Nanotechnology Perceptions
ISSN 1660-6795
www.nano-ntp.com

Isolation and Molecular Identification of Lipolytic Bacteria from Oil Contaminated Soil

E Julie, Nishi Ann

Department of Zoology, Providence Women's College, Kozhikode, Kerala.
Email.: juliee@providencecollegecalicut.ac.in

Soil, the upper layer of the Earth's surface, a complex and dynamic ecosystem that supports plant growth, provides water and nutrients, and purifies the air and water. Comprising a mixture of organic matter, minerals, gases, liquids, and organisms, soil is a natural resource that plays a vital role in many ecosystem functions. However, human activities such as land use change, urbanization, deforestation, and intensive agriculture can lead to soil degradation and loss of soil resources. Soil organisms, including bacteria, fungi, and earthworms, contribute to organic matter decomposition, nutrient cycling, and soil structure improvement, but can also compete with plants for nutrients. Soil pollution, caused by industrial activities, agricultural chemicals, and waste disposal, poses a significant threat to human health and the environment. Bioremediation, using microorganisms to break down pollutants, offers a promising solution for soil remediation. This study focuses on the isolation and identification of lipolytic bacteria from oil-contaminated soil, highlighting the importance of these microorganisms in degrading lipids and pollutants.

In this study, the soil sample was examined for the presence of lipase-positive strains using a screening method and strains were identified based on the standard physical, biochemical and molecular tests. The 16S rRNA gene sequence analysis used identified the strains as Acinetobacter juniiand Bacillus tropicus.

Keywords: Lipolytic bacteria, Enzymes, *Acinetobacter junii, Bacillus tropicus.*

1. Introduction

Lipases, also known as triacylglycerol acylhydrolases, are critical enzymes in various industrial applications due to their ability to catalyze important reactions, such as hydrolysis, interesterification, esterification, alcoholysis, and aminolysis. These enzymatic reactions are vital for many industrial processes. Microbial lipases are often regarded as more advantageous than those derived from animals and plants, offering diverse activities, ease of genetic modification, higher availability, rapid production,

and the ability to thrive on inexpensive substrates (Guerrand, 2017; Chandra et al., 2020).

Microbial lipases are important in numerous industries including oleochemicals, detergents, textiles, polymers, flavors, pharmaceuticals, paper and pulp production, cosmetics and biodiesel. They are also used in leather tanning and waste management (Choudhury & Bhunia, 2015). As the demand for lipases grows, particularly in the industrial sector, interest in discovering novel microbial lipase-producing strains is increasing.

Lipolytic microorganisms can degrade lipids such as fats and oils, inhabiting various environments, including soil, water, and animal digestive tracts. This group includes bacteria, yeast, and fungi, all of which produce lipases that catalyze the breakdown of fats into smaller molecules, such as fatty acids and glycerol. These microorganisms are crucial for environmental lipid biodegradation and are employed in industrial applications, including biofuel production, food additives, and cosmetic ingredients. Additionally, lipases are essential in producing fermented foods like cheese, yogurt, and pickles, and they assist in bioremediation efforts by breaking down harmful pollutants such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs).

Research conducted by Sirisha et al. (2010) focused on isolating and optimizing bacterial strains from oil-contaminated soils, identifying bacterial lipases as highly sought-after enzymes in the market. Furthermore, Mobarak- Qamsari et al. (2011) isolated a novel lipase-producing bacterium, Pseudomonas aeruginosa KM110, through 16S rRNA sequencing, confirming it as a new strain capable of tripling lipase production (0.76 U mL^{-1}) under improved conditions.

The majority of commercially available lipases are derived from microbial sources, found in varied habitats such as industrial waste, vegetable oil processing facilities, dairy farms, oil-contaminated soils, oilseeds, decaying organic matter, compost heaps, coal mines, and hot springs (Sztajer et al., 1988; Wang et al., 1995; Kulkarni et al., 2013; Sharma et al., 2014). Microbial lipases have earned significant industrial interest due to their stability, selectivity, and broad substrate specificity (Dutra et al., 2008; Griebeler et al., 2009). Compared to plant and animal enzymes, microbial enzymes are generally more stable and safer, making them preferable for various applications (Hasan et al., 2006).

