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# Characterization of Lipase Producing *Ochrobactrum ciceri* SW3 Collected from Untapped Oil Contaminated Soil

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## ABSTRACT

The study aimed to explore the industrially important oil-based substrates degrading bacteria from untapped oil-contaminated soils, especially bacteria exhibiting potential for bioremediation. A total of 25 samples from five groups of unexplored oil-contaminated soil sites (workshop, oil exchange, truck stand, hotels, and generator soil site) were first collected in Faisalabad to investigate. Tween20 (1%) agar plates were used to isolate and screen lipid substrates utilizing bacteria. Total colony-forming units (8.26×107) and the capacity of lipid substrates utilization by bacteria were determined through several lipase zones (434) found in 25 soil samples. Maximum CFUs (1.96×107) and lipase zones (124) around colonies appeared in workshop soil. Bacterial isolate SW3, with largest lipase zone showed maximum growths at optimum pH 6 (4.541±0.06, OD) and lipase activity (11.66±0.3LU/mL), while growth (4.48±0.08, OD), lipase activity (10.58±0.53LU/mL) at optimum temperature 37˚C, was calculated in the presence of 1% olive oil after 48 h of incubation respectively. SW3 showed resistance up to 5% NaCl concentration. The truck stand contaminated soil site was slightly more diverse in lipase-producing bacteria (1.54) compared to the other four sites by Shannon diversity index (H') analysis. All results were statistically analyzed through ANOVA. Isolate SW3 showed 99% sequence similarity to *Ochrobactrum ciceri* through 16S rRNA gene analysis. This study is an effort to report the initial screening of indigenous bacteria with degradation potential, especially the SW3 isolate, which works under a wide range of environmental conditions and may be considered in the future to reduce environmental issues.

**Keywords:** Oil-contaminated soil; microbial enzymes; Ochrobactrum ciceri; optimization; Bioremediation

## INTRODUCTION

Lipids are present in our environment, including soil, water, and air, and negatively impact ecosystems and living organisms. Agricultural runoff, industrial discharges without any proper treatment, and atmospheric deposition are the sources of lipid contamination. Now, there is a question of how contaminated soil and the potential of indigenous microbial flora of various contaminated soils can be exploited to address environmental and industrial problems. Contaminated soil harbors plenty of beneficial microbes, these microorganisms degrade contaminated materials of the soil through metabolic mechanisms by using their enzymatic machinery. Microbial enzymes are biological catalysts that help the exchange of contaminated substrates into less contaminated products or can completely degrade into simple inorganic compounds, providing constructive conditions that lower the energy needed to activate the reaction. Microbial enzymes are preferable in comparison to plant and animal-based enzymes because microbial enzymes have great diversity and are easily manipulated at the genetic level and grow rapidly on inexpensive media, high yield possibility, continuous production due to absence of seasonal variations (El-Batal et al., 2016; Gonzalez Henao and Ghneim-Herrera, 2021; Meyer et al., 2023; Munawar et al., 2023).

Microbes that are producing different types of enzymes like lipases, amylases, proteases, and phosphatases etc., are *Acinetobacter baylyi, Aspergillus oryzea, Burkholderia multivorans, Staphylococcus auricularis, Rhodococcus cercidiphylli, Penicillium expansum, Pseudomonas aeruginosa* etc., from various environments. Microorganisms that produce lipase have been present a variety of habitats such as industrial wastes, dairies, soil contaminated with oil, fruit, household, industrial wastes, decaying food, and vegetable oil processing factories (El-Batal et al., 2016; Javed et al., 2018; Munawar et al., 2023).

Bacterial lipases depend on different factors (nutrients, temperature, pH, dissolved oxygen concentration, nitrogen, carbon, inorganic salts, agitation speed, etc. Most of the lipase-producing bacteria are basic, acidic, and neutral (Kumar et al., 2023; Oliveira et al., 2016). Lipid-based substrates such as various oils or inducers, such as olive oil, Tween20, Tween80, glycerol, bile salts etc. However, nitrogen and carbon sources, essential micronutrients, and ions are proven best for the growth and production of lipase-producing microorganisms (Hasan et al., 2018).

