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Isolation and Characterization of Lipase Producing Bacteria in Hydrocarbon Contaminated Sites in Kano Metropolis

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Abstract

Lipase (Triacylglycerol hydrolases EC 3.1.1.3) is an enzyme capable of hydrolyzing lipids into fatty acids and glycerol. It possesses numerous industrial applications such as pharmaceuticals, food, detergents, paper and pulp, agrochemicals, biosurfactants and bioremediation, etc. Many attempts have been made to isolate lipase producing microorganisms since this enzyme is used in many biotechnological processes. This study was aimed at isolation and characterization of lipase producing bacteria in hydrocarbon contaminated soil sites in Kano metropolis. Bacterial isolation was done through serial dilution technique; and screened on tributyrin agar plates for lipase production. A total number of 49 pure bacterial culture were isolated from hydrocarbon contaminated soil samples from the three different hydrocarbon contaminated soil sites; eighteen distinct isolates indicated lipase production after lipase screening. Lipolytic activity assay revealed no significant difference in the lipase hydrolysis capacity (HC) of the isolates. Furthermore, the lipolytic bacteria isolates were characterized by morphological and biochemical tests and identified by molecular tool through sequencing method. Three distinct selected isolates were morphologically characterized as gram-negative, rod and coccus cells and 16S rRNA sequence analysis revealed isolate A4 to have 94.91% similarly to the Pseudomonas spp. (Pseudomonas azotoformans strain LMG 21611), isolate B4 also exibited 87.99% similarity to the Pseudomonas genus (Pseudomonas fluorescens strain PF-7) while isolate C1 showed 97.57% similarity to Acinetobacter genus (Acinetobacter baumannii strain NWPRD). These bacterial strains can be used for the production of lipase enzyme for various industrial applications and bioremediation.

Keywords: Hydrocarbon, Hydrolysis Capacity, Lipolytic Bacteria, Soil, 16SrRNA

INTRODUCTION

Lipases are glycerol ester hydrolases that act on acylglycerols to liberate fatty acids and glycerol. Lipases can hydrolyze long chain water-insoluble triglycerides into diglycerides, monoglycerides, glycerol and fatty acids (Gilham & Lehner, 2005; Angkawidjaja & Kanaya, 2006). Lipases are almost present in all enzymes that are widely distributed in plants, animals and microbes (Dutta & Ray, 2009).

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The potentiality of lipases to do very specific chemical transformation (biotransformation) has make them increasingly popular in the food, detergent, starch, pulp and paper, leather, baking, cosmetic, organic synthesis, and pharmaceutical companies and industries (Park *et al*., 2005; Gupta *et al*., 2007; Grbavcic *et al*., 2007; Vijayalakshmi *et al*., 2011; Imran *et al.,* 2012). Lipases are produced by various microorganisms and higher eukaryotes. Most of the commercially used lipases are of microbial origin. Lipase-producing microorganisms have been found in various habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food, compost heaps, coal tips, and hot springs (Sztajer *et al*., 1988; Wang *et al*., 1995; Kulkarni *et al*., 2013; Sharma & Kanwar , 2014).

Microbial lipases have earned tremendous industrial attention due to their stability, selectivity, and broad substrate specificity (Dutra *et al*., 2008; Griebeler *et al*., 2011). Microbial enzymes are also more stable than plant and animal enzymes and their production is more convenient and safer (Hasan *et al*., 2006; Thomas *et al.,* 2015). A vast variety of extracellular lipases of bacterial origin with different properties and specifications have been described and characterized. Extracellular lipase was isolated from many different bacterial species, which includes *Bacillus sp., Pseudomonas sp.* and *Burkholderia sp.,* (Ertuğrul*et al*., 2007; Kiran*et al*., 2008; Wang *et al*., 2009).

This research is also aimed at isolation and identification of these important lipase producing bacteria from hydrocarbon contaminated soil in Kano metropolis.

MATERIALS AND METHODS

Study Area

Soil samples were collected from three different hydrocarbon contaminated sites in Kano metropolis. These sites are; (i) Site A; BUK New Site (11°57′50″N, 8°25′55″E) (ii) Site B; Kabuga (11°58′17″N, 8°26′43″E) and (iii) Site C; Tal'udu (11°59′21″N, 8°29′06″E).

Collection of Samples

The sampling sites were auto-mechanic workshops where all forms of hydrocarbons are used. A soil auger was used in collecting the soil samples. The auger was used to make a depth of 30cm. The soil samples were collected in an unused plastic bag sealed with heavy-duty rubber bands (Veerapagu *et al*., 2013).

