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eswari beeram

eshu.sonu@gmail.com

Mohan babu university <https://orcid.org/0000-0002-6055-6241>

Vishnu vardhan S

Mohan babu university

Siva Priya

Mohan babu University

SriVidhya

Mohan babu university

Research Article

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Isolation of lipase producing strains *Acinetobacter pittii* and *Enterobacter asburiae* from the oil mill sample and induced oil environment and their characterization for Bioremediation

Eswari beeram^{*1}, Vishnu Vardhan^{1a}, Srividhya^{1b}, Siva Priya^{1c}

Corresponding author email: eswaribeeram@gmail.com, ph: 9700277136

Eswari beeram^{*1}, Assistant Professor, DBCS, SoLAS, Mohan Babu University-517102.

Vishnu Vardhan^{1a}, Srividhya^{1b}, Siva Priya^{1c}, BSc., Microbiology DBCS, SoLAS, Mohan Babu University-517102.

Novelty statement:

The current study demonstrates a novel approach for isolation and characterisation of lipase-producing microbial strains *A. pittii* & *E.asburiae* from oil mill soil samples and using an induced oil-rich environment to selectively enhance lipolytic activity in the particular desired strains. Unlike conventional isolation methods, this strategy allows provision for the isolation of microorganisms growing in lipid rich environment. The research further emphasizes on exploration of oil mill soil sample as a low utility potent source for industrially significant microbes yet. Detailed characterization of the isolates provides insights on their enzymatic efficacy and degradability of metal oxides. Additionally, the study provides industrial application orientation of bioremediation of oil-contaminated environments. This integrated approach contributes to sustainable waste management by identification and application of robust strains for the purpose of bioremediation of oil waste.

Abstract:

A. pittii & *E.asburiae* are the gram-negative rod-shaped bacteria isolated from the oil mill sample and induced oil environment by using gram staining method. The isolated bacteria are characterised biochemically using IMVIC test procedures. Both the strains showed positive reaction with methyl red, catalase reaction and citrate utilisation test where as negative for gelatin hydrolysis, urease and oxidase reaction. *Enterobacter asburiae* is a motile bacterium with motility away from the stab line where as *A.pittii* is an immotile with motility along the stab line in the semisolid motility agar. Microbial lipases are isolated from the *A. pittii* & *E. asburiae* using chromato-focussing and the isoelectric pH of the lipases isolated from the bacteria is found to be 4.4. The isolated lipase is separated using SDS PAGE for the purity assessment. The bacterial strains are confirmed as *A. pittii* & *E. asburiae* by 16srRNA sequencing and BLAST assembly. The resulted genomic data is deposited in Gen Bank of NCBI and are provided with the accession numbers PZ167599 (*E.asburiae*) & PZ167600 (*A.pittii*).

Keywords: *Enterobacter asburiae*, *Acinetobacter pittii*, gelatin hydrolysis, oxidase reaction, catalase reaction, SDS PAGE.

Introduction:

Industrial development in terms of growth and expansion has led to release of lipid-rich effluents in large quantities into the environment. These wastes are not readily degradable due to their high oil and grease content, finally leading to negative global impact through soil and water pollution. Conventional treatment methods which are currently under usage are often expensive and inefficient, there by necessary alternative need for sustainable oil waste treatment ⁽²⁾. In this context, microbial bioremediation can serve as an effective and alternative eco-friendly approach, there by utilizing the naturally occurring microorganisms capable of degrading the biohazardous oil waste through their enzymatic action ⁽¹¹⁾.

Lipase-producing bacteria play important role in degradation of oil waste and complex lipids in to harmless fats. Species such as *Acinetobacter pittii* and *Enterobacter asburiae* are already known for their metabolic adaptability and ability to survive in harsh and oil-contaminated environments. These microorganisms produce extracellular lipases that facilitate the hydrolysis of triglycerides ⁽¹²⁾, adding them as a source of valuable resource for the environment clean up procedures ^(5,8). Their resilience under stress conditions further adds an advantage of using the organisms in Bioremediation applications.

Oil mill waste serves as a rich and underexplored source of lipolytic microorganisms ⁽¹⁾. By using induced environment, we can potentially enrich the lipolytic strains ⁽¹²⁾. This method allows identification of strains with superior enzymatic activity compared to conventional methods. In the present study, *Acinetobacter pittii* and *Enterobacter asburiae* isolated from oil mill samples and induced environments using enrichment strategies, ensure the selection of efficient lipid-degrading strains present in the environment.

The isolated strains are subjected to characterization to evaluate their biochemical properties and lipase activity ⁽¹⁶⁾. Their potentiality in degradation of oil pollutants highlights their applicability in oil contaminated environments for the process of Bioremediation ⁽¹⁵⁾. This study contributes to the development of cost-effective and sustainable waste management solutions there by identification of potential candidate strains that are capable of efficient degradation of lipid contaminants.

Despite the significant research and advancements in the field of isolation of lipase producing strains that are capable of lipid degradation several research gaps remain on strains such as *Acinetobacter pittii* and *Enterobacter asburiae* usage for bioremediation. Most of the available methodologies on basic isolation and screening and little emphasize on oil induced environment conditions and lack of studies on the adaptability and stress tolerance to maximize lipase yield and efficiency is limited in context. There is a lack of standardised methodologies for the enrichment procedures, selection and comparison of different methods under different conditions.

Materials and methods:

Isolation and biochemical characterisation of *A. pittii* and *E.asburiae* collected from oil mill sample and induced oil environment:

Soil sample was collected from local oil mill located at stone house peta, Nellore. The collected sample was serially diluted and plated on the agar plate for overnight incubation

and observed for colonies on the next day. The plates with observed growth were selected and the culture is tested for biochemical characterization like motility, sugar fermentation, gelatin hydrolysis, urease production, catalase production and oxidase production and the results were recorded and tabulated.

Development of induced oil environment in the laboratory conditions:

Soil sample is obtained from the herbal garden of School of pharmaceutical sciences, MBU, Tirupati and every day the soil is allowed to saturated with either palm oil or coconut oil up to 15-30 days and this source soil sample is used for serial dilution and plating on nutrient agar. After suitable growth, the colonies grown are used for biochemical characterisation procedures and other studies.

