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Isolation and Characterization of Lipase Producing *Bacillus Sonorensis* and *Bacillus Halotolerans* from Oil Contaminated Soil of North Telangana

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Abstract
In the present observation, isolation and primary screening were conducted for the lipolytic activity of various bacterial species with reference to different oil-contaminated soils. The soil samples were collected from oil-contaminated effluent in Warangal region, North Telangana, India. The soil samples were analyzed for the presence of lipolytic bacterial strains and screened on Tributyrin agar plates for detection of lipolytic activity. Out of 70 bacterial colonies, 40 isolates were found to be lipase positive. Of the isolates, *Bacillus sonorensis* had shown maximum lipase production activity, whereas the *B.holotolerans* showed minimum activity. During the screening process of the bacterial strains, the existence of different predominately rod-shaped and cocci (SP5 and SP9) species were noted. Even though positive results for starch hydrolysis was observed, whereas the oxidase test was found negative for SP4, SP5, and SP9 and other samples. The SD2 strain had shown the highest lipolytic activity (4.75 m/ml) after 48 hrs. of incubation, whereas SD1 strain had shown lowest lipolytic activity (3.95 m/ml) followed by SB2 and SH3. Molecular identification was carried out based on 16s rDNA sequences and a phylogenetic tree was constructed to specifically identify the species. We were successful in identifying the two bacterial strains, namely, *Bacillus sonorensis* RCPS1, and *Bacillus halotolerans* strain RCPS2, responsible for lipase production from oil effluent contaminated soils of Warangal.

Keywords
Lipase; *Bacillus sonorensis*; *Bacillus halotolerans*; microbial lipase production.

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INTRODUCTION
White biotechnology revolution would not have been possible without the contributions of numerous enzymes that are irreplaceable in the present industry. Microbial enzymes are an essential part of various hydrolysis, acidolysis, oxidation, reduction, alcoholysis, esterification process and relevant applications [1]. Bacterial enzymes are serving as the prime source of such requirement of the industry. Plentiful application of several enzymes produced from the microbial sources, specifically of bacterial origin, are available in the modern industry. This include, enzymes used in the food technology [2], detergent production, cosmetic, pharmaceutical, leather, paper and pulp industry, and textile industry [3]. Enzymes such as protease, lactase, lipase, amylase, catalase, xylanase, cellulase, phytase, protease, and many others are predominantly

produced from microbial sources and extensively used in the industries. All these enzymes perform specific catalytic activity and are of paramount importance due to their unique role in various product developments.

Among all these biocatalysts, lipases are appreciated across the biotechnological industry due to its efficient catalytic impact in mild conditions, greater tolerance towards a range of organic solvents, and a broad range of substrate specificity [4].

Lipase is an abundant esterase that is important for the lipid hydrolysis and other specific functions. Modern biotechnological science has been successful in orchestrating the precise application of lipase according to the production demand. Lipases function through hydrolysis of triglycerides to fatty acid and glycerol, numerous types of lipases play an important role in lipid metabolism in different organisms [5].

Structural insight of Lipase enzyme suggests that it contains hydrolase folds, both α, and β type, along with specific histidine base and serine nucleophile that aids in the catalytic activity [6]. Considering the diversity of the enzyme, phylogenetic analyses have been conducted on different types of lipases belonging to the superfamily [7]. Lid domain is structurally important for lipase enzymes as it differs structurally and functionally depending on the enzyme activity, substrate specificity and thermostability [8]. Further, based on the enzymatic activity, lipases are categorized as nonspecific; 1, 3 specific, and fatty acid specific. Thus, depending on the industrial requirement, a lipase type could be selected and produced [9].

To attain industry level almost homogenous lipase quality, a stepwise standardized purification process should be followed for the extracellular enzyme [9]. In addition to the purification, physicochemical property assessment, analysis of the kinetic properties of the extracted and purified enzyme, and modification of the enzyme following a site-directed mutation or rational designing are part of the enzyme enrichment process [10].

The different technical procedure is considered to maximize the production of the enzyme. Submerged fermentation has been employed for a better selection of the lipase producing microorganisms [11]. In comparison to bacterial lipase, solid-state fermentation process opted for better yield of lipase from the fungal origin [12]. In another attempt, shea nut cake has been used for lipase production from *Aspergillus niger* [13]. Apart from the experimentation with the media and production process, detail exploration has been done for the cultural conditions, especially to produce thermostable lipase [14] with reference to specific industrial objectives, such as detergent making [15]. Improving measures including statistical tools have been implemented to optimize the media and enzyme production [16].

Considering the multifaceted role of lipase enzyme and its industrial demand, the present work was carried out. Isolation and characterization of novel bacterial strains that can produce ample lipase was the primary objective of the study. The source was chosen as oil mill effluent contaminated soil sample from the surrounding industrial area and to have the efficient utilization of the waste contaminated soil.

2. MATERIALS AND METHODS

This study was focused on the identification and characterization of the lipase producing bacterial species present in the oil effluents containing soil in North Telangana region of India. The following methodology was used.

Sample collection:

The sample collection spots were determined through identifying the presence of dairies and oil refineries and samples were collected from the oil and fat contaminated soils of those dairies and oil refineries present around North Telangana region of India.

2.1 *Isolation of bacterial species through spread plating*

The standard protocol of bacterial isolation was followed to identify the present bacterial species in the collected soil samples. Serial dilution was conducted followed by plating in tributyrin agar base containing 0.3 % (w/v) yeast extract, 0.5% (w/v) peptone, 1% (v/v) tributyrin and 2% agar. Spread plate technique was followed for this purpose. The pH was maintained at 7.0. All the plates were incubated for two days at 37^0C. Furthermore, Tributyrin agar plates with bacterial culture were sub-cultured for every 15 days for better maintenance of the sample source.