Microbial lipases have high biotechnological applications. Lipase breaks triglycerides into glycerol and fatty acids (Adetunji and Olaniran, 2021). Microbial lipases have been added to detergents as a major part, because they can remove fatty stains in laundry, dishwashers etc. (Haniya Mazhar et al., 2018; Ohadi et al., 2017). Indigenous microorganisms-based technology is one such important technology, and these organisms inhabit the soil with the abilities of biodegradation, bioleaching, biocomposting, nitrogen fixation, and improving soil fertility (Salwoom et al., 2019; Ulwiyyah et al., 2019). The oil-degrading fungi with bioremediation potential have also been isolated from petroleum-contaminated soil from Iran (Sadeghian and Mohammadi-Sichani, 2023). Different types of *Aspergillus sp*., with insecticide degradation and enzymatic potential from soil samples, were reported by (Mohapatra et al., 2024).
Due to amazing natural potential of bacteria for lipids degradation and speedy involvement in bioremediation and other industrial applications of lipase producing bacteria from contaminated soil (Yao et al., 2021), hence current work was design to explore new bacterial species, to investigate whether bacteria residing for long time in contaminated soil have developed enzymatic potential to degrade various contaminants and to determine that enzymatic specially lipolytic potential of these local bacteria to utilize or degrade lipid substrates from different oil contaminated sites in Faisalabad under wide range of growth conditions.

## MATERIALS AND METHODS

### Sample collection

For sample collection, five groups of contaminated soils sites (workshop, truck stand, hotel, generator and oil exchange) from Faisalabad were developed by collecting soil samples from five different types of workshop soil sites, five different truck stands, five hotels, five generators and five oil-exchange contaminated soil sites were placed in total five group as mentioned above. Furthermore, these all-contaminated sites have been selected for the first time to investigate microbiological research. In this study, only 25 soil samples have been mentioned. The collected samples were transported to the Research Lab, Department of Zoology, Government College University, Faisalabad, and were stored at -4 °C and -20 °C for further study.

### Isolation and screening of lipid-utilizing bacteria

Lipid substratum-utilizing bacteria were isolated from oil-contaminated soil samples by applying the standard serial dilution method. Screening of these bacteria was determined by measuring the potential of lipid substrate utilization through qualitative analysis using 1% olive oil and Tween 20 as substrates, and agar plates were used under sterilized conditions. Total colony forming units, morphology, and lipid utilization were measured through lipase zones of hydrolysis after 24 h of incubation at 37 °C, pH 7. Lipids substrates utilization by isolates was further confirmed quantitatively by using broth media containing 2% inoculum and 1% substrates at 150 rpm shaking incubation. The supernatant was separated from bacterial cells after regular intervals of 24 h and was placed at 4 °C for further lipase assay.

### Lipase assay

Lipase activity was calculated by the titrimetric method (Cherry and Crandall Jr, 1932). The reaction mixture containing 1 mL of supernatant (crude enzyme), 1 mL of olive oil, and 2 mL of phosphate buffer (pH 7) was incubated at 37 °C for 60 min. The reaction was stopped, and fatty acids were extracted by the addition of 1.0 mL of acetone and ethanol solution (1:1). The amount of fatty acid liberated was estimated by titrating with 0.05M NaOH until pH 10.5 using phenolphathelin as an indicator. One unit of enzyme activity (i) was defined as the amount of enzyme required to liberate 1 µ/mol of equivalent fatty acid under the standard assay conditions.

$$\text{Amylase activity (U/mL)} = \frac{\mu g \text{ of maltose released}}{\text{Volume of enzyme taken} \times \text{Time of incubation}} \quad \text{(i)}$$