Synthesis of substrate for lipase assay:

Ester of PNP (p-nitro phenyl stearate) is subjected to organic synthesis by addition of 1g of p-nitro phenol and 1g of stearic acid to 100ml of toluene by continuous stirring on a magnetic stirrer by without applying the heat. 10ml solution of thionyl chloride is added slowly in a drop wise manner followed by 5ml of Dimethyl formamide and diethyl amine. The solution is allowed for stirring up to 1hr and allowed to evaporate at room temperature. The resulting 5 ml aqueous phase solution is collected and used as substrate for lipase assay.

Lipase assay:

1ml of the 5ml collected substrate is diluted to 100ml with methanol. 1ml of the diluted substrate and 5ml of 0.1M Tris EDTA buffer is added to the test tube followed by addition of 1ml of methanol. To the solution 0.5 ml of supernatant enzyme is added and allowed for incubation of 15min and after required time period the reaction is stopped by addition of 1ml 0.1N NaOH and the reading is recorded at 420nm. The blank is prepared by without addition of enzyme. 5ml of bacterial culture is taken in a centrifuge tube and centrifuged at 5000 rpm for 15 min and the resulted supernatant is used as a source of enzyme.

Calibration of standard curve of PNP:

100mg of PNP is weighed and dissolved in 100ml of distilled water and few drops of conc. HCL is added to convert the solution in to colourless and 10ml of the above solution is taken and diluted to 100ml with distilled water and used as standard. 0.2-1.0ml of standard is taken in 5 different test tubes and made up to 1ml with distilled water. 1ml of 0.1M NaOH is added to the test tube and the colour intensity is recorded at 420nm against blank. Blank is prepared by without addition of PNP. Concentration of PNP liberated is calibrated using standard curve.

Calibration of standard curve of protein by Biuret method:

1ml of supernatant is taken and adjusted to 2ml with distilled water and 3ml of biuret reagent is added and incubation for 15 min. purple colour developed is read at 540nm against blank and concentration of protein is calibrated using standard curve. A standard curve is

constructed by taking 0.2-1.0 ml of protein solution and 3ml of biuret reagent is added and the purple colour developed is read against blank at 540nm.

Isolation and purification of Lipase from *A. pittii* and *E. asburiae* using Chromatofocusing:

CM - cellulose is dissolved in tris HCl buffer and made in to slurry. A tidy and clean glass column is taken and sterilised with ethanol and washed with water and made ready to use. The prepared slurry is loaded in to the glass column and the gel is continuously washed with the buffer until the residues are removed and free flow is detected. Centrifuged supernatant of *A. pittii* and *E. asburiae* is loaded on to the surface of the gel loaded separate columns and allowed for separation of enzyme. Flow through is collected and the bounded enzyme is separated with series of buffers with different pHs ranging 4.4, 4.8, 5.0 and 5.4 and the collected fractions is analysed for lipase presence using UV- Visible spectrophotometer. The fraction with highest protein concentration is used for Ammonium sulphate precipitation (60%) and centrifuged and redissolved in tris buffer. The protein stored is used for SDS PAGE analysis for checking of the purity and the fraction sample is assayed for lipase activity by PNP assay and concentration of protein lipase present in the sample is determined using biuret method.

SDS PAGE:

Column separated fraction (pH: 4.4) of lipase from *A. pittii* and *E. asburiae* is used for evaluation of purity by SDS PAGE. Procedure of SDS PAGE is followed as per protocol of (Laemmli, 1970).

16srRNA sequencing:

The procedure is carried out accordingly from the collected literature of Castresana, J. (2000), Dereeper A et al., (2008), Edgar RC (2004), Talavera, G., and Castresana, J. (2007) & Edwards U et al., (1989).

Results:

A. pittii and *E. asburiae* is isolated from the oil sample and induced palm oil environment. From the fig.1A,1B &1C Colonies were observed on the nutrient agar and from 1D the bacterial strains were characterised using biochemical tests. From the Figure 1A and 1D *Acinetobacter pittii* showed positive reaction for methyl red and citrate utilization test and negative for Indole and Voges- Proskauer tests. From the figure 1B and 1D *E. asburiae* reacted positive for methyl red and citrate utilization test and showed negative for both Indole and Voges- Proskauer tests.