2.2 *Biochemical characterization*

This was done through the measurement and estimation of indole, hydrogen sulfide production, nitrate reduction, oxidase, gelatin hydrolysis, catalase activity, Voges-Proskauer, methyl red, and citrate. Sugar fermentation test was also carried out using the protocol described for identification of bacteria by MacFaddin [17]. The total number of samples was 10 which are represented by SP1 to SP10 in Table 2. The characteristics analyzed were morphological appearances, response to gram staining, the shape of the bacteria and motility. The specific analysis was also done where the response of the bacteria presents in the sample against Indole

production test, Methyl Red test, Voges Proskauer test, H2S production test, Urease test, Catalase test, Oxidase test, Starch Hydrolysis test, and Nitrate reduction test were measured. Other assessments pertaining to the carbohydrate formation, glucose, sucrose, dextrose, maltose, fructose, and lactose production were also considered (Table 2).

2.3 *Screening of the bacterial species present in the sample*

The lipolytic ability of the organisms was determined through the qualitative plate assay method. All the screened isolates were grown for 2 days on tributyrin agar plates and incubated at 36^0 C. Estimation of the zone of inhibition or hydrolysis (Eq.1) was performed to understand the activity of the bacterial strains. By observing the zone of clearance, the lipid hydrolyzing efficiency of each isolate was calculated by following method.

Hydrolyzing efficiency = $\pi R_1^2 - \pi R_2^2 / \pi R_1^2 \times 100$**….. Eq 1**

Further strict selection of the potent strains was performed for the Isolates having hydrolyzing efficiency of more or equal to 60%. Selected lipase producing bacteria strains were subjected to lipase assay to estimate the lipase activity.

2.4 *Isolate selection for lipase production*

The best lipase producing bacteria were screened by agar well diffusion method. Sterilized tributyrin medium with olive oil as substrate was made for the agar well diffusion assay. These tributyrin agar plates were punched aseptically with sterile cup borer to obtain well of 4mm diameter. The isolates were grown in sterile nutrient broth for 24 hours. Pouring of 50 µl sample in each well was done separately and was incubated at 37°C for 48 hours. The appeared clear zone near each well was measured and the obtained information was used for the analysis of further enzyme production. Clear zones developed around the wells were measured and the data was used for further production of the enzyme.

2.5 *Lipase production*

The isolates that produced Lipase hydrolytic zones on tributyrin agar plate were considered for further production analysis. Inoculum media was prepared using 10g yeast extract, 10g Peptone, 20 g glucose, 10 g sodium acetate (CH3COONa), 3H2O, 0.09 g magnesium sulfate (MgSO4), 0.03 g Manganese sulfate (MnSO4), 1.5 g Copper sulfate (CuSO4), 0.5 g potassium chloride(KCl), 5 ml olive oil, 1000 ml distilled water). pH was maintained at 10.8. 15 ml of the preparation was used for the inoculation of the cultured isolate. The inoculum flasks were incubated at 37°C temperature for overnight on rotary Shaker at 120 rpm.

The composition of production medium used was 2 g peptone, 1 g Ammonium dihydrogen phosphate ($NH_4H_2PO_4$), 2.5 g Sodium Chloride (NaCl), 0.24 g Magnesium sulfate (MgSO4), 0.24 g Calcium Dichloride Hydrate ($CaCl_2H_2O$), olive oil 12 ml, and tween 20, 2-3 drops for every 1000 ml of water. The culture was incubated in inoculum media for overnight. 1 ml of such cultures were incubated in 250 ml of Erlenmeyer Flasks containing 100 ml of production medium, kept on a rotary shaker at 120 rpm, and incubated at 37°C.

Every 24 hours, an inspection of the incubated cultures was done for consecutive 5 days. Every day, 1 ml of culture filtrate was centrifuged at 8000 rpm for 60 minutes at 4°C. The supernatant of cell-free culture was used as the crude enzyme of Lipase. The Lipolytic activity of the supernatant was determined by the titrimetric method.

2.6 *Lipase assay*

The lipase activity of the isolated bacterial strains was estimated by a titrimetric method using olive oil as substrate. To obtain the outcome, 2 ml of phosphate buffer was added to the reaction mixture (at pH 7.0), containing 1 ml of culture supernatant and 1ml of olive oil and incubated at 37^0C for 60 minutes. After incubation, the reaction was terminated by adding 1 ml of acetone: ethanol solution in 1:1 proportion. Later, the amount of fatty acid released was measured by titrating with 0.05M Sodium Hydroxide (NaOH) at pH 10.0 by using phenolphthalein indicator.

2.7 *Molecular Identification*

To pursue the molecular identification and characterization of the cultured isolates, DNA was isolated from the culture by Agarose gel electrophoresis method. The quality of the isolated DNA was evaluated through 1.0% Agarose gel.

Further, the fragment of the 16S rDNA gene was amplified by 27F and 1492R primers ((5´ 3´) forward strand: "AGAGTTTGATCMTGGCTCAG" and (3´5´) reverse strand: "AAGTCGTAACAAGGT"), and the PCR amplicon band was developed in Agarose gel. Purification was done for the DNA sample to eradicate any possibly present contamination. PCR amplification was carried out by using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. A six-frame (3 forward and 3 reverse) sequence was

generated and the consensus sequence of the 16S rDNA gene was derived through aligner software. The consensus sequence was used for further nucleotide-nucleotide BLAST [18] analysis with the Gene Bank database and the best alignments were identified based on the E-value, maximum identity score, and percent query coverage. Later, based on maximum identity score, top ten sequences were selected and aligned using multiple sequence alignment program ClustalW [19]. Phylogenetic analysis and distance matrix were constructed using MEGA 7 [20]. The selected nucleotides were submitted to the National Center for Biotechnology Information (NCBI) nucleotide data bank.

3.0 RESULTS AND DISCUSSION

The objective of this study was to identify and characterize from different oil effluent contaminated soil extract around north Telangana region. The obtained outcome of the experimentation is described in the following sections in detail.

3.1 *Isolation and screening*

Analysis of the collected soil samples resulted in nearly 70 bacterial colonies in the laboratory. These bacterial colonies were subjected to screening on Tributyrin agar plates for detection of lipolytic strains. Among the 70 bacterial colonies, 40 isolates were found to be lipase positive.

These lipase positive bacteria of different strains showed lipolytic activity by producing clear zones around the bacterial growth (Fig.1.). The observation suggested that the large zone of clearance represented higher lipolytic activity whereas the small clear zone represented comparatively lower lipolytic activity.