Laboratory Procedures

Bacterial isolates from the samples were made through serial dilution technique on nutrient agar medium. One gram of soil each from the samples were separately suspended in 9ml of distilled water in test tubes and vortexed. Each suspension was serially diluted from 10^{-1} to 10^{-5}. Plate pouring technique was used to inoculate 1ml of the serially diluted samples from test tubes 10^{-3}, 10^{-4} and 10^{-5} from each of the three soil samples and incubated at 37°C for 24 hours. The most prominent colonies observed after 24 hours were sub-cultured on nutrients agar slants for further studies (Chitra *et al*., 2014).

Pure isolated bacterial cultures were screened for lipase activity using tributyrin agar medium. The media was sterilized for 15 minutes by autoclaving under pressure at 121°C and cooled. Sterile methylene blue was added on the media as dye to give contrast. The aliquot was then transferred to petri dishes and allowed to solidify. A loopful of each pure culture was streaked each onto the tributyrin agar plates and incubated at 37°C for 24 hours. A clear opaque zone of hydrolysis around the colonies indicates the presence of lipase activity. The hydrolysis capacity (HC value); i.e the ratio of diameter of clear zone of hydrolysis and colony diameter of the isolates were calculated (Shelley *et al*.1987; Sharma *et al*.,2001 Sirisha *et al*., 2010; Harnvoravongchai *et al*., 2020)

Lipase producing bacteria were characterized by several conventional microscopic and biochemical tests according to Bergy's manual. Three distinct lipase producers from each sampling sites were further characterized by 16S rDNA sequencing. 16SrDNA fragment was amplified by PCR. The PCR reaction was carried out using KAPATaq DNA polymerase. The Total reaction volume was 25 µL. Reaction mixture comprised of 2 µL each of the genomic DNA, 2.5 µL of 10 TaqA Buffer, 0.4 M (0.85 µL) of each of forward and reverse primers (forward 5 TGGAGAGTTTGATCCTGGCTCAG-3 and reverse primer 5-TACCGCGGCTGCTGGCAC -3) 1.25mM (1.5 µL) of $MgCl_2$, 0.25 mM (0.2 µL) of dNTP mixes and 0.2 µL of Taq DNA polymerase, in ddH2O. Amplification was carried out using the following conditions: initial denaturation of 5 min at 95 °C, followed by 35 cycles each of 30 s at 94 °C (denaturation), 30 s at 60 °C (primer annealing) and 1 min at 72 °C (extension) then followed by 10 min of final extension at 72 °C for 16S rRNA (Brahmbhatt, 2012). The data obtained were analyzed using one way analysis of variance ANOVA, Tukey method was used to determine significant differences between means ± standard deviation. Significance difference were all at $p<0.05$.

RESULTS AND DISCUSSION

Lipase Bacteria Screening and Plate Assay (HC Value)

Table 1 showed a total number of 18 lipolytic bacteria isolated and screened on tributyrin agar plates from sites A, B & C and their various lipase hydrolysis capacity. There was no significant difference at $p<0.05$ in the lipase hydrolysis capacity values recorded between the bacterial isolates. Several researchers have studied the qualitative lipolytic activity in bacterial isolates and determined it on tributyrin containing agar plates (Mobarak-Qamsari*et al*., 2011; Ilesanmi *et al*., 2020). Production of lipase by microorganisms is enhanced by the presence of natural oils such as olive oil (Bharathi *et al*., 2019; Fatima *et al*., 2020). In the present study olive oil was equally used to stimulate lipase production.Screening of lipolytic microbes on agar plates using tributyrin is usually favored as the substrate in plate assay technique (Abd Samad *et al*., 1989). In the present study, distinct bacterial isolates A4, A8, B4, B9, C1 and C7 recorded higher lipase activities as in the study of Shaini and Jayasree (2016) where isolates WCS1C2, WCS3C2, WCS5C1, WCS5C3, WCS6C4 and WCS6C3 recorded higher lipolytic activity on tributyrin agar plates.

Table 1: Lipase Hydrolysis Capacity (HC) of Lipolytic Bacterial Isolates (Sites A, B & C)

Lipase HC	Bacterial Isolate	Mean HC ± Standard Deviation
SITE A	A3	1.27 ± 0.07^{b}
	A4	1.68 ± 0.11^{a}
	A5	1.35 ± 0.08^{b}
	A6	1.18 ± 0.06^{b}
	A7	1.37 ± 0.10^{b}
	A8	1.60 ± 0.13^{a}
SITE B	B3	1.80 ± 0.60^{a}
	B4	1.45 ± 0.05^{ab}
	B6	1.27 ± 0.07^{ab}