### Optimization of culture conditions

Based on qualitative and quantitative analysis, bacterial isolate SW3 from workshop sample (W-3) was selected for maximum lipolytic product through (SF) fermentation by utilizing different lipids. All growth media were autoclaved at 121 °C, 15psi for 20 min. Various culture conditions pH (6, 7, and 8), NaCl concentration (0.5, 1, 2, 3,4,5,6 and 7%), temperature (25, 37, and 45 °C), substrates (olive oil and Tween20), incubation period (initial time, 24, 48, 72 and 96 h), were optimized for isolate SW3 growth and lipase production. The bacterial isolate SW3 was grown in 1000 mL Erlenmeyer flasks, containing 250 mL basal medium with 1% olive oil and Tween 20 at 150 rpm shaking incubation. Samples were collected after 24, 48, 72, and 96 h of incubation, and bacterial growth was measured at 620 nm, using optical density. The cell-free supernatant had been separated by centrifugation at 10000 rpm for 20 min at 4 °C and was used as the crude lipase for the determination of lipase activity.

### Diversity of bacteria

Shannon diversity index (H') was used to calculate diversity (ii) of lipase-producing bacteria from different oil-contaminated soil samples (Weaver and Shannon, 1949).

$$H' = -\sum_{i=1}^{s}(p_i \ln p_i) \qquad \text{(ii)}$$

### Statistical analysis

Experimental data were statistically studied by using the ANOVA test; results with a probability value less than P 0.05 were considered significant.

### Molecular identification

Bacterial isolate SW3 (PQ013652) was identified through the 16S rRNA gene sequencing method

from Macrogen (Korea). BLAST was used for sequence similarity, ClustalW for multiple sequence alignment, and MEGA X for phylogenetic tree.

## RESULTS AND DISCUSSION

### Lipid-utilizing bacteria

Total colony-forming units ($8.26\times10^7$, CFUs/ml) of lipid-utilizing bacteria were calculated from oil-contaminated soil samples on (1%) Tween 20 agar plates. Among twenty-five oils contaminated soil samples, highest number of bacteria ($6.4\times10^6$) were found in workshop soil sample (W-3), while, ($5.4\times10^6$) in hotel sample (H-1), in oil exchange soil sample (OE-4), ($4.3\times10^6$), ($4.1\times10^6$) in truck stand sample (TS-2) and ($3.8\times10^6$) in generator sample (G-4) bacteria were found. Overall, maximum colony-forming units (CFUs/ml) of bacteria ($1.96\times10^7$) were found in truck stand soil, and the lowest numbers of bacteria ($1.37\times10^7$) were found in oil exchange soil sites (Table 1). The potential of indigenous bacteria from different habitats has been investigated for various industrial purposes (Zhuang et al., 2020) like production of industrially important enzymes (lipases, proteases, amylases etc.), biodegradation of industrial waste, bio-mining, biomedical, heavy metal removal, enhanced oil recovery, food and brewery industry etc. (Aslam et al., 2020; Pambudiono et al., 2018).

### Lipase zones

The highest numbers ($1.24\times10^7$) of lipase zones around various bacterial isolates were found in workshop soil samples. In contrast, in the truck stand ($9.2\times10^6$), in the generator ($8.1\times10^6$), oil exchange ($7.0\times10^6$), and in the hotel ($6.7\times10^6$), lipase zones were found. As concerns the size of zone of hydrolysis, maximum lipase zone (4.9mm) around bacterial isolate was found in workshop soil sample (W-3), while, (3.6mm) in hotel soil sample (H-4), (3.4mm) in oil exchange sample (OE-2), in truck stand (TS-2) and generator (G-1) soil samples, size of lipase zone was (3.2mm), (Table 1), while in Table 2, only soil samples with large lipase zones are shown. In the present study, indigenous bacteria collected from different oil-contaminated sites in Faisalabad showed enzymatic potential for lipases based on the utilization of Tween 20 and olive oil as lipid substrates. The zone of hydrolysis is a suitable method to screen lipase on agar substrate plates. Clear zones were considered as a result of the breakdown of oil substrates due to the release of lipase enzyme from bacterial colonies (Carrazco-Palafox et al., 2018; Ramnath et al., 2017). Similarly, lipase detection on Tween80 agar plates through clear zones of 28.6 to 29.1 mm around the lipase-producing cultures was observed in contaminated soil with cooking oil and engine oil.