Factors such as nitrogen sources, pH, temperature, aeration, and inoculum size significantly influence lipase production. In the fields of biotechnology and organic chemistry, lipolytic enzymes are widely applied across diverse industries, including dairy, detergents, fine chemicals, textiles, food processing, pharmaceuticals, surfactant synthesis, polymers, paper manufacturing, cosmetics, leather, and wastewater treatment (Gupta et al., 2004). Optimizing enzyme production often requires adjusting different nutritional and physicochemical factors to achieve the best outcomes.

The metabolic activities of bacteria are significantly impacted by substrate composition. Strategies for enhancing enzyme production involve careful selection and adjustment of various growth conditions, including the use of diverse nutritional

elements and physical factors. The specific culture media used can also affect the efficiency of lipase production (Dhiman & Chapadgaonkar, 2013). Generally, lipases are activated in the presence of lipid sources such as oils, fatty acids, and triacylglycerols. Industrial wastes provide a range of elements that support the growth of specific bacteria. Thus, lipid-rich environments have been strategically chosen for isolating indigenous bacteria with the potential to produce lipases. This study emphasizes the isolation of lipolytic bacteria from oil-contaminated workshop soils, utilizing morphological, biochemical, and phylogenetic analyses for identification.

2. Materials and Methods

Collection of Soil Sample: Soil samples were gathered from the workshop area located in Puthiyapalam, within the Kozhikode District Fig. 01. The sample were collected from a depth of 5-10 cm using a sterile spatula and stored in sterile glass vials following collection, the sample ware immediately transferred to the laboratory for examination and subsequent analysis (Alhamdani et al., 2016).

Figure 01. Sample collection site

Sample Mixing: A 20-gram portion of the collected soil was measured and thoroughly mixed with 90 ml of distilled water in a beaker. This mixture was then incubated for 15 minutes to facilitate bacterial growth.

Isolation of bacteria: The sample mixture was serially diluted up to 10^{-6}dilutions. 100µl of last three dilution was spread on nutrient agar plate (NA), and incubated at 37℃ for up to 24hr. Microbial colonies, which appeared on nutrient agar plates ware isolated and transferred to another NA plate by streak method and kept for incubation (Veerapagu et al, 2013; Sagar et al 2013). This plate used as master plate and isolated colonies were then transferred to nutrient broth for further study.

Isolation of lipase producing bacteria: The isolated colonies were inoculated on Tributyrin agar medium (Tributyrin 0.5ml. Peptone 0.15g, Yeast extract 0.15g, NaCl 0.25g, Agar 1.25g and distilled water 50ml. The pH of the medium was adjusted to 7.0) by spot or streak plate method. The plates were incubated at 37 °C for 24 h. After incubation, the plates were exposed to UV light (350 nm) for determination the lipase activity of the isolated strains (Rabbani et al., 2013).

Morphological and biochemical characterization of isolates: Two colonies with fluorescence [PV3A and PV3B] were sub cultured on NA media and subjected to

various tests to identify the bacterial strain. According to the Bergey's manual of systemic bacteriology, these tests were selected and were performed in triplicate (Claus & Berkeley, 1986). The tests included: Gram staining reaction, spore position and shape, and the biochemical test such as IMViC test, oxidase, catalase, starch test. The cell morphology was examined by light microscopy and biochemical characteristics were investigated at 37 °C (Rabbani et al., 2013).

Characterization and molecular identification of bacteria: The genomic DNA isolation of two isolated lipolytic bacterial strainsPV3A and PV3B was done by the ORIGIN genomic DNA isolation Kit as per the manufacturer's instruction. 16S rRNA gene of isolated strains was amplified. Agarose gel electrophoresis is used for the conformation of the presence of DNA.