	B7	$1.17±0.07^{b}$
	B8	$1.19±0.08^{b}$
	B9	$1.81±0.14^{a}$
SITE C	C1	$1.30±0.15^{bc}$
	C2	$1.31±0.09^{bc}$
	C4	$1.20±0.08^{c}$
	C5	$1.16±0.09^{c}$
	C6	$1.50±0.14^{ab}$
	C7	$1.78±0.16^{a}$

Means that do not share a letter are significantly different at p<0.05

Morphological and Biochemical Tests

Table 2 showed morphological and biochemical test results of a total number of 18 lipolytic bacterial isolates from sites A, B & C. A total number of 6 isolates were gram negative rods and cocci cells while 12 were gram positive cells. All the bacterial isolates were oxidase positive with the exception of B8. Similarly, all the lipolytic isolates were catalase positive. All the isolates were also starch positive with the exception of A3. This result is similar to the work of Mathew and Izomor (2023) who recorded oxidase positive, catalase positive, starch positive and lipase positive in various morphological and biochemical test results conducted on 5 bacterial isolates screened for lipase production.

Table 2: Cultural and Biochemical Characteristics of The Bacterial Isolates from The Sites

Isolate	Gram's Reaction	Oxidase Test	Catalase Test	Starch H. Test	Lipase Test	Presumptive Bacteria
A3	+ single rods	+	+	-	+	*Bacillus cereus*
A4	- single rods	+	+	+	+	*P. aeruginosa*
A5	+ rods in pairs	+	+	+	+	*Bacillus subtilis*
A6	+ rods in chains	+	+	+	+	*Bacillus cereus*
A7	+ rods in cluster	+	+	+	+	*C. xerosis*
A8	+ rods in pairs	+	+	+	+	*Rhodococcus equi*
B3	+ rods in pairs	+	+	+	+	*Rhodococcus equi*
B4	- cocci in cluster	+	+	+	+	*P. azotoformans*
B6	+ rods in cluster	+	+	+	+	*R. mucilaginosa*
B7	- rods in cluster	+	+	+	+	*P. putida*
B8	+ cocci in chains	-	+	+	+	*Micrococcus luteus*
B9	- rods in pairs	+	+	+	+	*Pseudomonas sp.*
C1	- rods in pairs	-	+	+	+	*Acinetobacter sp.*
C2	+ single rods	+	+	+	+	*Bacillus subtilis*
C3	+ rods in cluster	+	+	+	+	*Bacillus cereus*
C4	+ rods in chains	+	+	+	+	*Bacillus pumilus*
C5	+ rods in pairs	+	+	+	+	*B. linens*
C6	- rods in cluster	+	+	+	+	*P. aeruginosa*

Molecular Identification of Lipolytic Bacteria

Gel electrophoresis image of the three bacterial isolates is shown in Figure 1. The image showed amplified band sizes of the three lipolytic bacterial isolates (A4, B4 & C1) at approximately 1500bp.

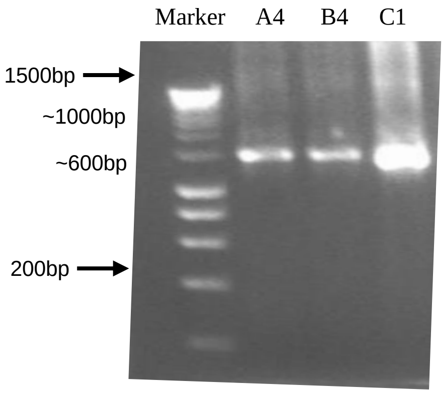


Figure 1: Agarose gel electrophoresis results show polymerase chain reaction (PCR) product sizes in bacteria (A4, B4 & C1). Using the DNA ladder marker as reference, DNA bands corresponding to A4 (1500bp), B4 (1500bp) and C1 (1500bp) are observed.

BLAST analysis revealed that all the amplified DNA sequences were bacteria in the phyla *Proteobacteria* or *Pseudomonadota* and belonged to two different genera. The 16S rRNA sequence and phylogenetic tree of the isolate A4 illustrated a high similarity and relation to the members of the genus *Pseudomonas*, showing 94.91% similarity with *Pseudomonas azotoformans*strain LMG 21611(GenBank Accession no. LT629702) and others (Fig. 5, 6 & 7). Isolate B4 also belongs to the *Pseudomonas* genus showing 87.99% similarly with *Pseudomonas fluorescens* strain PF-7 (GenBank Accession no. MF838652) and others (Fig. 8, 9 & 10). Isolate C1 demonstrated a high similarity and relation to the members of the *Acinetobacter* genus with 97.57% similarity to *Acinetobacter baumannii* strain NWPRD (GenBank Accession no. MW720652) as shown in Figure 11, 12 and 13.