Fig.1. Isolates producing lipase enzyme were screened by streaking on tributyrin agar plates. Clear zones of hydrolysis were observed on these plates with bacterial growth showing positive results.

During the screening process, *pseudomonas* had shown maximum lipase production compared to the other strains that were part of this study. Following the isolation strategy, the bacterial strains showing maximum Lipase production were further screened and identified by morphological, biochemical characteristics. As mentioned in the methodology, during the screening process of the bacterial strains found to have better lipase production were grown on different media, such as tributyrin agar medium, tween 20 and tween 80, and Rhodamine B agar media. Ample literature evidence is present regarding the abundant use of Tween media in detecting the lipase producing microorganisms on agar media [21].

The observation of the zone size (mm) developed by the samples in 24 hrs and 48 hrs respectively is represented in Table 1. It was noticed that the range of zone size produced by the samples in the first day was ranging from 9 mm to 20 mm (Table 1). The two days (48 hrs.) incubation displayed the range of zone size from 14 mm to 25 mm. The largest zone creation during the first 24 hrs was observed by the Sample 4 isolate (SD2, 20 mm) whereas after 48 hrs. incubation the largest zone was observed for the same sample (25 mm) followed by another sample (SD1) of the same isolate.

Followed by the identification of the samples in the isolates that created the largest zone size, detail biochemical analysis, lipase activity assessment was conducted.

Table 1 Representation of the zone size observed (in mm) for the considered isolates.

Isolate No.	Zone size (in mm)	
Sample 1	24 hrs.	48 hrs.
SA 1	10mm	14mm
SA 2	9mm	15mm
Sample 2		
SB 1	15mm	20mm
SB 2	16mm	21mm
Sample 3		
SC 1	15mm	19mm
SC 2	14mm	17mm
Sample 4		
SD 1	17mm	22mm
SD 2	20mm	25mm
Sample 5		
SE 1	10mm	15mm
SE 2	11mm	16mm
Sample 6		
SF	10mm	16mm
Sample 7		
SG	12mm	17mm
Sample 8		
SH 3	11mm	20mm
SH 4	12mm	19mm
Sample 9		
SI 5	14mm	21mm
Sample 10		
SJ 1	14mm	19mm
SJ 2	12mm	17mm

3.2 *Biochemical analysis*

As mentioned earlier, detail morphological and biochemical characterization was conducted for the selected samples as shown in Table 2. The morphological analysis suggested that all the samples were found to be Gram-positive except SP4, SP5, SP9, and SP10. The observed shapes were predominantly rod-shaped and cocci (SP5 and SP9) as presented in Table 2. Most of the samples showed positive motility in motility test except SP5 and SP9. Methyl Red test was found positive for SP2, SP8, and SP10. Hydrogen Sulfide (H_2S) production was observed for sample SP5 and SP9. Urease test was found positive in most of the cases excluding SP4 and SP9. In the case of the catalase test, only SP3 did not show any outcome, all other samples were found positive. No result was found for the oxidase test for SP4, SP5, and SP9, all other samples were found positive towards this test. Except for SP5 and SP9, all other samples provided positive results for starch hydrolysis analysis. Every sample showed positive results for Nitrate reduction test. Table 2 also represents the analysis of the sugars for the samples where “AG” refers to Agar Glucose and “A” is Agar. The analyses suggested the varying biochemical characterization of the samples considered for the study and hinted that SP5, SP9 are having different characteristics compared to the other samples. Similarly, SP4 and SP3 also showed slight disagreement with the outcome of other samples in a certain analysis as shown in Table 2.

Table 2 Biochemical characterization through Gram staining and other tests for the best 10 samples isolated.

Characteristics	SP1	SP2	SP3	SP4	SP5	SP6	SP7	SP8	SP9	SP10
Morphology										
Gram Stain	Positive	Positive	Positive	-	-	Positive	Positive	Positive	-	-
Shape	Rods	Rods	Rods	Rods	Cocci	Rods	Rods	Rods	Cocci	Rods
Motility	Positive	Positive	Positive	Positive	-	Positive	Positive	Positive	-	Positive
Bio-chemical Characteristics										
Indole production test	-	-	-	-	-	-	-	-	-	-
Methyl Red test	-	Positive	-	-	-	-	-	Positive	-	Positive
Voges Proskauer test	-	-	-	-	-	-	-	-	-	-
H2S production test	-	-	-	-	Positive	-	-	-	Positive	-
Urease test	Positive	Positive	Positive	-	Positive	Positive	Positive	Positive	-	Positive
Catalase test	Positive	Positive	-	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Oxidase test	Positive	Positive	Positive	-	-	Positive	Positive	Positive	-	Positive
Starch Hydrolysis test	Positive	Positive	Positive	Positive	-	Positive	Positive	Positive	-	Positive
Nitrate reduction test	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Carbohydrate Formation										
Glucose	AG	AG	A	A	AG	A	AG	A	AG	AG
Sucrose	AG	AG	-	A	AG	-	AG	-	AG	AG
Dextrose	AG	-	A	A	AG	A	AG	A	AG	AG
Maltose	-	-	A	A	-	A	-	A	-	-
Fructose	-	-	A	A	-	A	-	A	-	-
Lactose	-	AG	A	A	-	A	-	A	-	-

3.3 *Determination of lipolytic activity*

Out of the 17 isolates considered so far, five (5) were separated and grown in a lipid-containing media. The strict observation was maintained for lipolytic activity every 12 hours until 96 hours. It was noted that among the samples, samples SD1 and SD2 showed maximum activity at 48 hr.

3.4 *Assay of lipase activity*

Further, the Lipolytic activity was determined by using Olive oil as substrate. The outcome was computed using the following formulae:

LA= (VA × SA) / (VS × t) ……. Eq.2

Where, LA is Lipase activity (units/ml), VA=Volume of Alkali consumed, SA= Strength of the Alkali, VS= Volume of the sample and t refers to the time in minutes.

