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Identification and characterization of novel lipase producing soil bacterial isolates B3 and B4 using 16S rDNA analysis

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ABSTRACT

Soil samples were screened for lipase producing soil bacteria by employing various screening methods to arrive at two best lipase producing soil bacterial isolates named B3 & B4. The genomic DNA extracted from these soil isolates was purified separately and used them as templates for the amplification of 16S rRNA gene sequences of B3 & B4 using Eppendorf thermal cycler. These 16S rDNA amplicaons were purified and subjected to automated DNA sequencing on ABI 3730xl genetic analyzer. The subsequent forward and reverse sequences of the 16S rRNA genes were aligned to obtain the consensus sequences that were analyzed with BLASTN using NCBI GenBank database. The pair wise multiple alignment analysis of the ten best chosen bacterial strain sequences with the respective B3 & B4 sequence was performed using ClustalW and the results were processed to make the phylogenetic tree. From these results, the bacterial soil isolates B3 & B4 were identified as *Pseudomonas* sp. SPSU B3 and *Aneurinibacillus migulanus* sp. SPSU B4 and the respective NCBI GenBank Accession numbers for 16S rRNA genes were obtained as JX524282 and JX564542. Further these organisms were found as gram negative (B3) and gram positive (B4) with no response to the performed IMViC tests.

Keywords: 16S rDNA analysis, Soil isolates, *Pseudomonas*, *Aneurinibacillus*, Lipase

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INTRODUCTION

Soil is the ultimate source of diverse microbial organisms depending on the geographical variations in terms of moisture, minerals, carbon sources, temperature etc. Since less than 1% of microorganisms available in the natural habitats [1-2] are cultivated using the formulated microbiological culture media and devised methods, there had been the greater possibility of finding novel microbial strains as the nature reveals to the mankind from time to time. So the soil had been the versatile storage facility [3] and it has been supplying the required microbial flora [4-11] through their revival as per the needs of complex molecules degradation [12-15] leading to the supply of vital nutrients [16-19] to plants & animals. In this way the microorganisms fulfil the major role in harnessing the energy and revitalizing the food chain in its cycle.

It has been found in the modern practices that, the decomposing sites and industrial effluents [3, 20-22] are chosen for isolating the novel microbial organisms for the vested interests and needs. In an alternative way, the semi moist layer of soil deposited by run-off water in the water streams can also be considered for isolation of diverse microbial strains. These sediment soils must have got the deposition of various microbial strains during runoff period in the rainy seasons. Such soils can also be used specifically to identify & isolate the microbial organisms as industrially important strains producing the secondary metabolites such as enzymes like proteases, lipases, cellulases, pectinases, amylases [23-29] etc. In the similar lines, the present study was conducted to screen & isolate the novel lipase producing bacterial strains from such soil samples.

For microbial identification of unknown sample strains, one can use either the traditional biochemical tests [30] or the recent molecular techniques such as pulsed field gel electrophoresis (PFGE) of whole chromosomal DNA, RAPD & AFLP assays, 16S rDNA analysis, Real Time PCR and microarray based bacterial identification methods [31-40]. The traditional bacterial identification methods practiced for over a century are based on biochemical and phenotypic characters of microbial organisms, however they may fail to detect the peculiar biochemical characters specific to the unknown genus and species. Additionally the methods are devised for cultured organisms and the procedures employed may also take longer duration for identification of bacterial strains. On the contrary the molecular techniques like 16S rDNA analysis provide the faster way to identify the unknown bacterial cultures to the nearest species [41].

MATERIALS AND METHODS

Soil samples

Soil samples were collected from the runoff water streams located in the city outskirts of Guntur (Andhra Pradesh), Nagpur (Maharashtra) and Udaipur (Rajasthan) India through a depth of 4-5cm. These soil samples were processed [42] in the Microbiology Laboratory,

Department of Biotechnology, School of Engineering, Sir Padampat Singhania University, Udaipur, Rajasthan, India.