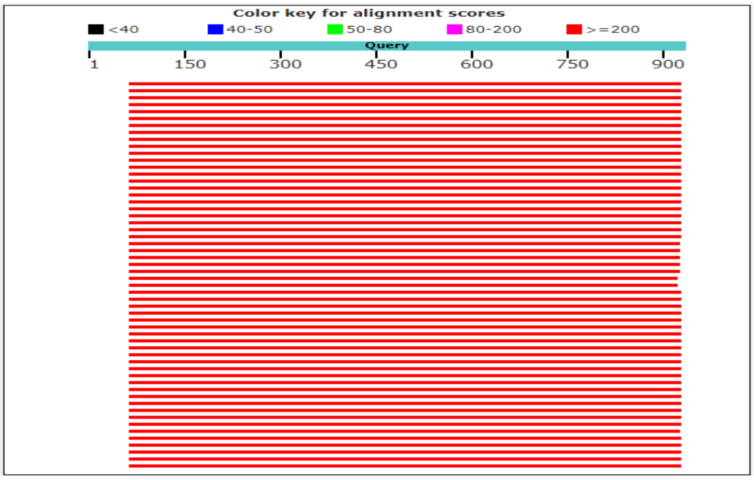
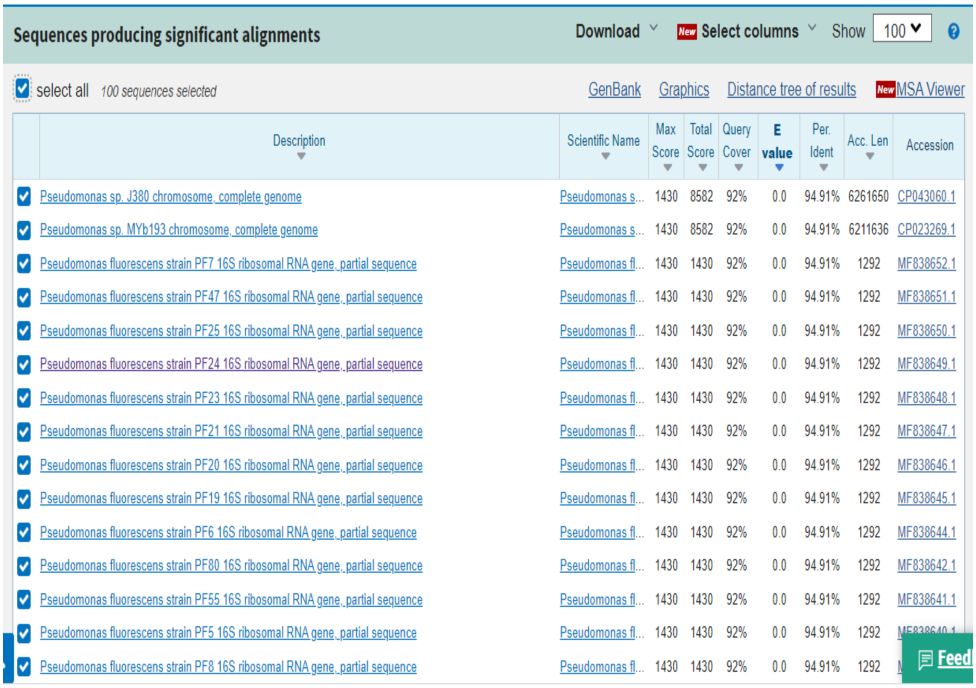


Figure 5: Alignment of BLAST Query of Isolate A4 (Query Id: IcI24733; Molecule type: DNA; Query length: 935)



Sequences producing significant alignments

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas sp. J380 chromosome, complete genome	Pseudomonas s...	1430	8582	92%	0.0	94.91%	6261650	CP043060.1
Pseudomonas sp. MYb193 chromosome, complete genome	Pseudomonas s...	1430	8582	92%	0.0	94.91%	6211636	CP023269.1
Pseudomonas fluorescens strain PF7 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838652.1
Pseudomonas fluorescens strain PF47 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838651.1
Pseudomonas fluorescens strain PF25 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838650.1
Pseudomonas fluorescens strain PF24 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838649.1
Pseudomonas fluorescens strain PF23 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838648.1
Pseudomonas fluorescens strain PF21 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838647.1
Pseudomonas fluorescens strain PF20 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838646.1
Pseudomonas fluorescens strain PF19 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838645.1
Pseudomonas fluorescens strain PF6 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838644.1
Pseudomonas fluorescens strain PF80 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838642.1
Pseudomonas fluorescens strain PF55 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838641.1
Pseudomonas fluorescens strain PF5 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	MF838640.1
Pseudomonas fluorescens strain PF8 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1430	1430	92%	0.0	94.91%	1292	[illegible]