Lipolytic activity of selected 5 strains is represented in Fig.3. During this exercise, the bacterial strain SD2 had shown the highest lipolytic activity (4.75 m/ml) within the 48 hours of incubation. This result was followed by the strain SD1, with an activity estimation of 3.95 m/ml. The other strains SB2 and SH3 showed quite similar activity values (3.2 m/ml and 3.1 m/ml) during incubation at the same temperature. It was also noted that SB1 and SI5 also provided similar lipolytic activity (2.05 m/ml and 2.55 m/ml) at the same temperature. Our observation suggested that SD2 was having the maximum lipolytic activity among all the isolates considered from the soil samples.

3.5 *Molecular identification of the samples*

Biochemical characterization of the samples was followed by their molecular characterization. The best two samples in terms of lipolytic activity and zone inhibition ability were selected for the molecular analysis.

To continue the molecular identification, the protocol mentioned in the methodology section was

followed. 16SrDNA gene amplification was considered for molecular identification and as specific marker considering the ubiquity, ample length of the gene, evolutionary conservation of the marker in bacterial species and its ability to distinguish among closely related species [22]. Moreover, the rDNA gene marker has been widely experimented and tested for its ability to distinguish the isolates [23, 24]. In both the cases, the comparative mobility of the sample DNA with reference to the ladder is shown by genomic DNA (gDNA), 16S PCR amplicon and the ladder specifications (in bp) in Fig.4. The obtained outcome suggested the approximate molecular weight and hinted the plausible length of the sample sequence.

3.6 *gDNA and 16S Amplicon QC data*

Fig.4. Molecular characterization of the genomic DNA extracted from the soil sample. (A) The first panel represents the comparative position of the sample genomic DNA and the ladder used for the first sample (SOSS). The second panel refers to the 16S PCR amplicon output and the third panel refers to the ladder specifications. (B) The first panel represents the comparative position of the sample genomic DNA and the ladder used for the second sample (COSS). The second panel refers to the 16S PCR amplicon output and the third panel refers to the ladder specifications.

3.7 *Sequencing and computational identification*

Sequencing of the DNA obtained after the gel electrophoresis analysis was done using ABI 3730xl Genetic Analyzer. The Aligner tool was considered for generating the consensus sequence for both the samples from the six-frame sequences. Computational characterization of pathogenic [25] and industrially important bacteria [26, 27] species sequences serve immensely to the scientific analysis of the species. Computation intensive analysis of the bacterial sequences belonging to various species and strains have come a long way from simple sequence similarity analysis to the use of intelligent techniques for the purpose of accurate identification and classification [28-30]. In the present study, the obtained consensus sequences were considered for further sequence analysis and phylogenetic studies.

3.8 *Sequence alignment and phylogenetic analysis*

Both the consensus sequences were found to be of 1438 bp (SOSS) and 1473 (COSS) bp long respectively. Separately, both the sequences were subject to the nucleotide-nucleotide BLAST (BLASTn) analysis. Megablast with highest possible similarity was opted along with the possible E-value threshold of zero (0). The obtained query coverage for both the samples is represented in Fig.5.

Fig.5. Observed query coverage of >=200 for (A) SOSS_Sample and (B) COSS sample.

The obtained BLAST alignment result is represented in Table 3 and Table 4 for both the samples respectively. In both the cases, higher scores were obtained with more than 97% query coverage and percent identity and zero (0) E-values suggesting the authenticity of the resultant hits. For the SOSS_7.1 sample, the proximity was observed with various *Bacillus sp.* including *licheniformis (*strain DSM 13, strain ATCC 14580, strain BCRC 11702), *haynesii (*strain NRRL B-41327), *sonorensis (*strain NBRC 101234), *aerius(*strain 24K*), swezeyi (*strain NRRL B-41294), *subtilis (*strain DSM 10), and *halotolerans (*strain DSM 8802).

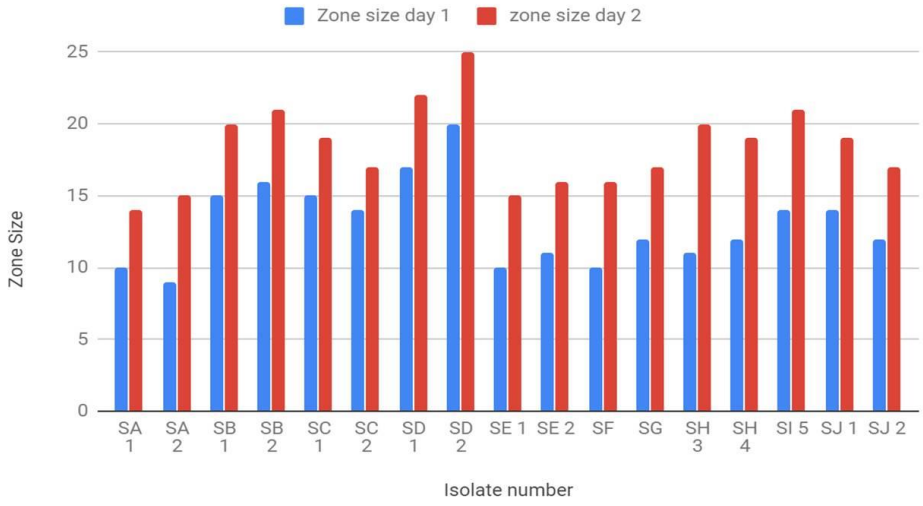

Fig.2. Showing the zone sizes for the different isolates and their respective samples found in Day 1 and Day 2. The "X" axis represents the isolate details along with the sample respective numbers whereas the "Y" axis represents the comparative Zone sizes observed in mm.

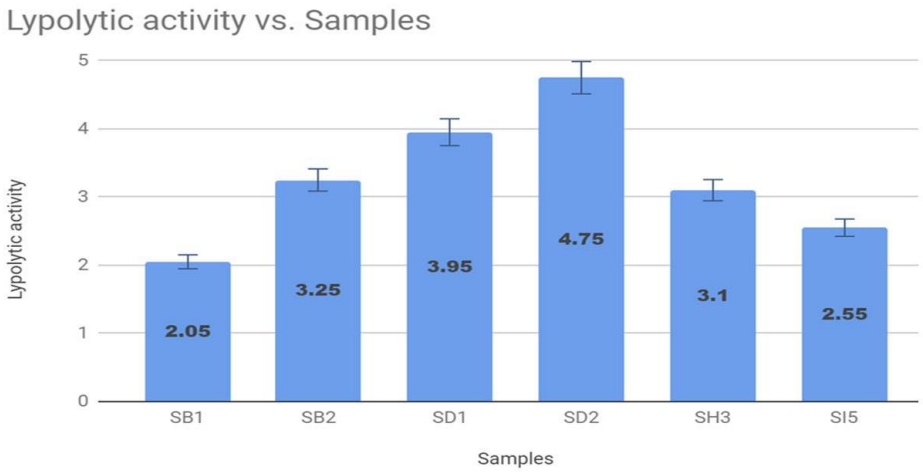

Fig.3. Lipolytic activity of the selected samples.