### Diversity of bacteria

Diversity of bacteria having lipid utilization potential from different oil-contaminated samples was determined by the Shannon diversity index (H'). It was observed that the truck stand oil-contaminated soil showed slightly more diversity (1.541) in terms of lipase-producing bacteria as compared to other soil samples (Table 1). Substrates bind to specific sites of the enzyme and influence growth and enzyme activity. Among various lipase substrates, olive oil has been reported as the most suitable and cost-effective substrate for lipase production (Ali AA et al., 2022; Jacob et al., 2022; Munawar et al., 2023; Salwoom et al., 2019).

**Table 1:** Total colony-forming units (CFUs), lipase zones, and diversity of lipase-producing bacteria in oil-contaminated soil samples

| Oil-contaminated soil sites | Total CFUs/mL | Total lipase zones | Shannon diversity (H') = -(pi*Inpi) |
|---|---|---|---|
| Workshop soil | 1.96 | 124 | 1.44 |
| Truck stand soil | 1.87 | 92 | 1.54 |
| Hotel soil | 1.56 | 67 | 1.48 |
| Generator soil | 1.50 | 81 | 1.47 |
| Oil exchange soil | 1.37 | 70 | 1.45 |
| Total = | $8.26\times10^7$ average= 1.652 | 434 | 7.38 average= 1.476 |

### Selection of SW3 Strain

Among various soil samples, bacterial isolate SW3 from workshop soil sample (W-3) showed maximum

lipid utilization potential through lipase zone (8 mm) after 48 h of incubation on 1%, Tween20 agar plates at 37°C, and was selected for further lipase studies. Substrates bind to specific sites of the enzyme and influence growth and enzyme activity. Among various lipase substrates, olive oil has been reported as the most suitable and cost-effective substrate for lipase production (Ali AA et al., 2022; Jacob et al., 2022; Munawar et al., 2023; Salwoom et al., 2019).

**Table 2:** Analysis of Cross-tabulation among EAT 26 and PSS 10

| Soil sites | Lipolytic potential (Lipase zones, mm) |
|---|---|
| Workshop soil samples | |
| W-1 | 4.0 |
| W-2 | 3.4 |
| W-3 | 8.9 |
| W-4 | 2.3 |
| W-5 | 2.2 |
| Truck stand soil samples | |
| TS-1 | 2.8 |
| TS-2 | 3.2 |
| TS-3 | 2.4 |
| TS-4 | 1.4 |
| TS-5 | 2.3 |
| Hotel soil samples | |
| H-1 | 3.4 |
| H-2 | 3.3 |
| H-3 | 2.2 |
| H-4 | 3.6 |
| H-5 | 1.8 |
| Generator soil samples | |
| G-1 | 3.2 |
| G-2 | 2.4 |
| G-3 | 1.3 |
| G-4 | 1.2 |
| G-5 | 1.4 |
| Oil exchange soil samples | |
| OE-1 | 2.1 |
| OE-2 | 3.4 |
| OE-3 | 2.6 |
| OE-4 | 3.2 |
| OE-5 | 3.1 |