Screening of soil samples

The soil samples of Guntur (G), Nagpur (N) and Udaipur (B) were processed by serial dilution method and the resulting soil suspensions were inoculated (1ml) on to the sterile tributyrin agar plates by pour plate method and the produced colonies with surrounding clear zones after 48hrs incubation period at 30^{o}C were isolated from each sample and processed them subsequently with *Rhodamine B* agar plates to identify and isolate true lipase producing isolates. The true lipase producing bacterial isolates from each soil sample were isolated and preserved for further processing [42].

The best lipase producing bacterial isolates

The isolated lipase producing isolates from the soil samples were subjected to agar well diffusion lipase assay and titrimetric lipase assay to arrive at a total of 22 (12 from B, 5 from N, 5 from G) best lipase producing bacterial lipase isolates. These isolates were further subjected to broth culture optimization studies with respect to temperature, incubation period [42] and media composition in terms of variable carbon source [43] to arrive at the best three lipase producing bacterial isolates as B1, B3 & B4 (from the soil samples of Bhatewar, the outskirts of Udaipur city). These isolates were further subjected to mutative strain improvement techniques, UV-irradiation, microwave irradiation and ethidium bromide treatment along with further media optimization parameters by varying substrate concentrations, nitrogen sources and pH [44] to finally arrive at two best lipase producing bacterial isolates as B3 & B4.

Characterization & identification of lipase isolates B3 & B4

Biochemical Characterization

The bacterial isolates B3 & B4 were tested with gram staining, followed by IMViC tests, followed by gelatin liquefaction test, phenol red broth tests of dextrose, maltose, mannitol, sorbitol and dulcitol along with urease test [30] to characterize the strains in terms of their phenotypic and physiological characters.

Genomic DNA preparation

The lipase isolates B3 & B4 were cultured for overnight in nutrient broth media at 150rpm & 30^{o}C and 1.0 ml of broth samples were withdrawn aseptically and centrifuged at 5000g for 10 min and the respective pellets were processed for genomic DNA extraction using XcelGen bacterial genomic DNA isolation kit, XG 2411-01, following the manufacturer's protocol. These bacterial genomic DNA were quantified by Nanodrop spectrophotometer as 500ng/µl approximately. The purity of the extracted DNA was confirmed by running 2.5µg DNA

separately from each sample on submarine gel electrophoresis set at 80V for 30 minutes at 25°C. The resultant DNA bands were visualized using UV- Trensilluminator.

PCR amplification

16S rDNA fragments of DNA samples of B3 & B4 were amplified by using universal 16S rRNA gene primers 8F: AGAGTTTGATCCTGGCTGAG as forward primer & 1492R: ACGGCTACCTTGTTACGACTT as reverse primer. The PCR amplification was carried out in Eppendorf Thermal cycler with 25 µl of final reaction volume containing 7.5 µl DNase-RNase free water, 12.5 µl 2X PCR master mix (MBI Fermentas), 1.0 µl forward primer 8F, 1.0 µl reverse primer 1492R and 3.0 µl diluted DNA (30ng/µl). The PCR was initiated with initial denaturation of DNA at 95°C for 2min and subsequently the number of cycles (94°C for 30s, 52°C for 30s and 72°C for 90s) were set to 30, and the final extension was performed at 72°C for 10min. 5 µl from the resulting PCR amplicons of B3 & B4 were mixed separately with 1µl of 6X gel loading dye and electrophoresed on 1.2% agarose gel containing ethidium bromide (0.1 µg/ml) at constant electric field of 5V/cm for 30min in 0.5X TAE buffer. The amplified PCR products 16S rDNA fragments of isolates B3 & B4 were confirmed as 1500bp compact single band DNA visualized separately under UV-light using gel documentation system (Biorad). These 16S rDNA fragments were further purified using Min Elute Gel Extraction kit (QIAGEN) following the manufacturer's protocol.