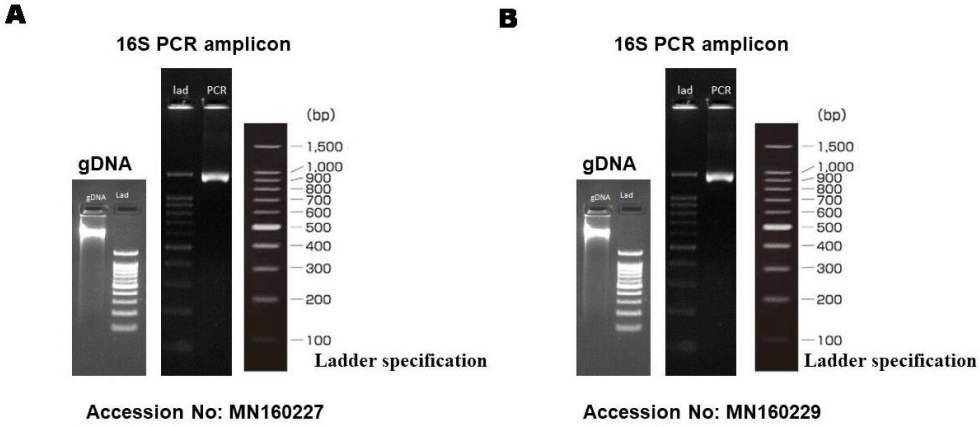

Fig.4. Molecular characterization of the genomic DNA extracted from the soil sample. (A) The first panel represents the comparative position of the sample genomic DNA and the ladder used for the first sample (SOSS). The second panel refers to the 16S PCR amplicon output and the third panel refers to the ladder specifications. (B) The first panel represents the comparative position of the sample genomic DNA and the ladder used for the second sample (COSS). The second panel refers to the 16S PCR amplicon output and the third panel refers to the ladder specifications.

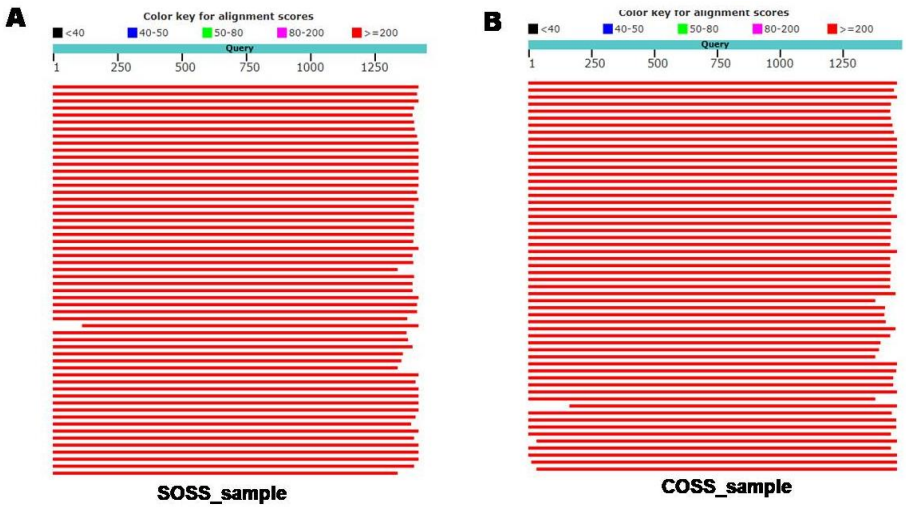

Fig.5. Observed query coverage of >=200 for (A) SOSS_Sample and (B) COSS sample.

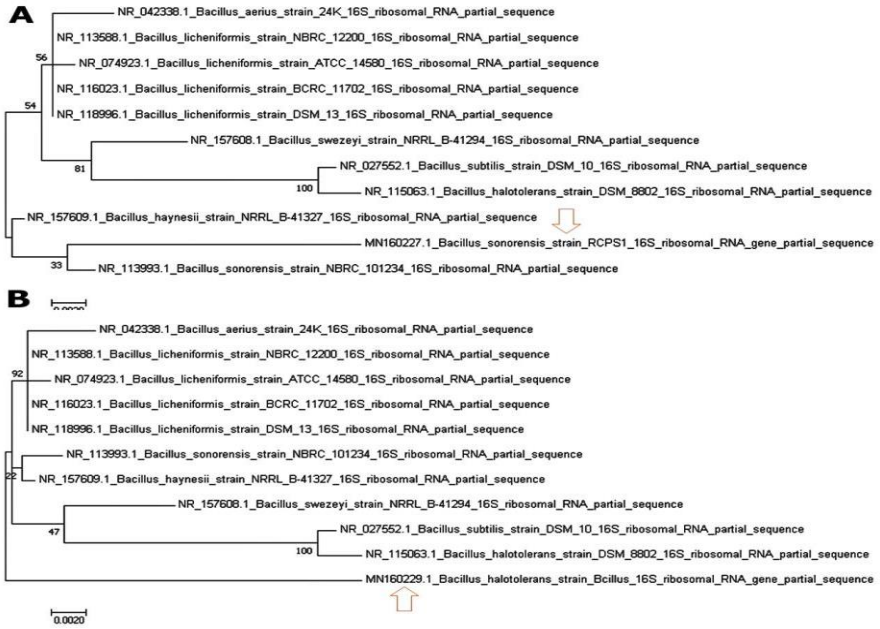

Fig.6. Reconstructed phylogenetic tree for (A) SOSS_7.1 sample (MN160227.1) and (B) COSS_7.4 sample (MN160229.1)