Figure 6: BLAST Score, Query Coverage & E Value for Query Sequence of Isolate A4

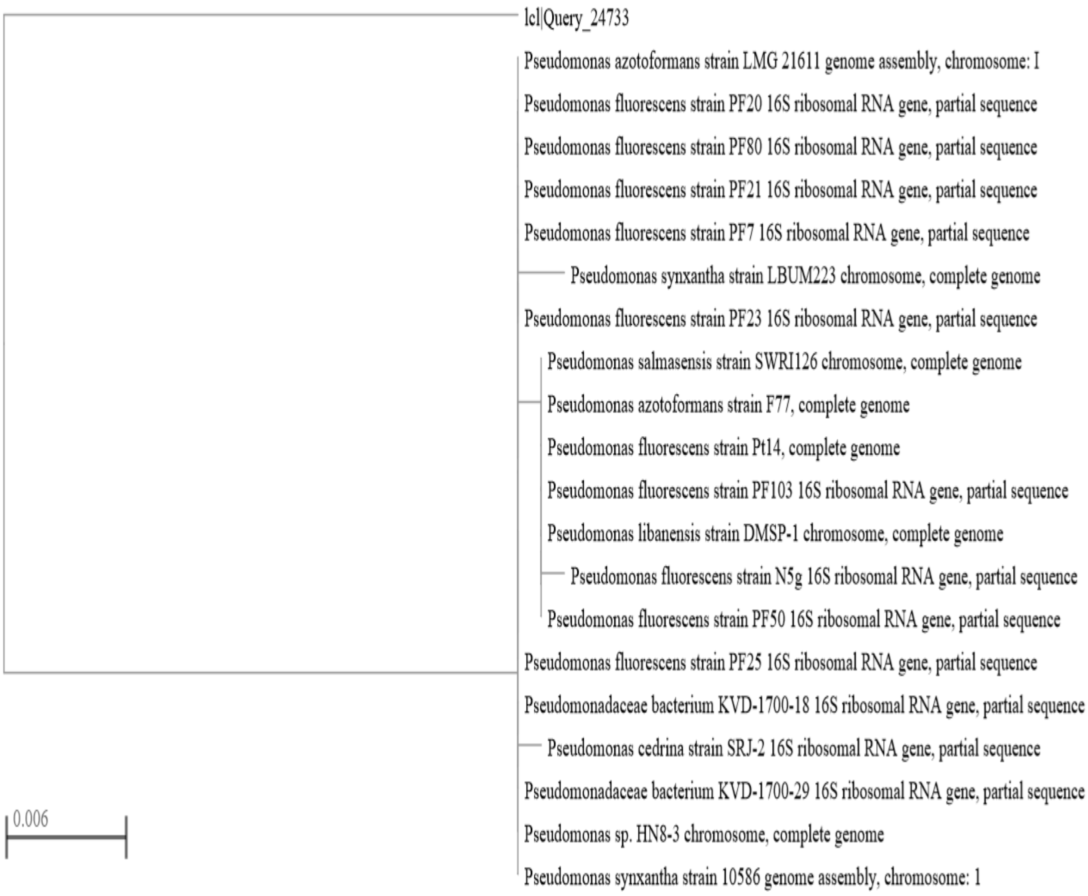


Figure 7: Phylogenetic Tree of Isolate A4 (*Pseudomonas* spp.)