Sequencing and analysis of 16S rRNA gene sequences

The purified 16S rRNA genes of isolates B3 & B4 were subjected to automated DNA sequencing on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). Sequencing was carried [45-48] out using BigDye Terminator v3.1 Cycle sequencing kit following the manufacturer's protocol, where sequencing cycle was set with the thermal ramp rate of 1°C per second for 25 cycles (96°C for 10s; 52°C for 5s and 60°C for 4min). The resultant forward and reverse sequences of 16S rRNA genes of isolates B3 & B4 were aligned with CAP3 v1.0 aligner programming tool [49] and the consensus 16S rRNA gene sequences were obtained as shown in Figures 2 & 3 respectively. These consensus gene sequences were used to identify the bacterial isolates with BLASTN analysis using NCBI GenBank nrdatabase and obtained the homology to the closest bacterial organisms with maximum similarity ranging from 99% - 100%. The resulting first ten best 16S rRNA gene sequences of the neighborhood bacterial cultures were chosen along with the respective B3 & B4 16S rRNA gene sequences for the analysis of homology match using Neighbor-Joining method [50] and aligned using multiple alignment software, Clustal W. These alignment results were used to construct phylogenetic tree using MEGA 4.0 software tool [51].

RESULTS AND DISCUSSION

The soil samples of Guntur (G), Nagpur (N) and Udaipur (B) were screened through various screening and optimization techniques to finally isolate two best lipase producing soil isolates as B3 & B4. The phenotypic, physiological and biochemical characterization of the soil

isolates B3 & B4 were performed and the results were tabulated in Table-1. The genomic DNA of the final isolates B3 & B4 were extracted and found to be of good purity as (OD260/OD280) ~1.91. The quality and purity of these DNA samples were further confirmed with agarose gel electrophoresis resulting in the single band of high molecular weight DNA as it was observed under UV illumination. The 16S rRNA genes of isolates B3 & B4 were amplified by PCR with 10pmole of both 8F and 1492R primer set separately using Eppendorf Thermal Cycler and the resulting PCR amplicons were visualized as a single compact band of expected 1500bp DNA using 1.2% agarose gel electrophoresis as shown in the Figure 1. These PCR amplicons were purified and subjected to automated DNA sequencing. The resultant forward and reverse sequences of 16S rRNA genes were aligned with CAP3 aligner software separately for isolates B3 & B4, and constructed the corresponding consensus sequences as shown in the Figures 2 & 3. These consensus sequences were analyzed with BLASTN search tool using nrdatabase of NCBI GenBank for the identification of bacterial isolates B3 &B4 and from the results the first ten best homology microorganisms were selected and shown in the Tables 2 & 3. The homologous 16S rRNA gene sequences of the selected strains with respect to isolates B3 &B4 were obtained from the microbial nucleotide databases through NCBI facility. These selected homology sequences of 16S rRNA genes along with the respective B3 & B4 16S rRNA gene sequences were aligned using multiple alignment software Clustal W and the obtained results were processed using Ribosomal Database Project-RDP [52] and generated the phylogenetic tree through MEGA 4.0 software tool as shown in the Figures 4 & 5.

Table 1: Physical and biochemical responses of the bacterial isolate B3 & B4

Biohemical/ biophysical parameter	Bacterial Isolates	
	B3	B4
Grams	Negative	Positive
Morphology	Rod	Rod
Indole	Negative	Negative
MR	Negative	Negative
VP	Negative	Negative
Citrate	Negative	Negative
Urea	Negative	Negative
Gelatin liquefaction	Negative	Negative
Phenol red Dextrose Broth	Negative	Negative
Phenol red Maltose Broth	Negative	Negative
Phenol red Mannitol Broth	Negative	Negative
Phenol red Sorbitol Broth	Negative	Negative
Phenol red Dulcitol Broth	Negative	Negative

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Table 2: Isolate B3 homology to the nearest known neighborhood bacterial strains