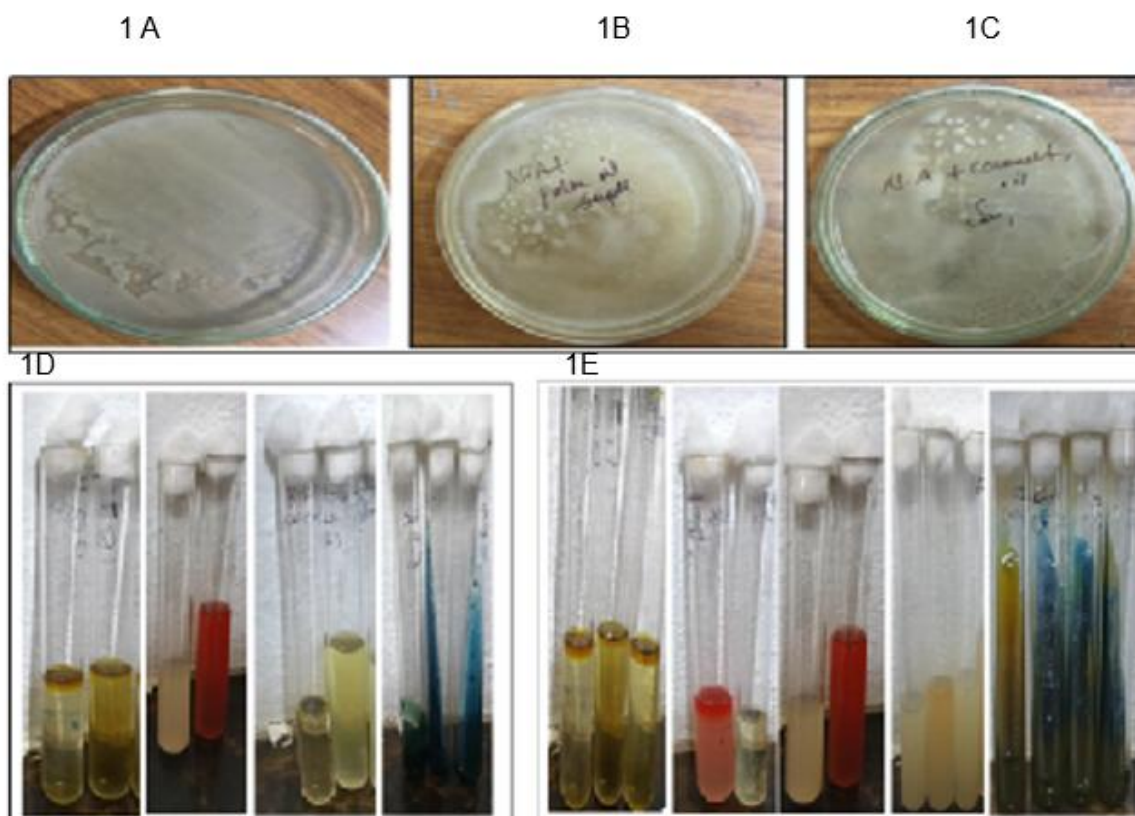


Figure: 1 Isolation and characterisation of *Acinetobacter pittii* and *E. asburiae* from oil mill sample and induced oil environment 1A: *A. pittii* from oil mill sample 1B: *E. asburiae* from induced palm oil environment .1C *A. baumanii* from induced coconut oil sample. 1D: characterisation of bacterial samples using IMVIC tests.

From the figure 2a(i), 2a(ii) and 2a(iii) *A. pittii*, *A. baumanii* and *E. asburiae* showed positive tests for catalase enzyme presence and from the figure 2b(i), 2b(ii) and 2b(iii) & 2c (i), 2c (ii) and 2c (iii) *A. pittii*, *A. baumanii* and *E. asburiae* shown negative for gelatin hydrolysis and urea hydrolysis.

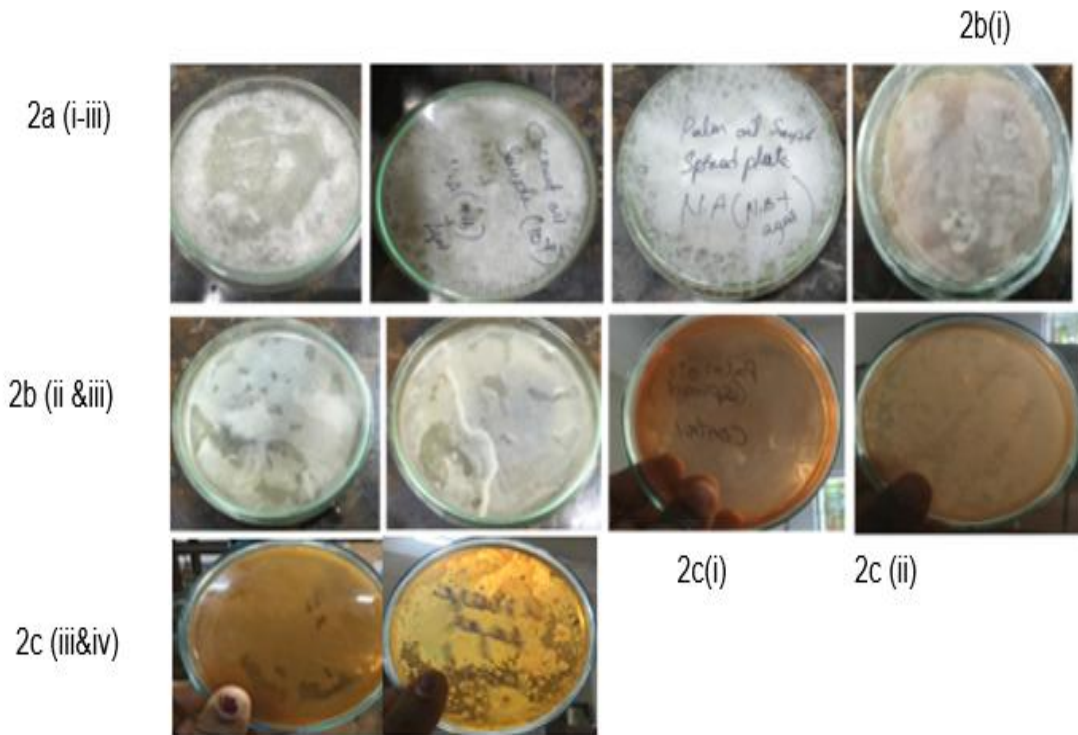


Figure:2 Biochemical characterisation of *A.pitti* and *E.asburiae*: 2a: Catalase test (2a(i): *A.pitti*, 2a(ii): *A.baumannii*, 2a(iii): *E.asburiae*) 2b: Gelatin hydrolysis test (2b(i): *A.pitti*, 2b(ii): *A.baumannii*, 2b(iii): *E.asburiae*) 2C: Urease test (2c (i): *A.pitti*, 2c (ii): *A.baumannii* , 2c (iii): *E.asburiae*).