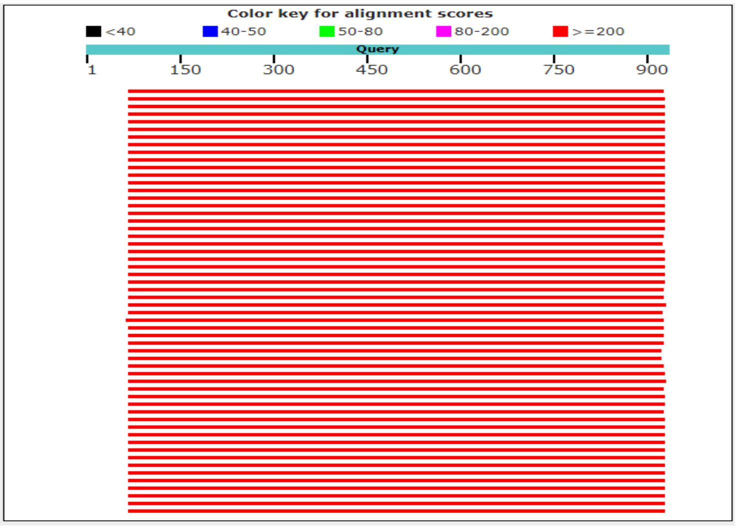
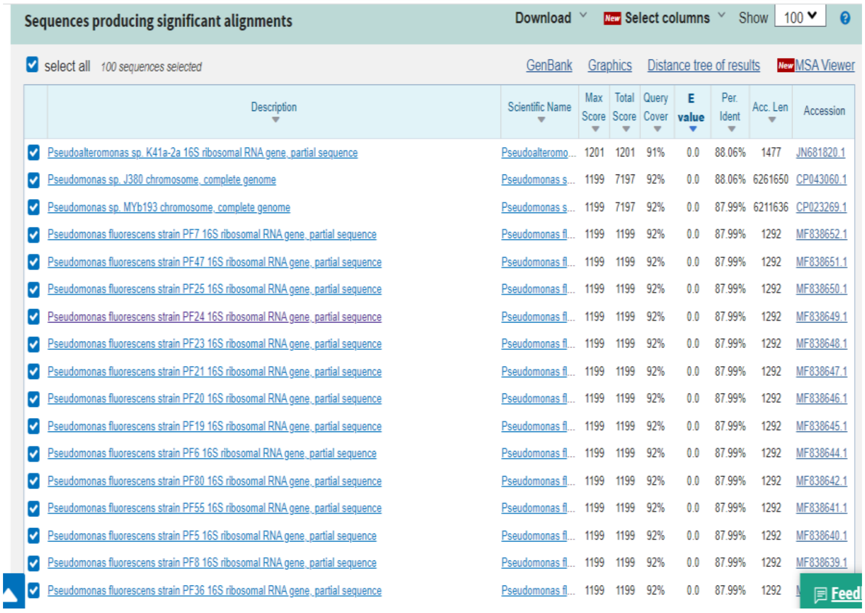


Figure 8: Alignment of BLAST Query of Isolate B4 (Query Id: IcI21175; Molecule type: DNA; Query length: 937)



Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank — Graphics — Distance tree of results — MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudoalteromonas sp. K41a-2a 16S ribosomal RNA gene, partial sequence	Pseudoalteromo...	1201	1201	91%	0.0	88.06%	1477	JN681820.1
Pseudomonas sp. J380 chromosome, complete genome	Pseudomonas s...	1199	7197	92%	0.0	88.06%	6261650	CP043060.1
Pseudomonas sp. MYb193 chromosome, complete genome	Pseudomonas s...	1199	7197	92%	0.0	87.99%	6211636	CP023269.1
Pseudomonas fluorescens strain PF7 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838652.1
Pseudomonas fluorescens strain PF47 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838651.1
Pseudomonas fluorescens strain PF25 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838650.1
Pseudomonas fluorescens strain PF24 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838649.1
Pseudomonas fluorescens strain PF23 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838648.1
Pseudomonas fluorescens strain PF21 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838647.1
Pseudomonas fluorescens strain PF20 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838646.1
Pseudomonas fluorescens strain PF19 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838645.1
Pseudomonas fluorescens strain PF6 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838644.1
Pseudomonas fluorescens strain PF80 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838642.1
Pseudomonas fluorescens strain PF55 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838641.1
Pseudomonas fluorescens strain PF5 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838640.1
Pseudomonas fluorescens strain PF8 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	MF838639.1
Pseudomonas fluorescens strain PF36 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1199	1199	92%	0.0	87.99%	1292	[illegible]