## Optimization of Culture Conditions

### Effect of pH

Maximum isolate SW3 growth (4.541±0.06, O.D) and lipid utilization through lipase production (11.66±0.3 LU/ml) were observed at pH 6 at 37°C after 48 h of incubation in the presence of olive oil substrate as compared to Tween20 substrate. However, strain SW3 also showed good growth and lipase activity at pH 7 and 8 (Figs. 1a and 1b, 2a, 2b). Various physicochemical incubation conditions, like pH and temperature, and substrates play a vital role in lipase enzyme production from a variety of bacteria. Lipase production from different types of bacteria *Bacillus, Pseudomonas* sp., *Staphylococcus* sp. etc., at various ranges of pH, temperature, and substrates has been reported by different researchers (Ahmad et al., 2019; Behera et al., 2019). pH is involved in the ionization and deionization of acidic and basic amino acid groups in the active centre of the enzyme structure (Munawar et al., 2023; Ullah et al., 2018).

### Effect of substrates

The maximum growth (optical density at 620nm) and lipids utilization activity of isolate (SW3) were determined (4.54±0.06 and 11.66±0.03 LU/mL) at optimum pH (6) and temperature (37°C) when 1% olive oil was used as substrate. However, growth and lipase activity of isolate (SW3) were observed (3.91±0.02) and (8.74±0.01 LU/mL) at optimum pH (6) and temperature (37°C) in the presence of 1% Tween20 used as substrate (Fig. 2).

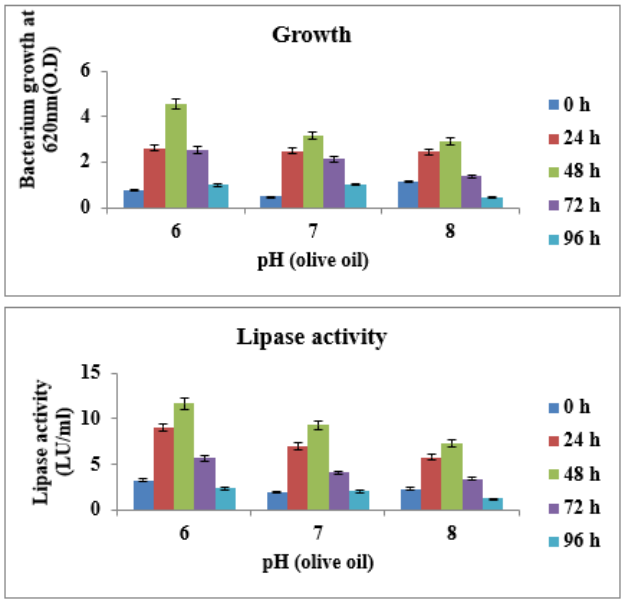


**Figure 1:** Effect of pH on growth and lipolytic activity of isolating SW3 in olive oil substrate

Substrates bind to specific sites of the enzyme and influence growth and enzyme activity. Among various lipase substrates, olive oil has been reported as the most suitable and cost-effective substrate for lipase production (Ali AA et al., 2022; Jacob et al., 2022; Munawar et al., 2023; Salwoom et al., 2019)