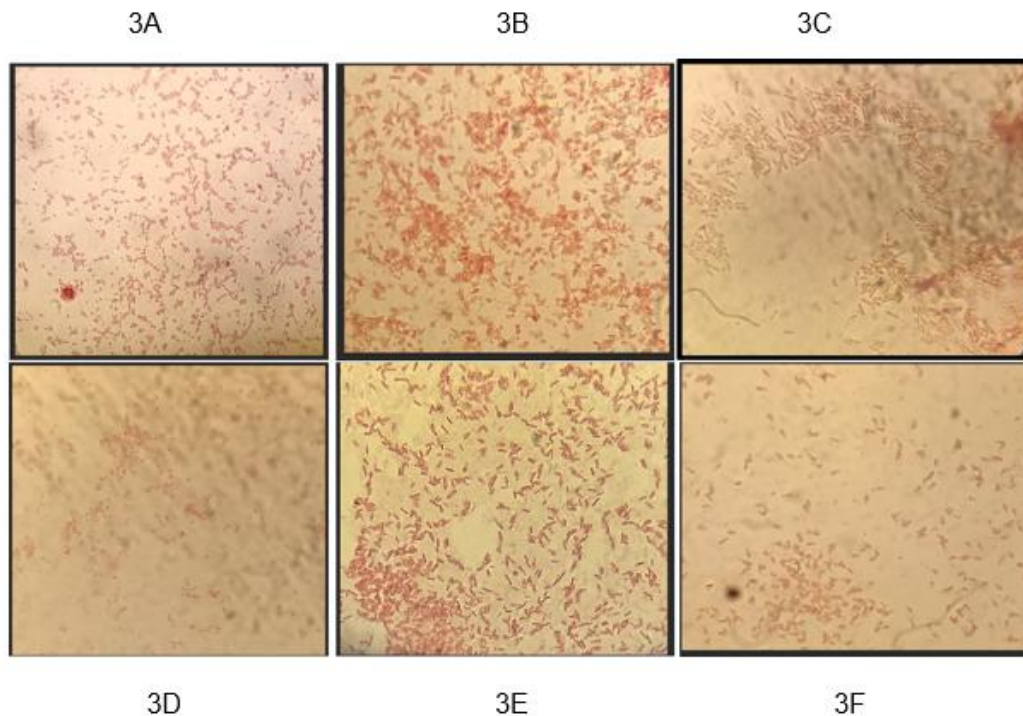


Figure : 3 Categorisation of bacteria as Gram +ve or Gram -ve using gram staining and spore staining techniques. 3a. Gram staining of *A.baumannii*, 3b. spore staining of *A.baumannii*, 3c. Gram staining of *A.pitti*, 3d. spore staining (*A.pitti*), 3e. Gram staining (*E.asburiae*), 3f. spore staining (*E.asburiae*).

From the figure 3a,3b&3c all the 3 strains *A.pitti*, *A. baumannii* and *E.asburiae* identified as gram -ve rod shaped bacteria and from the figure 3d,3e and 3f *A.pitti*, *A. baumannii* and *E.asburiae* stores lipids in the presence of high concentration of lipids and from the results the strains *A.pitti*, *A. baumannii* and *E.asburiae* are confirmed as non-sporulating bacteria and the lipid granules are found to be colourless with malachite green stain (spore staining).

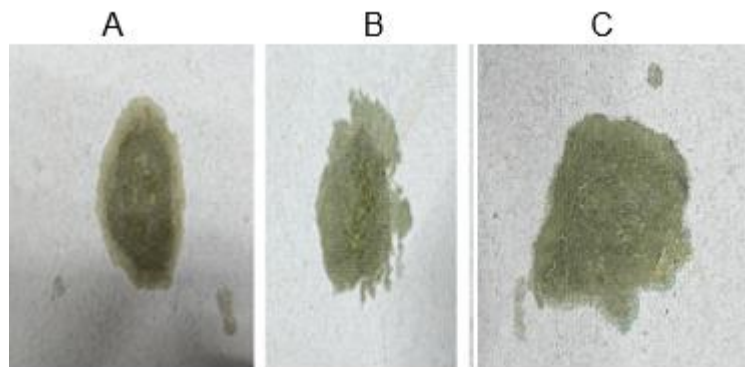


Figure:4 Oxidase test for *A.pitti* and *E.asburiae* and *A.baumannii*

A.pitti and *E.asburiae* and *A.baumannii* are found to be negative for oxidase test and characterised as aerobic (*A.pitti* and *E.asburiae*) and facultative anaerobic bacteria (*E.asburiae*) (Fig:4 & 7D).



Figure: 5 Widal test to confirm the bacterial strain: (a) Coconut oil sample (b) Palm oil sample (c) oil mill sample. Cross reaction was reported with *A.pitti* and *E.asburiae* and it is not observed with *A.baumannii*

From the figure 5 *E.asburiae* and *A.pitti* shows similar antigens to H&O present in *S. typhi* and *paratyphi* and showed cross reaction with widal test where as *A.baumannii* does not cross react with antibodies of *S.typhi* and *paratyphi*. *A.pitti* has formed clear zone on TWEEN20 agar and with stearic acid but does not with cholesterol or coconut oil (Fig 6A,6C). In control acetone and water is used instead of bacterial strain. *E.asburiae* and *A.baumannii*

growth is inhibited in the presence of complex lipids (fig.6D and 6E) and partial clearance zone is seen in TWEEN20 agar with the both bacteria (fig.6F and 6G) which confirms the lipase production is minimal with the 2 strains.