Table 3 BLAST alignment outcome for SOSS_7.1 sample (Top 10 sequences) along with their respective scores, query coverage, E-values, percent identity, and Accession numbers.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus licheniformis strain DSM 13 16S ribosomal RNA, partial sequence	2566	2566	97%	0	99.57%	NR_118996.1
Bacillus haynesii strain NRRL B-41327 16S ribosomal RNA, partial sequence	2564	2564	97%	0	99.71%	NR_157609.1
Bacillus licheniformis strain ATCC 14580 16S ribosomal RNA, partial sequence	2555	2555	97%	0	99.43%	NR_074923.1
Bacillus sonorensis strain NBRC 101234 16S ribosomal RNA, partial sequence	2542	2542	96%	0	99.71%	NR_113993.1
Bacillus licheniformis strain BCRC 11702 16S ribosomal RNA, partial sequence	2538	2538	96%	0	99.78%	NR_116023.1
Bacillus licheniformis strain NBRC 12200 16S ribosomal RNA, partial sequence	2536	2536	96%	0	99.57%	NR_113588.1
Bacillus aerius strain 24K 16S ribosomal RNA, partial sequence	2512	2512	96%	0	99.21%	NR_042338.1
Bacillus swezeyi strain NRRL B-41294 16S ribosomal RNA, partial sequence	2497	2497	97%	0	98.86%	NR_157608.1
Bacillus subtilis strain DSM 10 16S ribosomal RNA, partial sequence	2449	2449	97%	0	98.08%	NR_027552.1
Bacillus halotolerans strain DSM 8802 16S ribosomal RNA, partial sequence	2444	2444	97%	0	98.01%	NR_115063.1

Table 4 BLAST alignment outcome for COSS_7.4 sample (Top 10 sequences) along with their respective scores, query coverage, E-values, percent identity, and Accession numbers.

Description	Max Score	Total Score	Query Cover	E-value	Percent Identity	Accession
Bacillus licheniformis strain DSM 13 16S ribosomal RNA, partial sequence	2577	2577	98%	0	98.56%	NR_118996.1
Bacillus haynesii strain NRRL B-41327 16S ribosomal RNA, partial sequence	2575	2575	97%	0	98.83%	NR_157609.1
Bacillus licheniformis strain ATCC 14580 16S ribosomal RNA, partial sequence	2566	2566	98%	0	98.43%	NR_074923.1
Bacillus sonorensis strain NBRC 101234 16S ribosomal RNA, partial sequence	2545	2545	97%	0	98.68%	NR_113993.1
Bacillus licheniformis strain BCRC 11702 16S ribosomal RNA, partial sequence	2542	2542	96%	0	98.75%	NR_116023.1
Bacillus licheniformis strain NBRC 12200 16S ribosomal RNA, partial sequence	2540	2540	97%	0	98.54%	NR_113588.1
Bacillus aerius strain 24K 16S ribosomal RNA, partial sequence	2516	2516	97%	0	98.20%	NR_042338.1
Bacillus swezeyi strain NRRL B-41294 16S ribosomal RNA, partial sequence	2501	2501	97%	0	97.93%	NR_157608.1
Bacillus subtilis strain DSM 10 16S ribosomal RNA, partial sequence	2494	2494	98%	0	97.54%	NR_027552.1
Bacillus halotolerans strain DSM 8802 16S ribosomal RNA, partial sequence	2488	2488	98%	0	97.47%	NR_115063.1

Table 3 BLAST alignment outcome for SOSS_7.1 sample (Top 10 sequences) along with their respective scores, query coverage, E-values, percent identity, and Accession numbers.

The result for the COSS_7.4 sample suggested that the sequence was in closeness with the similar bacterial species as SOSS_7.1.

Table 4 BLAST alignment outcome for COSS_7.4 sample (Top 10 sequences) along with their respective scores, query coverage, E-values, percent identity, and Accession numbers.

The obtained fasta sequences of the top 10 hits from the BLAST analysis was further used for phylogenetic analysis using MEGA7.0 software. The nucleotide frequency (T(U), C, A, G) distribution of both the sequences in comparison to their closely associated species is shown in Table 5 and Table 6.

The average frequency of the nucleotides observed were 19.9% (T(U)),23.9%(C), 24.7% (A), and 31.5%(G), whereas our SOSS_7.1 sample represented the same frequencies as 20.0%, 24.1%, 24.8% and 31.0% respectively (Table 5). The respective abundance of the four nucleotides in first, second and third codon position is represented in the same Table along with the comparison representation of the closely related species of the genus Bacillus.

Table 5 Frequency of nucleotides in SOSS_7_1 and closely related sequences.

Accession ID	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
MN160227.1	20.0	24.1	24.8	31.0	1438.0	21	24.5	27.7	26.6	481.0	19	22.5	24.0	34.2	480.0	19	25.4	22.9	32.3	477.0
NR 027552.1	19.8	23.5	24.9	31.8	1517.0	21	22.7	27.5	28.7	506.0	19	22.9	23.9	33.9	507.0	19	25.0	23.4	32.7	504.0
NR 042338.1	19.7	24.3	24.6	31.4	1494.0	21	24.1	27.7	27.3	498.0	19	23.6	23.0	34.5	499.0	20	25.2	22.9	32.4	497.0
NR 074923.1	20.0	23.9	24.5	31.6	1545.0	21	23.4	27.1	28.2	517.0	19	23.6	22.9	34.5	516.0	20	24.8	23.4	32.0	512.0
NR 113588.1	19.6	24.0	24.8	31.7	1472.0	21	23.8	27.6	27.6	492.0	19	23.2	23.6	34.6	491.0	19	24.9	23.1	32.7	489.0
NR 113993.1	19.7	23.9	24.8	31.5	1475.0	21	23.7	27.6	27.6	493.0	19	22.9	23.7	34.3	493.0	19	25.2	23.1	32.7	489.0
NR 115063.1	20.2	23.7	24.7	31.4	1545.0	22	23.1	26.9	28.3	516.0	19	23.4	23.6	33.7	516.0	20	24.6	23.6	32.2	513.0
NR 116023.1	19.7	24.0	24.7	31.5	1468.0	21	23.9	27.6	27.8	490.0	19	23.2	23.6	34.2	491.0	19	25.1	23.0	32.6	487.0
NR 118996.1	20.0	23.9	24.6	31.5	1545.0	21	23.4	27.3	28.0	517.0	19	23.6	23.1	34.3	516.0	20	24.8	23.4	32.0	512.0
NR 157608.1	19.8	23.9	24.6	31.7	1507.0	21	24.1	27.2	28.0	503.0	19	22.6	23.8	34.3	504.0	20	25.0	22.8	32.6	500.0
NR 157609.1	20.0	23.7	24.9	31.4	1508.0	21	23.4	28.0	27.4	504.0	19	22.6	23.6	34.5	504.0	19	25.0	23.2	32.4	500.0
Average	19.9	23.9	24.7	31.5	1501.3	21	23.6	27.5	27.8	501.5	19	23.1	23.5	34.3	501.5	19	25.0	23.2	32.4	498.2