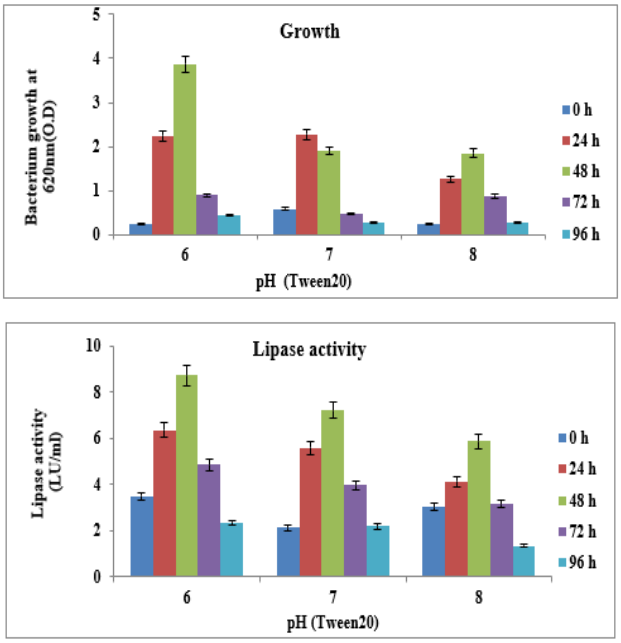


**Figure 2:** Effect of pH on growth and lipolytic activity of isolate SW3 in Tween 20 substrate.

**Effect of temperature**

Maximum bacterial growth (4.541±0.06, optical density) from isolate (SW3) was found at 37°C after 48 hours of incubation using olive oil substrate. But bacterial growth and lipolytic activity were low in Tween 20 as compared to olive oil (3.91±0.02, optical density) and (8.01±1.1LU/mL), respectively (Figs. 3a, 3b, 4a, 4b). High and low temperatures change the kinetic energy of the substrate to the enzyme binding complex (Femi-Ola et al., 2018; Hasan et al., 2018). These results are nearly similar to Bharathi et al. (2019), who reported that eight bacterial strains from petrol-spilled soil were isolated by the serial dilution technique, and maximum lipase production by *Bacillus* sp. at pH 6 at 37, 48 h using olive oil as substrate, while maximum growth was 3.4 by measuring optical density at 610 nm. Lipase activity from isolate AD2, *Staphylococcus* spp, was measured by the Titrimetric method using olive oil as a substrate. Maximum activity of 4.27 U/ml obtained at 37°C for 96 h at 120 rpm and pH 7.0 Lipase production from *Pseudomonas aeruginosa* from soil samples using the culture 1% of corn oil, pH 7, at a temperature of 37 °C after period of 24 h incubation at 151 rpm has been reported from Baghdad (Ali AA et al., 2022; Patel and Desai, 2018)

**Effect of incubation time on bacterial growth and lipase activity**

Both growth and lipase production of bacterial isolate (SW3) were gradually increased (0.78±0.05, 2.61±0.05, and 4.541±0.06) and (3.27±0.1, 9.05±0.1, and 11.66±0.3 LU/ml) from initial hours to forty-eight hours of incubation, respectively. But growth and enzyme production declined (2.53±013 and 1.00±0.11) and (5.69±0.2 and 2.37±0.2) after 72 and 96 h of incubation in the presence of olive oil and Tween 20. (Jacob et al., 2022) studied different conditions and reported bacterial species *Proteus* sp., *Corynebacterium* sp., *Rhodococcus* sp., *Pseudomonas* sp., *Streptococcus* sp., and *Bacillus* sp., with oil-degradation potential from petroleum oil-contaminated areas.

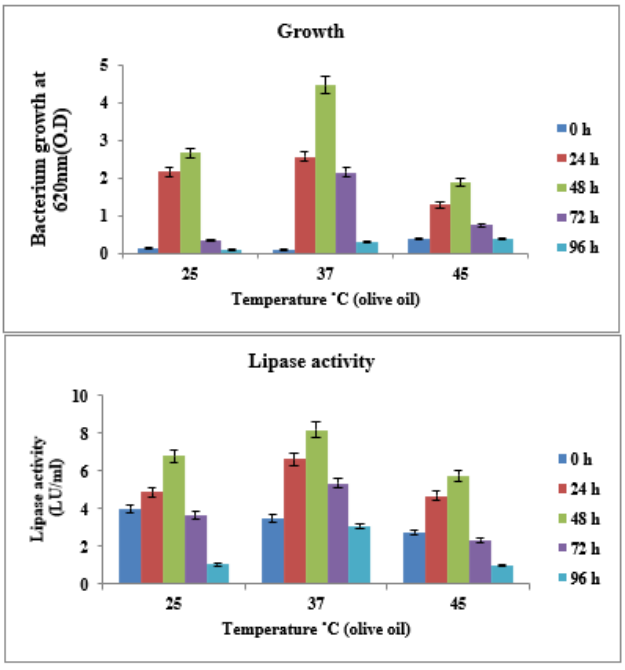


**Figure 3:** Effect of temperature on growth and lipolytic activity of isolate SW3 in olive oil substrate.

The optimum pH and temperature were 7 and 37°C for the strain *Pseudomonas* sp., on 1% olive oil in

comparison to other substrates, coconut oil, sunflower oil, and mustard oil, and *Bacillus safensis* (Patel and Parikh, 2022).