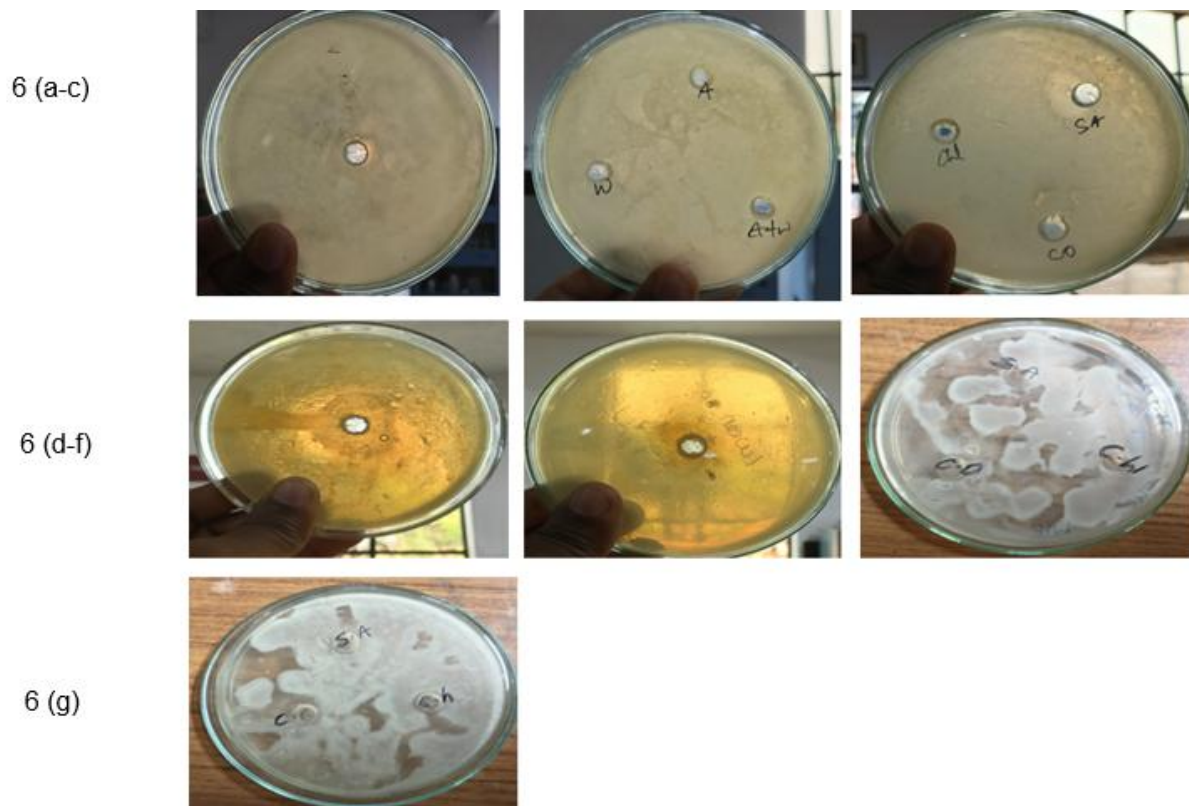


Figure:6: Characterization of lipase production from *A.pitti*, *A.baumannii* and *E.asburiae* using lipase plate assay: 6a: Lipase activity of *A.pitti* on Tween20 agar, 6b: Contol,6c: Lipase assay by using agar diffusion method. 6d&6e: Lipase activity on Tween 20 agar (*E.asburiae* (6d), *A.baumannii* (6e)), 6f&6g: Lipase assay by using agar diffusion method (*E.asburiae* (6f), *A.baumannii* (6g)): SA- Stearic acid, C.O – Coconut oil, CHL: Cholesterol.

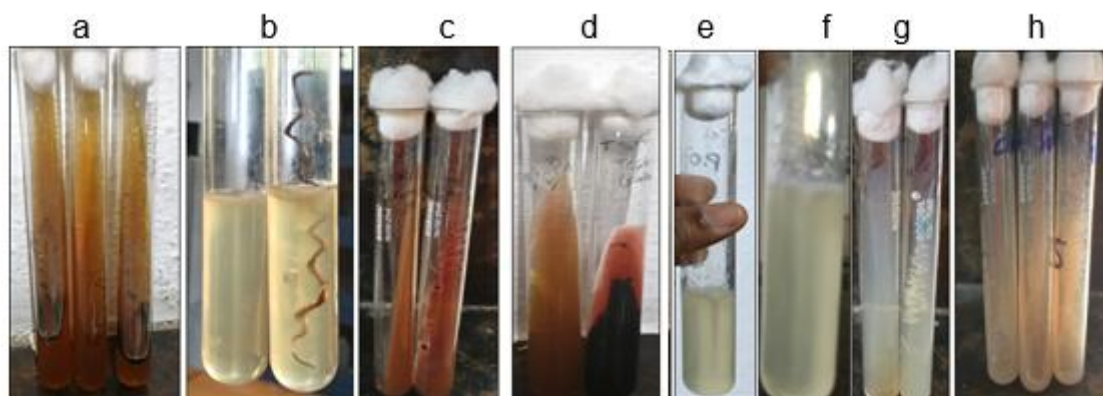


Figure:7: Biochemical characterization of *A.pitti*, *A.baumannii* and *E.asburiae* using Triple Sugar Agar, Semisolid motility agar and Gelatin Iron agar. (a) Sugar fermentation by *A.pitti* on TSA, (b) Motility test for *A.pitti*, (c) sugar fermentation by *A.baumannii* on TSA, (d) sugar fermentation by *E.asburiae* on TSA, (e) Motility test for *E.asburiae*, (f) Motility test for *A.baumannii*, (g) Gelatin iron agar slant of *A.baumannii*,(h) Gelatin iron agar slant of *A.pitti* and *E.asburiae*

From the fig.7 *E.asburiae* is a motile, fermentative and non H₂S producer but certain strains of *E.asburiae* can produce H₂ which reduce sodium thiosulfate or ferrous sulfite producing H₂S resulting in the blackening of the butt. *A.pitti* and *A.baumanii* is non motile, non-fermentative and non H₂S producer with negative tests on motility agar, TSA agar and gelatin iron agar media.

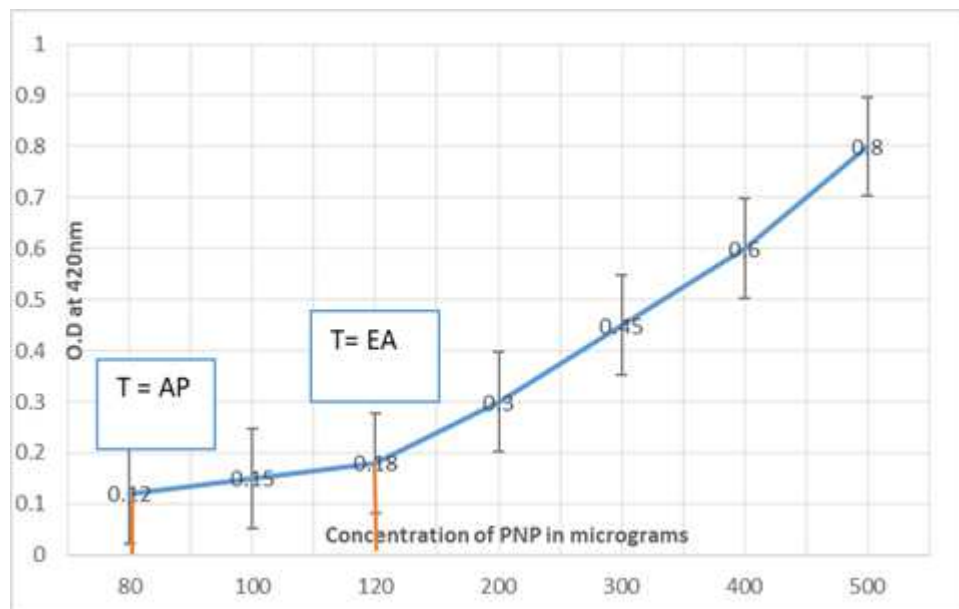


Figure:8 Standard curve of PNP along with test sample enzyme activity (Lipase) from *A. pittii* and *E.asburiae* cut in the curve.