Accession Number	Bacterial Strain	Max score	Total score	Query coverage	E value	Max identity
HQ143645.1	Pseudomonas sp. strain WCH22	2518	2518	100%	0	99%
GQ280063.1	Pseudomonas sp strain BJ-53	2470	2470	99%	0	99%
GQ280016.1	Pseudomonas sp. BJ-6	2470	2470	99%	0	99%
JQ963329.1	Pseudomonas stutzeri strain K-2-7	2468	2468	99%	0	99%
JX223818.1	Uncultured bacterium clone EMIRGE_OTU_s6b4a_1432	2468	2468	99%	0	99%
JQ858222.1	Uncultured Pseudomonas sp. clone TJ-7	2468	2468	99%	0	99%
JQ858221.1	Uncultured Pseudomonas sp. clone TJ-6	2468	2468	99%	0	99%
JQ858217.1	Uncultured Pseudomonas sp. clone TJ-2	2468	2468	99%	0	99%
JF461537.1	Pseudomonas stutzeri strain SN1	2468	2468	99%	0	99%
JN645996.1	Pseudomonas sp. 1410	2468	2468	99%	0	99%

Table 3: Isolate B4 homology to the nearest known neighborhood bacterial strains

Accession Number	Bacterial Strain	Max score	Total score	Query coverage	E value	Max identity
AB210964.1	Aneurinibacillus aneurinilyticus strain: SSCT74	2440	2440	100%	0	99%
NR_036798.1	Aneurinibacillus aneurinilyticus strain Murayama	2435	2435	100%	0	99%
AB680012.1	Aneurinibacillus aneurinilyticus strain: NBRC 3115	2431	2431	100%	0	99%
AB271755.1	Aneurinibacillus aneurinilyticus	2431	2431	100%	0	99%
AB112724.1	Aneurinibacillus aneurinilyticus strain:DSM 5562T	2431	2431	100%	0	99%
AB101592.1	Aneurinibacillus aneurinilyticus	2431	2431	100%	0	99%
GU549488.1	Aneurinibacillus aneurinilyticus strain BS-1	2429	2429	100%	0	99%
AB211028.1	Aneurinibacillus aneurinilyticus strain: SSCS14-1	2422	2422	100%	0	99%
HQ670752.1	Aneurinibacillus aneurinilyticus strain RPS5	2407	2407	100%	0	99%
NR_036799.1	Aneurinibacillus migulanus strain B0270	2407	2407	100%	0	99%

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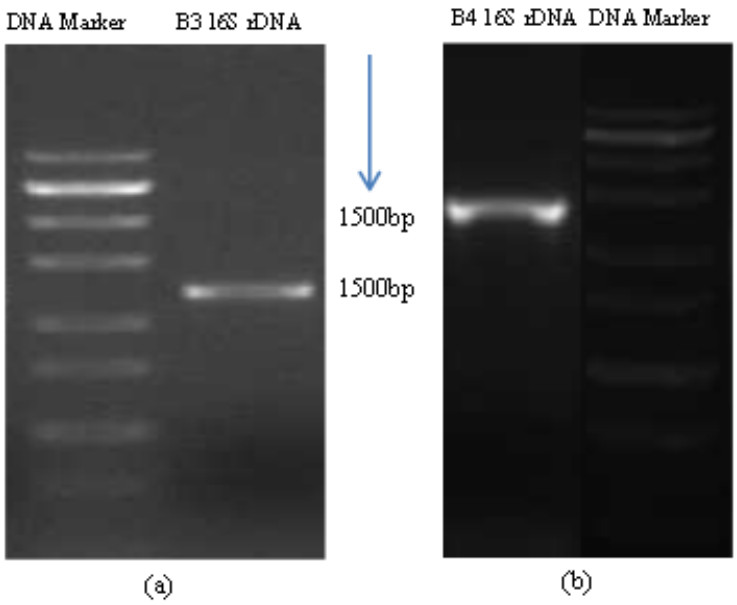