PCR amplification of 16s rRNA the isolates: About 2ng of isolated bacterial DNA was amplified for 16s rRNA genes using universal primers targeting the 16S rRNA gene, specifically employing forward primer 27F (5'-GAGTTTGATCCTGGCTCAG-3') and reverse primer 1429R (5'-GAATTACCGCGGCGCGGCG-3´). The PCR reaction mixture consisted of 2ng of 1µl genomic DNA, 1µl each forward and reverse primer at a concentration of 10µM, 2µl dNTPs (2Mm), 10µl of 10X reaction buffer, 1µl *Taq* polymerase (5U/µl) and 84µl water. After an initial denaturation at 95°C for 5 minutes, amplification was made through 30cycles, each consisting of a denaturation at 95°C for 10 seconds, annealing at 50°C for 1minutes, extension step at 72°C for 45 seconds and a final extension at 72°C for 3 minutes. The PCR product was resolved on a 2% TAE – agarose gel, stained with Ethidium Bromide, for the confirmation of the target gene amplification. Ethidium bromide acts as an intercalating agent on the bases of DNA molecules and imparts an orange colour to DNA under ultraviolet light which can be viewed on the gel (Sambrook & Russell, 2001).

Agarose Gel Electrophoresis: The PCR products were resolved on 2 % TAE agarose gel (Serwer, 1983) stained with EtBr (Sambrook &Russell, 2001) and photographed using a gel documentation system Figure 02. A Gene Ruler (Thermo Scientific; GeneRuler 500bpDNA Ladder. #SM0242) was used to determine the size of the product. EtBr act as an intercalating agent on the bases of DNA molecules and imparts an orange colour to DNA under ultraviolet light.

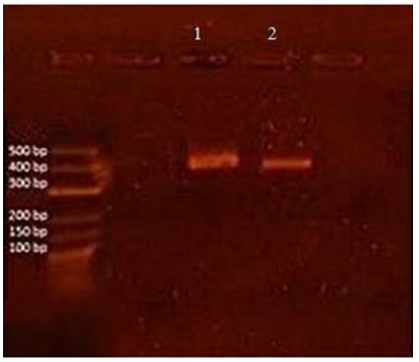

Figure 02. Agarose Gel Electropherogram of 16S rRNA amplicon:

1. *Acinetobacter junii* isolate, 2. *Bacillus tropicus* isolate
Sequencing of PCR product: The purified PCR product was sequenced from both ends using forward and reverse primers by Sanger's dideoxy chain termination sequencing method (Sanger & Coulson, 1975) at Gene Spec Labs Private Ltd., Cochin with ABI 3730XL Automated Sequencer. The forward and reverse sequences were trimmed for the primer sequences and then assembled by using ClustalW and the consensus was taken for the analysis (Thompson et al., 1994).The final sequences were subjected to ncbiBLAST search tool to detect sequence similarity.

3. Result

In this study, the soil sample was examined for the presence of lipase-positive strains using a screening method that is suitable for the detection of lipase producers as described in the methods. The bacterial species were isolated by culturing on nutrient agar. Two bacterial colonies appeared on the plate which was further streaked on a nutrient agar plate to get pure culture. All the bacterial isolates were further processed for lipolytic activity. All the isolated bacterial colonies were screened on tributyrin agar plates for their lipolytic activity. For this, a loopful of each bacterial isolate was separately inoculated on a lipolytic agar plate and incubated at room temperature for about 24 hours. Lipase producer strains were identified by the formation of colonies that showed orange fluorescent colour when exposed to UV light. The step of the study consisted of lipolytic bacteria isolation and identification, based on the standard physical and biochemical test (Table 01). The 16S rRNA gene sequence of isolate PV3A showed 100% similarity to *Acinetobacter junii* strain ATCC 17908 isolated from Saudi Arabia and PV3B showed 100% similarity to *Bacillus tropicus* strain MCCC 1A01406 isolated from USA on the basis of these tests as well as the 16S rRNA sequencing technique, the isolates were identified as *Acinetobacter junii* and *Bacillus tropicus.*

Table1: Biochemical and morphological characteristics

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Tests	PV3A	PV3B
Gram staining	Positive	Positive
Shape	Coccobacilli	Rod shape
Motility	Non motile	Motile
Indole	Negative	Negative
Methyl red	Negative	Negative
Citrate	Positive	Positive
Oxidase	Negative	Negative
Catalase	Positive	Positive

4. Discussion

Oily soil provides a good and rich media for lipase producing bacteria. So, it attracts a lot of interest among scientists (Sirisha et al.,2010; Bharathi et al.,2019., Ilesanmi et al., 2020). In the present study, the soil sample were collected from mechanic's workshop at

Puthyapallam of Calicut District, were petrol, diesel and engine oil were spilled. (Dutta &Ray 2009; Ghaima et al.2014). Sample were examined for the presence of lipase-positive strains using a screening method that is suitable for the detection of lipase producers as described in the methods. Lipase producer strains were identified by the formation of colonies that showed orange fluorescent colour when exposed to UV light. Lipase producing bacteria forms orange fluorescent halos around their colonies under UV light. (Ameri et al., 2015; Mohammed, 2013).