Table 6 Frequency of nucleotides in COSS_7.4 and closely related sequences.

Accession ID	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
MN160229.1	20.2	23.8	24.6	31.4	1473.0	22	23.8	27.3	27.3	491.0	20	22.7	23.3	33.9	493.0	19	24.7	23.1	33.1	489.0
NR 027552.1	19.8	23.5	24.9	31.8	1517.0	21	22.9	27.4	28.6	507.0	19	22.7	23.9	34.0	506.0	19	25.0	23.4	32.7	504.0
NR 042338.1	19.7	24.3	24.6	31.4	1494.0	21	24.1	27.7	27.3	498.0	19	23.6	23.0	34.5	499.0	20	25.2	22.9	32.4	497.0
NR 074923.1	20.0	23.9	24.5	31.6	1545.0	21	23.4	27.1	28.2	517.0	19	23.6	22.9	34.5	516.0	20	24.8	23.4	32.0	512.0
NR 113588.1	19.6	24.0	24.8	31.7	1472.0	21	23.8	27.6	27.6	492.0	19	23.2	23.6	34.6	491.0	19	24.9	23.1	32.7	489.0
NR 113993.1	19.7	23.9	24.8	31.5	1475.0	21	23.7	27.6	27.6	493.0	19	22.9	23.7	34.3	493.0	19	25.2	23.1	32.7	489.0
NR 115063.1	20.2	23.7	24.7	31.4	1545.0	22	23.2	26.9	28.2	517.0	19	23.3	23.7	33.8	515.0	20	24.6	23.6	32.2	513.0
NR 116023.1	19.7	24.0	24.7	31.5	1468.0	21	23.9	27.6	27.8	490.0	19	23.2	23.6	34.2	491.0	19	25.1	23.0	32.6	487.0
NR 118996.1	20.0	23.9	24.6	31.5	1545.0	21	23.4	27.3	28.0	517.0	19	23.6	23.1	34.3	516.0	20	24.8	23.4	32.0	512.0
NR 157608.1	19.8	23.9	24.6	31.7	1507.0	21	24.2	27.2	28.0	504.0	19	22.5	23.9	34.4	503.0	20	25.0	22.8	32.6	500.0
NR 157609.1	20.0	23.7	24.9	31.4	1508.0	21	23.4	28.0	27.4	504.0	19	22.6	23.6	34.5	504.0	19	25.0	23.2	32.4	500.0
Average	19.9	23.9	24.7	31.5	1504.5	21	23.6	27.4	27.8	502.7	19	23.1	23.5	34.3	502.5	19	24.9	23.2	32.5	499.3

3.9 *Phylogenetic analysis*

To obtain a better phylogenetic tree, initial model selection was tested in MEGA 7.0. The model selection test was conducted using Maximum Likelihood (ML) statistical method while considering all the sites. The initial tree was generated through the Neighbor-joining method.

For SOSS_7.1, a total of 24 models were generated with varying number of parameters (19 to 29), BIC and AIC scores. The obtained best model based on the BIC (Bayesian Information Criterion) and AICc (Akaike Information Criterion, corrected) assessment [31] was Tamura-Nei(T92) model having 21 parameters and BIC score of 5570.57 and AIC score of 5408.68.

Similarly, for the COSS_7.4 sequence, following the similar parameters models were tested and 24 best plausible models were reported. Tamura-Nei (T92+G) model along with a discrete Gamma distribution (+G) was selected as the best model with 22 parameters, 5526.62 BIC and 5356.97 AICc scores. The generated trees are represented in Fig.6. (A and B).

The obtained tree evidently revealed that the SOSS_7.1 sample belongs to the *Bacillus sonorensis* and COSS_7.4 belongs to *Bacillus halotolerans* species. The computed evolutionary distances between the considered samples and the respective best 10 BLAST hits are presented in Table 7 and Table 8.

Table 7 Estimates of evolutionary divergence between top BLAST hits and SOSS_7.1 sample sequence. The upper diagonal side represents the observed standard error (SE) values and the lower diagonal displays the distance values.

MN160227.1(Bacillus sonorensis strain RCPS1)		0.004	0.002	0.001	0.001	0.001	0.004	0.001	0.001	0.003	0.001
NR 027552.1 (Bacillus subtilis strain DSM 10)	0.015		0.004	0.004	0.004	0.004	0.001	0.004	0.004	0.004	0.004
NR 042338.1 (Bacillus aerius strain 24K)	0.004	0.015		0.002	0.001	0.002	0.004	0.001	0.001	0.003	0.002
NR 074923.1 (Bacillus licheniformis strain ATCC 14580)	0.003	0.014	0.003		0.001	0.001	0.004	0.001	0.001	0.003	0.001
NR 113588.1 (Bacillus licheniformis strain NBRC 12200)	0.002	0.013	0.002	0.001		0.001	0.004	0.000	0.000	0.002	0.001
NR 113993.1 (Bacillus sonorensis strain NBRC 101234)	0.003	0.015	0.004	0.003	0.002		0.004	0.001	0.001	0.003	0.001
NR 115063.1 (Bacillus halotolerans strain DSM 8802)	0.017	0.003	0.017	0.015	0.015	0.017		0.004	0.004	0.004	0.004
NR 116023.1 (Bacillus licheniformis strain BCRC 11702)	0.002	0.013	0.002	0.001	0.000	0.002	0.015		0.000	0.002	0.001
NR 118996.1 (Bacillus licheniformis strain DSM 13)	0.002	0.013	0.002	0.001	0.000	0.002	0.015	0.000		0.002	0.001
NR 157608.1 (Bacillus swezeyi strain NRRL B-41294)	0.010	0.016	0.010	0.008	0.007	0.008	0.016	0.007	0.007		0.003
NR 157609.1 (Bacillus haynesii strain NRRL B-41327)	0.002	0.013	0.004	0.002	0.001	0.002	0.015	0.001	0.001	0.009	