Figure 1. Visualization of amplified16S rDNA fragments of 1500bp of microbial isolates B3 (a) & B4 (b).

gagccagcggatgagtggagcttgctcctgattcagcggcggacgggtgagtaatgcctaggaatctgcctggtagt
gggggacaacgtttcgaaaggaacgctaataccgcatacgtcctacgggagaaagtgggggatcttcggacctcacg
ctatcagatgagcctaggtcggattagctagttggtgaggtaaaggctcaccaaggcgacgatccgtaactggtctga
gaggatgatcagtcacactggaactgagacacggtccagactcctacgggaggcagcagtggggaatattggacaat
gggcgaaagcctgatccagccatgccgcgtgtgtgaagaaggtcttcggattgtaaagcactttaagttgggaggaag
ggcagtaagttaataccttgctgttttgacgttaccaacagaataagcaccggctaacttcgtgccagcagccgcggtaa
tacgaagggtgcaagcgttaatcggaattactgggcgtaaagcgcgcgtaggtggttcgttaagttggatgtgaaagc
cccgggctcaacctgggaactgcatccaaaactggcgagctagagtatggcagagggtggtggaatttcctgtgtagc
ggtgaaatgcgtagatataggaaggaacaccagtggcgaaggcgaccacctgggctaatactgacactgaggtgcg
aaagcgtggggagcaaacaggattagataccctggtagtccacgccgtaaacgatgtcgactagccgttgggatcctt
gagatcttagtggcgcagctaacgcattaagtcgaccgcctggggagtacggccgcaaggttaaaactcaaatgaatt
gacgggggcccgcacaagcggtggagcatgtggtttaattcgaagcaacgcgaagaaccttaccaggccttgacatg
cagagaactttccagagatggattggtgccttcgggaactctgacacaggtgctgcatggctgtcgtcagctcgtgtcgt
gagatgttgggttaagtcccgtaacgagcgcaacccttgtccttagttaccagcacgttaaggtgggcactctaaggag
actgccggtgacaaaccggaggaaggtggggatgacgtcaagtcatcatggcccttacggcctgggctacacacgt
gctacaatggtcggtacaaagggttgccaagccgcgaggtggagctaatcccataaaaccgatcgtagtccggatcg
cagtctgcaactcgactgcgtgaagtcggaatcgctagtaatcgtgaatcagaatgtcacggtgaatacgttcccgggc
cttgtacacacctctcgtcacaatctgggagtgggtgagagct

Figure 2. Consensus sequence constructed from the forward and reverse sequences of 16S rDNA fragments of isolate B3.

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gcggttagcggcggacgggtgagtaacacgtaggcaacctgcctgtacgactgggataactccgggaaaccggag
ctaataccggatacgtttttcagaccgcatggtctgaaagagaaagaccttggtcacgtacagatgggcctgcggcgc
attagctagttggtggggtaacggcctaccaaggcgacgatgcgtagccgacctgagagggtgatcggccacactgg
gactgagacacggcccagactcctacgggaggcagcagtagggaatcttccgcaatggacgaagtctgacggagc
aacgccgcgtgaacgatgaaggttttcggatcgtaaagttctgttgttagggaagaaccgccgggatgacctcccggt
ctgacggtacctaacgagaaagccccggctaactacgtgccagcagccgcggtaatacgtaggggg caagcgttgt
ccggaattattgggcgtaaagcgcgcgcaggcggcttcttaagtcaggtgtgaaagcccacggctcaaccgtggagg
gccacttgaaactgggaggcttgagtgcaggagaggagagcggaattccacgtgtagcggtgaaatgcgtagagat
gtggaggaacacccgtggcgaaggcggctctctggcctgtaactgacgctgaggcgcgaaagcgtggggagcgaa
caggattagataccctggtagtccacgccgtaaacgttgagtgctaggtgttggggactccaatcctcagtgccgcagc
taacgcaataagcactccgcctggggagtacggccgcaaggctgaaactcaaaggaattgacggggacccgcaca
agcggtggagcatgtggtttaattcgaagcaacgcgaagaaccttaccagggcttgacatcccgctgaccctcctaga
gataggagctctcttcggagcagcggtgacaggtggtgcatggttgtcgtcagctcgtgtcgtgagatgttgggttaag
tcccgcaacgagcgcaacccttgtccttagttgccagcattcagttgggcactctagggagactgccgtcgacaagac
ggaggaaggtggggatgacgtcaaatcatcatgccccttatgtcctgggctacacacgtgctacaatggatggaacaa
cgggcagccaactcgcgagagtgcgccaatcccttaaaaccattctcagttcggattgcaggctgcaactcgcctgca
tgaagccggaatcgctagtaatcgcggatcagcatgccgcggtgaatacgttcccgggtcttgtacacaccgcccggc
acaccacgagagt