Figure 9: BLAST Score, Query Coverage & E Value for Query Sequence of Isolate B4

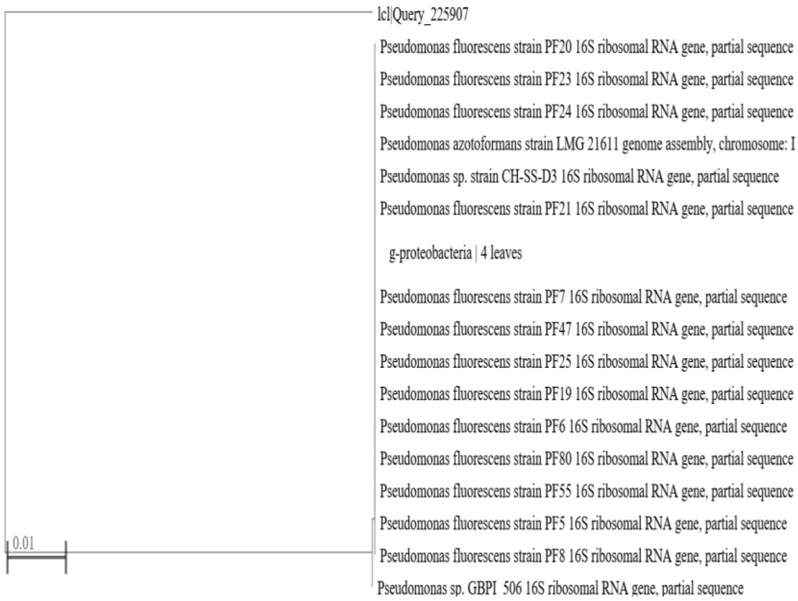
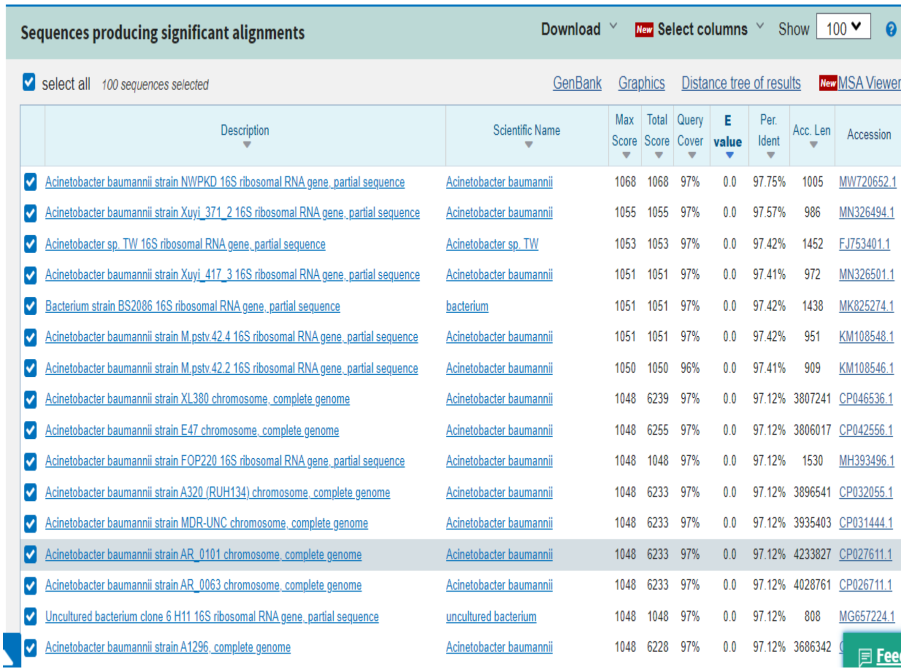


Figure 10: Phylogenetic Tree of Isolate B4 (*Pseudomonas*spp.)

Figure 11: Alignment of BLAST Query of Isolate C1 (Query Id: IcI2145; Molecule type: DNA; Query length: 633)



Sequences producing significant alignments

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Acinetobacter baumannii strain NWPKD 16S ribosomal RNA gene, partial sequence	Acinetobacter baumannii	1068	1068	97%	0.0	97.75%	1005	MW720652.1
Acinetobacter baumannii strain Xuyi_371_2 16S ribosomal RNA gene, partial sequence	Acinetobacter baumannii	1055	1055	97%	0.0	97.57%	986	MN326494.1
Acinetobacter sp. TW 16S ribosomal RNA gene, partial sequence	Acinetobacter sp. TW	1053	1053	97%	0.0	97.42%	1452	FJ753401.1
Acinetobacter baumannii strain Xuyi_417_3 16S ribosomal RNA gene, partial sequence	Acinetobacter baumannii	1051	1051	97%	0.0	97.41%	972	MN326501.1
Bacterium strain BS2086 16S ribosomal RNA gene, partial sequence	bacterium	1051	1051	97%	0.0	97.42%	1438	MK825274.1
Acinetobacter baumannii strain M.pstv.42.4 16S ribosomal RNA gene, partial sequence	Acinetobacter baumannii	1051	1051	97%	0.0	97.42%	951	KM108548.1
Acinetobacter baumannii strain M.pstv.42.2 16S ribosomal RNA gene, partial sequence	Acinetobacter baumannii	1050	1050	96%	0.0	97.41%	909	KM108546.1
Acinetobacter baumannii strain XL380 chromosome, complete genome	Acinetobacter baumannii	1048	6239	97%	0.0	97.12%	3807241	CP046536.1
Acinetobacter baumannii strain E47 chromosome, complete genome	Acinetobacter baumannii	1048	6255	97%	0.0	97.12%	3806017	CP042556.1
Acinetobacter baumannii strain FOP220 16S ribosomal RNA gene, partial sequence	Acinetobacter baumannii	1048	1048	97%	0.0	97.12%	1530	MH393496.1
Acinetobacter baumannii strain A320 (RUH134) chromosome, complete genome	Acinetobacter baumannii	1048	6233	97%	0.0	97.12%	3896541	CP032055.1
Acinetobacter baumannii strain MDR-UNC chromosome, complete genome	Acinetobacter baumannii	1048	6233	97%	0.0	97.12%	3935403	CP031444.1
Acinetobacter baumannii strain AR_0101 chromosome, complete genome	Acinetobacter baumannii	1048	6233	97%	0.0	97.12%	4233827	CP027611.1
Acinetobacter baumannii strain AR_0063 chromosome, complete genome	Acinetobacter baumannii	1048	6233	97%	0.0	97.12%	4028761	CP026711.1
Uncultured bacterium clone 6 H11 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1048	1048	97%	0.0	97.12%	808	MG657224.1
Acinetobacter baumannii strain A1296, complete genome	Acinetobacter baumannii	1048	6228	97%	0.0	97.12%	3686342	[illegible]