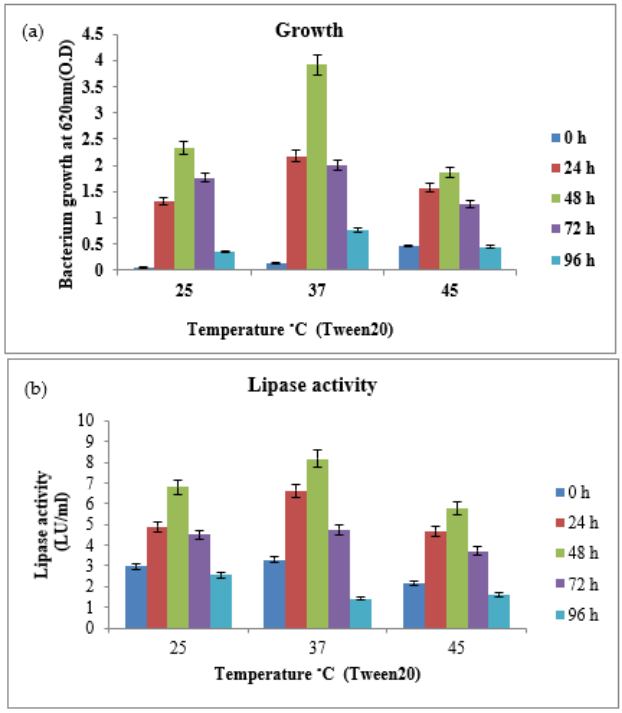


**Figure 4:** Effect of temperature on growth and lipolytic activity of isolate SW3 in Tween 20 substrate.

## NaCl resistance

Bacterial isolate SW3 showed resistance against NaCl because the *Ochrobactrum ciceri* SW3 was determined to grow in sodium chloride concentrations from 0.5% up to 7%. The bacterium showed growth upto 5% NaCl concentration, while no growth of SW3 was shown in the presence of 6% and 7% of sodium chloride (Table 3). (Hu et al., 2020) reported that *Ochrobactrum teleogrylli* sp. nov., was grown upto 7% NaCl concentration and the salt tolerance range for the bacterium growth was 0–4.5 % (w/v). Lipase production was indicated by zone (2.3cm) around colony of *Bacillus halotolerans* (VSH 09) on the (1%) tributyrin agar plates from oil-contaminated soils of Hubballi-Dharwad in Karnataka, India, lipase

**Table 3:** Isolate SW3 resistance to NaCl concentrations.

| NaCl (%) | SW3 growth |
|---|---|
| 0.5 | + |
| 1 | + |
| 2 | + |
| 3 | + |
| 4 | + |
| 5 | + |
| 6 | - |
| 7 | - |

production was achieved at (29.68 ± 0.18 IU/mL) pH 7.0 and (27.28 ± 0.44 IU/mL) 35°C after 48 h of incubation as result of batch submerged fermentation.