Figure 3. Consensus sequence constructed from the forward and reverse sequences of 16S rDNA fragments of isolate B4.

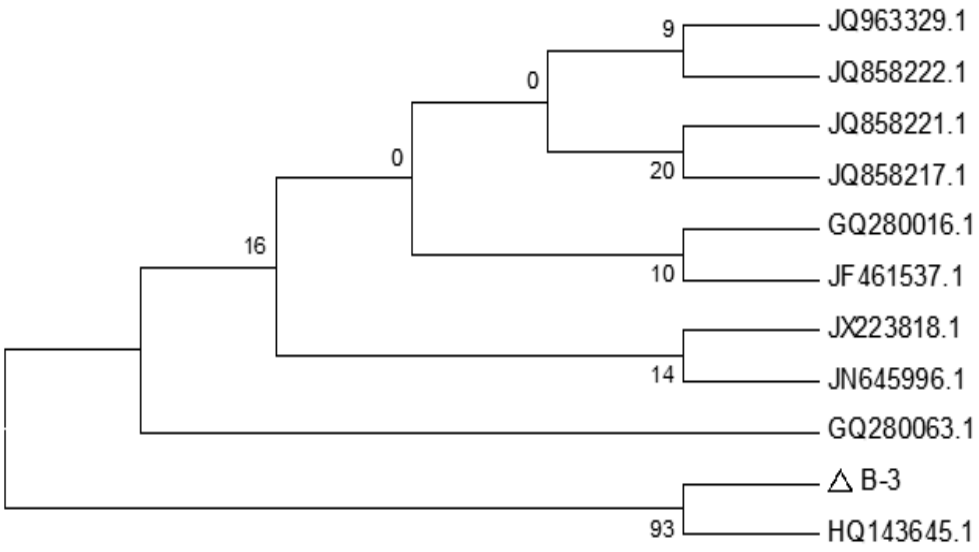

Figure 4. Phylogenetic tree of the isolate B3 with the selected best homologous known bacterial strains