From the PNP standard assay the amount of lipase isolated from *A.pitti* is calculated as 80µg and for *E.asburiae* it is around 120µg (fig:8). From fig. 9 the isoelectric pH of the lipase isolated from both *A.pitti* and *E.asburiae* is found to be 4.4 with highest enzyme activity (fig.9& 11) isolated using CM cellulose by chromatofocussing technique using pH gradient ranging from 4.4 to 5.4 and salt gradient ranging from 0.5M to 2.0M NaCl.

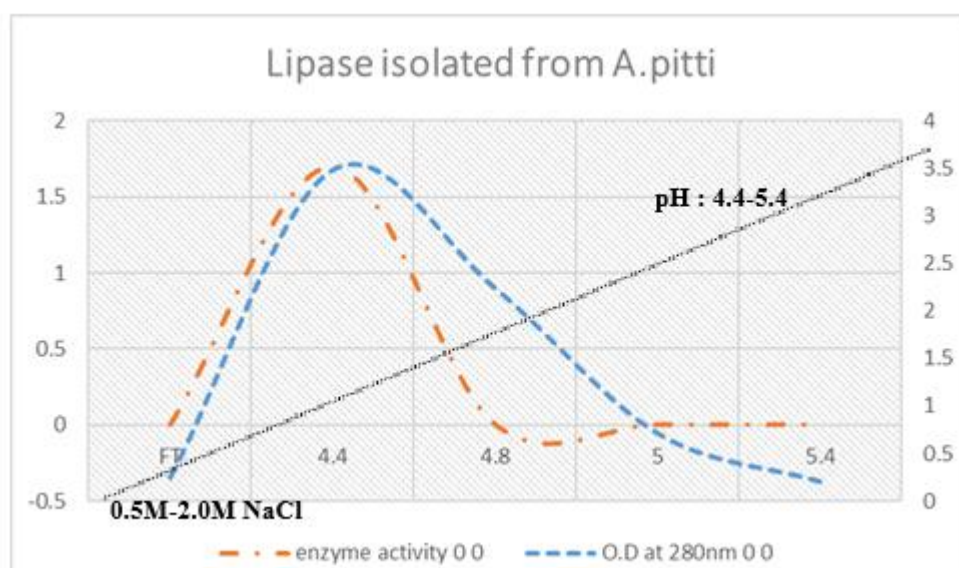


Figure: 9: A characterisation graph plotted with fractions and flow through of lipase sample of *A. pittii* collected from ion exchange column chromatography (CM- Cellulose) with pH gradient ranging from 4.4 – 5.4 and salt concentration gradient ranging from 0.5M NaCl to 2.0M NaCl.

From figure 10 the amount of protein concentration present in test is calculated as 3mg for *E.asburiae* and 4.3 mg for *A.pitti* using standard curve of protein by biuret assay.

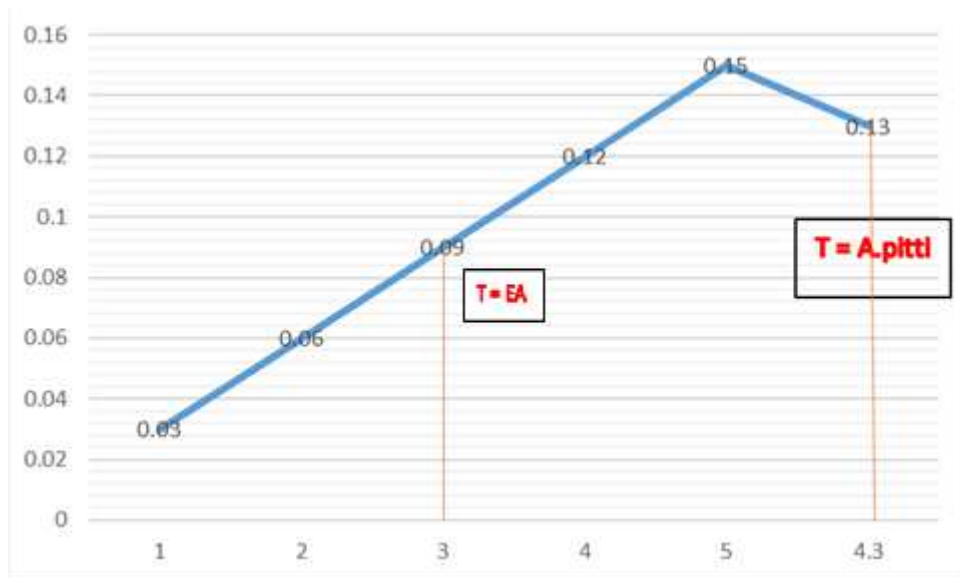


Figure:10 Determination of Protein in *A.pitti* and *E.asburiae* along with lipase activity cut in the standard curve from samples of *A.pitti* and *E.asburiae*

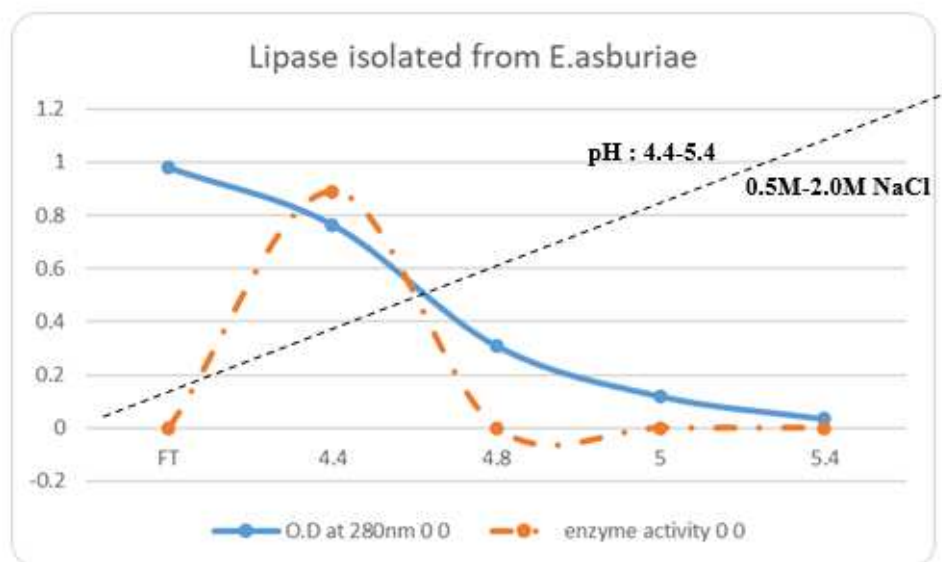


Figure:11 A characterisation graph plotted with fractions and flow through of lipase sample of *E.asburiae* collected from ion exchange column chromatography (CM- Cellulose) with pH gradient ranging from 4.4 – 5.4 and salt concentration gradient ranging from 0.5M NaCl to 2.0M NaCl.