Lipase enzymes are the most important industrial enzymes, accounting for a major volume of total worldwide enzyme sales. It is produced by animals, plants, and microorganisms. Microbial lipases have gained special industrial attention due to their stability, selectivity, and broad substrate specificity. Many microorganisms are known as potential producers of extracellular lipases, including bacteria, yeast, and fungi (Chandra et al., 2020).

The industrial demand for new sources of lipases with different catalytic characteristics was stimulated the isolation and selection of new lipase-producing strains, lipases producing bacteria are distributed in diverse habitats in soils, water and plants in the fields, these organisms are occupy different locations as vegetable oil processing factories (Veerapagu et al., 2013), industrial wastes (Sirisha et al., 2010), dairies, soil contaminated with oil decaying food, oilseeds, compost heaps, coal tips, and hot springs (Wang etal.,1995; Sharma et al., 2001). The study of (Sagar et al.,2013) was suggests that waste contaminated sites such as dumped with kitchen wastes, which are usually comprised of numerous lipid leftovers from processes of cooking and non-cooking, can be serve as excellent breeding grounds for the isolation of lipolytic bacteria of industrial significance.

According to (Sarkaret al., 1998) Tributyrin is considered the most suitable material for the Screening of lipase-producing microorganisms in the present study it was taken into account for screening. Screening and selection of a particular isolate, is a tedious task, especially when commercially competent enzyme yields are to be achieved. It has been reported that Microorganism provide high yields of lipase when organic nitrogen sources are used (Fogarty et al., 2012).

The study consisted of lipolytic bacteria isolation and identification based on the standard physical and biochemical test. On the basis of these tests as well as the 16S rRNA sequencing technique, the isolates were identified as *Acinetobacter junii* and *Bacillus tropicus.* The isolates showed 100% similarity. In the present study, isolates belonging to Bacillus species were identified as lipase producers. This is consistent with various studies, which mentioned that bacterial *Bacillus* species are the prominent source of lipases enzyme (Patel &Shah 2020). The earlier results confirmed that the two strains were potent to produce lipase and also indicate that lipolytic bacteria are widespread in the oil- contaminated environments (Yoon et al.,2004).

The soil is a reservoir of a huge and diverse microbial population, which is considered a rich source of many types of microbial strains which can afford a particular group of microbial strains necessary for the degradation of different contaminants thrown into the soil. Hence the soil samples maybe used to isolate the novel strains that can be used as a part of the microbial collection for the production of lipase at research labs and industries.

Lipase-producing bacteria have a biotechnological and ecological importance that attract lots of scientists to find new and more efficient strains. Oil and fuel contaminated soil is a good source for the isolation of lipase-producing bacteria.

Utilization of enzymes synthesized from biological organism for industrial application is gaining global attention due to their numerous industrial and biotechnology applications. Increased enzyme production from biological sources can be achieved by culture media optimization.

5. Conclusions

The soil is a reservoir of a huge and diverse microbial population, which is considered a rich source of many types of microbial strains which can afford a particular group of microbial strains necessary for the degradation of different contaminants thrown into the soil. Hence the soil samples may be used to isolate the novel strains that can be used as a part of the microbial collection for the production of lipase at research labs and industries. All the bacterial isolates as *Acinetobacter junii* and *Bacillus tropicus* were isolated during the survey from oil contaminant soil. The isolates exhibited the lipase producing activity. So, the using of 16SrDNA sequence database provides excellent identification at the species levels and it can lead to the recognition of novel species of bacteria.

Acknowledgement

Authors would like to acknowledge the service rendered by Amplicon BioLabs on bacterial screening, molecular study and manipulation of sequencing data.

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