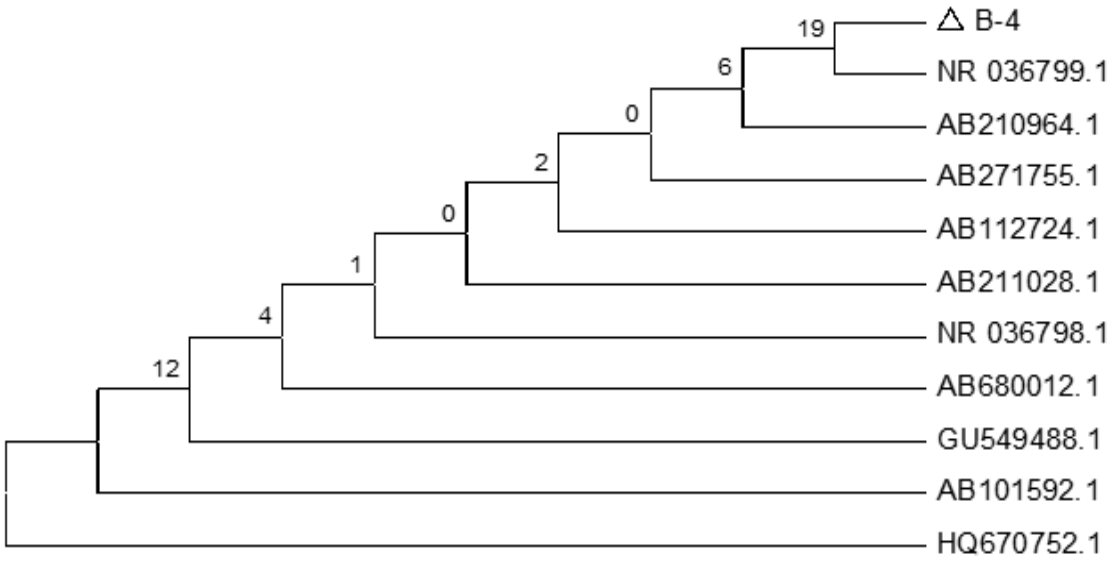

Figure 5. Phylogenetic tree of the isolate B4 with the selected best homologous known bacterial strains

The homologous organisms for isolate B3 as shown in the Table-2 were of maximum similarity (99%-100%) to the genus *Pseudomonas* having the similar characters to its nearest neighbors *Pseudomonas* sp. strain WCH22 (HQ143645.1) & *Pseudomonas stutzeri* strain K-2-7 (JQ963329) that are isolated from soil samples and were used for the degradation of oils and thus reduce the surface tension for the free flow of fluids. The other nearest neighbors could be the *Pseudomonas* sp. strains BJ-53 & BJ-6 (GQ280063.1 & GQ280016.1) that were isolated from the industrial effluents from the western India. However the final results of the most probable nearest neighborhood strain may be considered as the *Pseudomonas* sp. stain WCH22, which is also evident from the phylogenetic tree shown in the Figure 4.

Conversely, the strain B4 has the maximum similarity of 99% with the homologous neighborhood bacterial organisms in the genus *Aneurinibacillus*. But then the culture characters were found more similar to the dynamic and the probable degenerative properties of *migulanus* strain and this may be the reason for the final results of phylogenetic tree as shown in the Figure 5. These results were also supported by the earlier observations made during the strain improvement techniques applied on the stain B4. The UV-irradiated B4 strain produced the better lipase producing mutant strains; but then the lipase productivity of these mutants were lost in the subsequent generations indicating dynamic and degenerative properties that are peculiar for the *Aneurinibacillus migulanus* strain [53].

The 16S rRNA gene sequences of these lipase producing isolates B3 & B4 were submitted to GenBank database using Bankit submission tool of National Center for Biotechnology Information (NCBI), Bethesda, USA. The GenBank accession numbers were assigned to the submitted sequences of B3 & B4 as JX524282 and JX564542 respectively.

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CONCLUSIONS

The soil samples from Guntur (G), Nagpur (N) & Udaipur (B) provided similar bacterial lipase producing isolates, and they were subjected to various lipase screening assays & optimization techniques along with strain improvement strategies, providing two best lipase producing bacterial soil isolates as B3 & B4. The 16S rRNA gene sequences of isolates B3 & B4 from their genomic DNA samples were generated and analyzed by using BLASTN, CLUSTALW & MEGA 4 software tools to identify the bacterial isolates as *Pseudomonas* sp. SPSU B3 & *Aneurinibacillus migulanus* sp. SPSU B4 respectively. The Accession numbers for the 16S rRNA gene sequences of identified strains B3 & B4 were obtained from the NCBI GenBank as JX524282 & JX564542. These novel isolated lipase producing bacterial strains may further be studied for their best possible services to the mankind and they may be deposited in culture collection facilities for their greater access & explorations.

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