From the figure 12 lipase isolated from *A. pitti* and *E.asburiae* is pure and does not find to contain any impurities. From the figures 13,14&15 through 16s rRNA sequencing the isolated organism is identified and confirmed to be *Enterobacter asburiae* where as from figure 16,17 &18 the organism isolated from induced oil environment is identified and confirmed as *Acenitobacter pitti*.

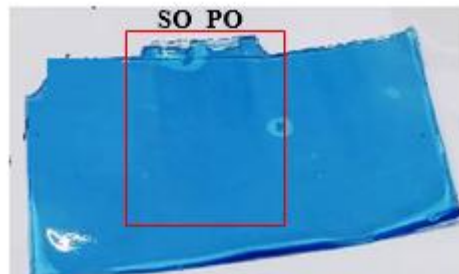


Figure:12 SDS PAGE of samples separated by column chromatography; SO: *A. pitti*, PO: *E.asburiae*

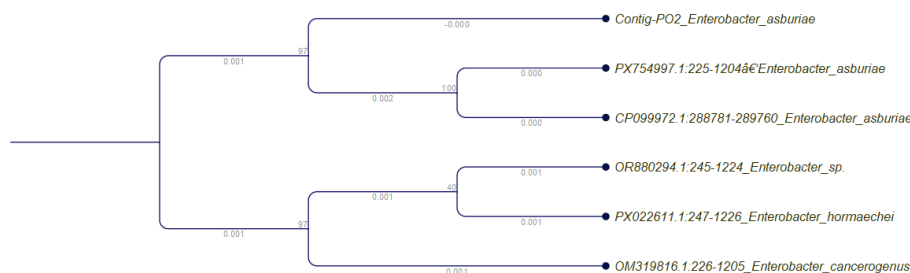


Figure:13 Phylogenetic tree of *Enterobacter asburiae* collected from induced palm oil environment in the lab.

GGATAACTAGTGGAAAGGGTCCCTAATACCGCACCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAGTGAAGACACGG
TCCAGACTCCTACGGGAGGCAGCAGTGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAA
GGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGTGCGATTGACGTTACCCGCAGAAGA
AGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCAC
GCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAAGTGCATTGCGAAACTGGCAGGCTAGAGTCTTGTA
GAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACA
AAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCTGGTAGTCCACGCCGTAAACGATGTCGACT
TGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAA
CTCAAATGAATTGACGGGGGCCCCGACAAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTT
GACATCCAGAGAACTTAGCAGAGATGGATTGGTGCCTTCGGGAACCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT
TGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTTCGGCCGGGAAGTCAAAGGAGAC
TGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATG
GCGCATACAAAGAGAAGCGACCCACTTCCCAACTGATCGTCTTTCTTA.

Figure:14 : Sequence of 16s rRNA of *Enterobacter asburiae* and the data is deposited in the NCBI database. [NCBI Gen Bank accession id: PZ167599]

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Enterobacter asburiae strain CMFR/FAS-01 16S ribosomal RNA gene, partial sequence	Enterobacter asburiae	1810	1810	94%	0.0	100.00%	1375	MK713758.1
Enterobacter hormaechei strain LMG 27195 16S ribosomal RNA gene, partial sequence	Enterobacter hormaechei	1810	1810	94%	0.0	100.00%	1410	MW228829.1
Bacterium strain B07 16S ribosomal RNA gene, partial sequence	bacterium	1810	1810	94%	0.0	100.00%	1412	OR905718.1
Bacterium strain B08 16S ribosomal RNA gene, partial sequence	bacterium	1810	1810	94%	0.0	100.00%	1412	OR905719.1
Bacterium strain B06 16S ribosomal RNA gene, partial sequence	bacterium	1810	1810	94%	0.0	100.00%	1407	OR905717.1

Figure: 15 NCBI – BLAST assembly of the sequenced sample : Enterobacter asburiae

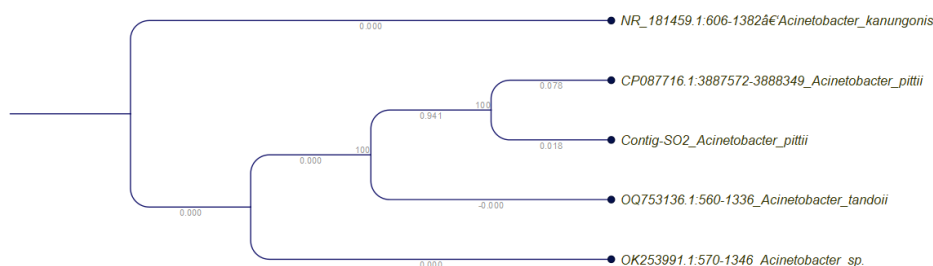


Figure: 16 Phylogenetic tree analysis of A.pitti

AAGGACCCGGTGAACGTATTCACCGTGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGTCGAGTTGCAGACTC
CAATCCGGACTACGATCGACTTTATCATATTAGCTTGCTCTCGCGAGGTCGCTTCCCTTTGTATCTACCATTGTAGCAGCTGTGTA
GCCCTACTCAAAAGGGACATGATGACTTGACGTCATCCCCACCTTCTCCGGTTTGTACACCGGCAGTCTCCTTAGAGTGCCCCGA
CATTACGAGCTGGAACAAAGGATAAGGTTTGCGCTCGTTGCGACTACTTAACCCAACATCTCAAGACACGATCTGACGACAG
CCATGCAGCACCTGTTTCAAAGTTCCCGAAGGCACCCATCTATCTCTGGAAGTTCTCTGTATGTCAAGAGCAGGTAAGGTTCT
TCGCGTTGCATCGAATTAACACATGCTCCACCGCTTGTCGCGCCCCCGTCAATTCAATTGAGTTTCAGCCTTGCGACCGTA
CTCCCCAGGCGGACTACTTATCGCGTTAGCTGCAGAACTAAAGGCTCAAGAGCCCAAACGACATAGTACACATCGTTTACGGC
CTGGACTACCAGGGTATCTAATCCTGTTTGTCTCCCATGCTTTCGCACCTGAGCGTCAGTGTTAGGCCAGATGGCTGCCTTCGC
CACTGTATTCTCCAAATCTCTACGCATTTACCGCTACACCTGGCAATTCCACCTCTCCTTTCCAGCACTCTAGCTACCCAGTT
TCCAATGAACCTCCAGGTTAAGCTC .