Figure 12: Blast Score, Query Coverage & E Value for Query Sequence of Isolate C1

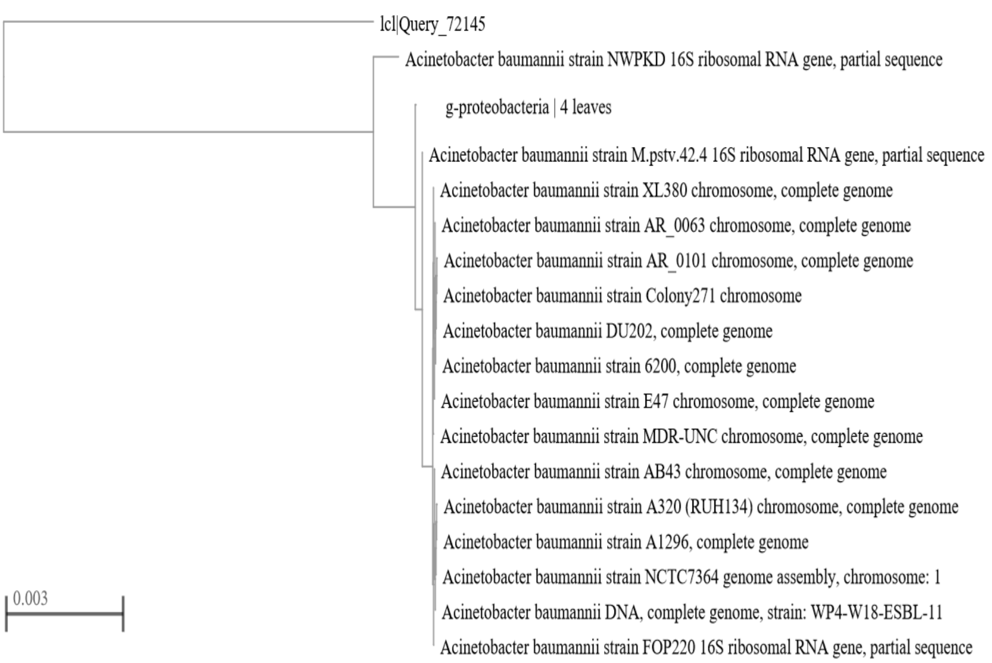

Figure 13: Phylogenetic Tree of Isolate C1 (*Acinetobacter sp.*)

16S rRNA gene sequencing is by far the most common, rapid and accurate method of targeting housekeeping genes to study bacterial phylogeny and genus/species classification (Abd Al-Wahid & Abd Al-Abbas, 2019). Several studies have reported the molecular identification of Lipase Producing *Pseudomonas sp.* and *Acinetobacter sp.* bacteria from oil contaminated environments (Veerapagu *et al.*, 2013; Alhamdani & Alkabbi, 2016). In the study of Mobarak-Qamsari *et al.*, (2011), 16S rRNA sequencing revealed a new strain of *Pseudomonas aeruginosa* (KM110) after isolation and screening of lipase producing bacteria from wastewater of an oil processing plant.

CONCLUSION

Results showed that lipase-producing bacteria can be obtained from oil-contaminated soil. Plate assay method can be a very good lipase production indicator. Lipolytic bacteria isolates recorded significant hydrolysis capacity after tributyrin agar plate assay. 16S rDNA gene sequencing; which is the contemporary and accurate tool used to identify and trace phylogenetic relationships between bacteria was used to identify three different strains of bacteria. *Pseudomonas sp.* and *Acinetobacter sp.* were established to be high lipase producers thus can be used in various industrial applications and importantly, bioremediation

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