, which is consistent with Omotoyinbo (2016). Salinity tolerating bacteria, according to DasSarma (2006), exhibit optimum growth in various saline environments, which are further classified into three classes: low salinities of 20-50 ppt, moderate salinities of 50-200 ppt, and high salinities > 200 ppt NaCl tolerating halophiles. The 16SrRNA gene sequences of potent bacterial strains were compared with available sequences in Gene Bank to identify bacterial strains. Damodarachari K., *et al* (2018) recorded similar findings with *Bacillus* spp. BLAST search results from NCBI revealed 98.76 percent similarity of L-PB424 with *Lysinibacillus acetophenoni* and 99.93 percent similarity of P-PB466 with *Pseudomonas stutzeri*.

Conclusions

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 use, and Siderophore production. **I am puzzled: no prior mention of siderophore and IAA production was made throughout the text! Either some paragraphs in Methods and Results are missing, or this part of the Conclusions should be removed!** 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 can be used as biofertilizers to combat abiotic stress and are a blessing for sustainable agriculture in stressful situations.

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