Table 7 Estimates of evolutionary divergence between top BLAST hits and SOSS_7.1 sample sequence. The upper diagonal side represents the observed standard error (SE) values and the lower diagonal displays the distance values.

Table 8 Estimates of evolutionary divergence between top BLAST hits and COSS_7.4 sample sequence. The upper diagonal side represents the observed standard error (SE) values and the lower diagonal displays the distance values.

Both the sequences were submitted to the GenBank database using the Maximum Likelihood method and the Accession number of MN160227.1 (SOSS_7.1) and MN160229.1(COSS_7.4) was obtained.

Advanced enzymology successfully led to the growth of white biotechnology [32]. Lipase (EC 3.1.1.3) plays a pivotal role in industrial applications through enhancing the fat or lipid hydrolysis [33]. Extensive applications of lipases are observed in the detergent industry, organic synthesis, leather industry, biotransformation, waste treatment and several other similar sectors [33]. The growing demand of the lipase allowed us to hunt for potential sources with great production yield. Out of abundant lipase sources, such as bacterial, fungal and others;

bacterial lipases [34] are the preferred ones due to low production and maintenance cost, great yield and ease of isolation and characterization.

Table 8 Estimates of evolutionary divergence between top BLAST hits and COSS_7.4 sample sequence. The upper diagonal side represents the observed standard error (SE) values and the lower diagonal displays the distance values.

MN160229.1 (Bacillus halotolerans strain Bcillus)		0.004	0.003	0.003	0.002	0.002	0.004	0.002	0.002	0.004	0.002
NR 027552.1 (Bacillus subtilis strain DSM 10)	0.015		0.005	0.004	0.004	0.005	0.001	0.004	0.004	0.004	0.004
NR 042338.1 (Bacillus aerius strain 24K)	0.008	0.019		0.001	0.001	0.002	0.005	0.001	0.001	0.003	0.002
NR 074923.1 (Bacillus licheniformis strain ATCC 14580)	0.006	0.017	0.003		0.001	0.002	0.005	0.001	0.001	0.003	0.001
NR 113588.1 (Bacillus licheniformis strain NBRC 12200)	0.006	0.016	0.002	0.001		0.002	0.004	0.000	0.000	0.003	0.001
NR 113993.1 (Bacillus sonorensis strain NBRC 101234)	0.006	0.019	0.006	0.004	0.004		0.005	0.002	0.002	0.003	0.001
NR 115063.1 (Bacillus halotolerans strain DSM 8802)	0.016	0.003	0.020	0.019	0.018	0.020		0.004	0.004	0.004	0.004
NR 116023.1 (Bacillus licheniformis strain BCRC 11702)	0.006	0.016	0.002	0.001	0.000	0.004	0.018		0.000	0.003	0.001
NR 118996.1 (Bacillus licheniformis strain DSM 13)	0.006	0.016	0.002	0.001	0.000	0.004	0.018	0.000		0.003	0.001
NR 157608.1 (Bacillus swezeyi strain NRRL B-41294)	0.014	0.019	0.011	0.010	0.009	0.008	0.019	0.009	0.009		0.003
NR 157609.1 (Bacillus haynesii strain NRRL B-41327)	0.005	0.016	0.004	0.003	0.002	0.003	0.017	0.002	0.002	0.010	

Bacillus subtilis is serving as a potential source of industrial lipase along with other bacteria and the exponential demands compel us to seek new strains continuously. Specificity and selectivity wise lipases are required in the industry, specifically for substrate-specific activity, regioselective activity, enantioselective activity [35]. Therefore, there is a large demand for hunting more microbial sources for lipase which could be further used for various hydrolysis, esterification, aminolysis, transesterification reactions in the global industry [36].

Stimulatingly, we were successful in isolating and molecular characterization of the bacterial species belonging to *Bacillus subtilis*. In literature, *Bacillus subtilis* and its closely related species were considered as excellent yield providing sources of lipase for industrial applications. The higher yield was achieved for the lipase source from *Bacillus licheniformis* [37].

In this study, the bacterial strains isolated and characterized by us are identified as *Bacillus sonorensis* strain RCPS1 (GenBank Accession number MN160227.1) and *Bacillus halotolerans* strain Bacillus (GenBank Accession number MN160229.1). Phylogenetically, both the strains are in proximity with *Bacillus subtilis*, the most potential lipase producing bacterial source.

Earlier reports suggest that *Bacillus sonorensis* was found the potential for lipase production mostly from marine sources [38]. Moreover, such lipases produced from the *Bacillus sonorensis* were mostly alkaline lipases which were found highly thermostable in some cases [39].

Recent reports suggest lipase production and great yield along with higher thermotolerance from *Bacillus halotolerans* species [40]. Greater operation range was also suggested for the lipases produced from strains of *Bacillus halotolerans* [41]. Thus, these evidences of better lipase producing capability of the isolated bacterial species suggests that such species from oil effluent contaminated soil could be up scaled for further industrial level.

5.0 CONCLUSIONS

According to the authors' knowledge, this is the first report of the isolation and characterization of the strain RCPS1 of *Bacillus sonorensis*, and strain of *Bacillus halotolerans* from oil effluent contaminated soil in the industrial area of North Telangana. Based

on our results, we recommend the use of these strains for further industrial lipase production and relevant applications. Consideration of these strains for direct industrial applications may require further experimentation and yield evaluation.

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