Figure:17: Sequence of 16srRNA of A. pitti and the data deposition of the sequence in public repository database NCBI. [NCBI Gen bank accession number: PZ167600].

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Acinetobacter pitti strain AP2044 chromosome, complete genome	Acinetobacter pitti	1042	5299	100%	0.0	90.83%	3921810	CP087716.1
Acinetobacter sp. SE63 16S ribosomal RNA gene, partial sequence	Acinetobacter sp. SE63	983	983	100%	0.0	89.41%	1389	KU353552.1
Acinetobacter sp. strain PS-1 16S ribosomal RNA gene, partial sequence	Acinetobacter sp.	976	976	100%	0.0	89.29%	1422	MK165130.1
Acinetobacter sp. strain D5 16S ribosomal RNA gene, partial sequence	Acinetobacter sp.	976	976	100%	0.0	89.29%	1433	OM959357.1
Acinetobacter sp. YA2 16S ribosomal RNA gene, partial sequence	Acinetobacter sp. YA2	976	976	100%	0.0	89.29%	1385	KU353555.1
Acinetobacter calcoaceticus 16S ribosomal RNA gene, partial sequence	Acinetobacter calcoaceticus	976	976	100%	0.0	89.29%	1339	KU097329.1

Figure:18: NCBI-BLAST assembly of the sequence of the strain A.pitti collected from oil mill sample.

Discussion:

The current study demonstrates successful isolation of efficient lipase-producing bacterial strains, namely *Acinetobacter pitti* and *Enterobacter asburiae*, from oil mill waste soil samples and from soils induced with oil-rich environment. The enrichment strategy proved successful isolation of efficient lipolytic strains as the oil-based substrates effectively induces and increases lipase production in the bacterial strains ⁽⁶⁾. This approach aligns with the recent research that isolation of lipase producing strains from oil mill waste contaminated soil can identify potent oil degraders therefore can be used effectively for oil cleaning or bioremediation purposes ⁽¹⁴⁾.

The observed lipase activity confirms the degradation of triglycerides into simpler fatty acids and glycerol and the enzyme production by the bacteria depends on the genetic makeup and adaptability of the bacteria to the oil rich environment to utilise the lipid substrates present in the oil waste ⁽⁶⁾. The induced environment represents the increase in enzyme production there by emphasizing the role of substrate in enhancing the lipase production. These findings correlate with the previous research on lipase that the enzyme is inducible with increase in gene expression of enzyme depending on the substrate availability.

Characterization of the isolates and their biochemical characteristics can help in practical bioremediation applications and bioengineering ^(9,10). *Acinetobacter pitti* and *Enterobacter asburiae* ability to survive and remain active under stressed conditions and their ecological adaptability testing under laboratory conditions remains uncertain. Availability of survivable traits can help in bioremediation as they are potential candidates that can survive and tolerate to varying conditions of metal oxides and pH.

Furthermore, the bioremediation potential can be proved by lipase production and oil degradation there by providing an eco-friendly and cost-effective alternative to conventional treatment methods of industrial applications ^(4,5). Overall, this study emphasizes the importance of selective isolation and enrichment methods of the strains and their characterisation for environmental cleanup there by effectively leading to pollution free environment in future ⁽³⁾.

Although lipase activity is already known reports on its characterization at molecular level and its gene expression including regulatory mechanisms is still under explored in *Acinetobacter pitti* and *Enterobacter asburiae*. Interactions of *Acinetobacter pitti* and *Enterobacter asburiae* with the already present complex microbial communities under natural environments is poorly understood and their insights into real-world performance in mixed consortia during bioremediation processes is still lacking.

Another major gap lies with the scaleup and application orientation in real world as the experiments are confined to the laboratory, with limited pilot-scale lack of insitu validation in actual contaminated sites rather than procuring the samples. Environmental factors including fluctuating temperatures, pH and presence of toxic compounds can influence the microbial efficiency ⁽⁶⁾ and the comprehensive studies evaluating these parameters is still not available up to the benchmark.

Additionally, there is no sufficient research available on the long-term survival, stability and reusability of these strains in natural bioremediation systems. Lack of research on genetic engineering or genetic makeup for the enhancing lipase activity in these strains is another major drawback in scientific research. Addressing these gaps will be crucial and beneficial for translating laboratory findings and practical, large-scale bioremediation solutions in to real time application by using lipase producing strains.

Conclusion:

The present study demonstrates the successful isolation of efficient lipase producing strains namely *Acinetobacter pittii* and *Enterobacter asburiae*, from oil mill waste samples and an induced oil-rich environment ⁽⁷⁾. The selective enrichment technique proved the recovery of effective lipase producing strains with significant enzyme activity. The isolates exhibited potency in degradation of lipid rich substrates confirming their role in successful and efficient degradation of oil contaminants.

Further characterization revealed that the biochemical and physiological characteristics of these strains play an important role in enabling them to work under varying environmental conditions. Their adaptability to work in oil-rich and stress-prone environments provides insights for their suitability for application in real-world bioremediation processes. The study also established that induced conditions may improve lipase production in these strains there by playing a key role in increasing the efficacy of microbial degradation.

Overall, the findings emphasize the critical role of *Acinetobacter pittii* and *Enterobacter asburiae* in bioremediation of oil-contaminated environments and also as promising candidates for cleaning of oil spills in the contaminated regions. This work contributes to the designing strategies for the development of sustainable and ecofriendly environment and management of oil waste while also providing a solution for problems in further research on large-scale application and process optimization ⁽¹³⁾.

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