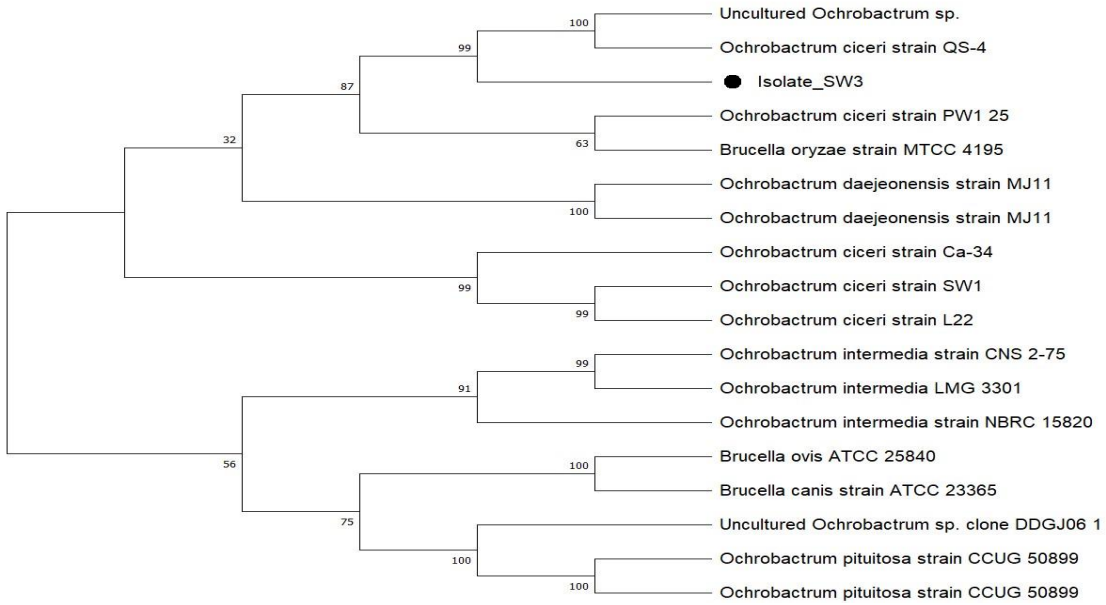


**Figure 5:** Neighbor-joining phylogenetic tree based on comparative 16S rRNA gene sequence analysis and phylogenetic relation of bacterial isolate SW3.

Lipase-producing isolate was identified as *Bacillus halotolerans* on 16S rRNA sequence analysis. The phylogenetic tree was constructed using the Neighbour-joining method (Mahnashi et al., 2022).

**Phylogenetic analysis**

BLAST analysis showed that isolate SW3 (PQ013652), showed 99% similarity to *Ochrobactrum ciceri*, through the *16S rRNA gene*. For phylogenetic relationship of SW3 is shown through the neighbour-joining tree *(*Fig. 5) (Saitou and Nei, 1987) by using a (1000 replicates) bootstrap test, p-distance method (Felsenstein, 1985; Nei and Kumar, 2000) in MEGA X (Kumar et al., 2023). Lipase-producing *Bacillus safensis* isolated and identified by 16S rRNA sequencing from different oil-contaminated industrial areas of Aravalli situated in Gujarat, India (Patel and Parikh, 2022)

This study is the first report about lipid utilization from isolate SW3 that has been identified as *Ochrobactrum ciceri*, *(*Figure 5) collected from oil-contaminated soil in Faisalabad. This species has not been reported before for lipase production. However, other species of this genus have been isolated from different industrial environments, rhizosphere plants, and reported lipase-producing *Ochrobactrum anthropi* from used engine oil contaminated soil sample and *Ochrobactrum intermedium* strain MZV101 with strong oil removal capability from Gheynarje Nir hot spring, Ardebil, Iran by using 16S rRNA technique (Ibrahim, 2018; Saeed et al., 2021; Zarinviarsagh et al., 2017).

**Conclusion**

Present studies conclude that all contaminated soil samples collected from contaminated sites, Faisalabad, exhibited bacterial diversity as well as degradation potential of lipid substrates. One of the bacterial isolates (SW3) from workshop sample number (W-3) showed more substrate utilization by releasing lipases. Maximum lipolytic activity from isolate (SW3) was observed at optimum pH 6 at a temperature of 37˚C after 48 h of incubation by utilizing olive oil as substrate. Furthermore, isolate SW3, as Ochrobactrum ciceri was able to work under different environmental conditions other than optimum. This lipid utilization potential can be enhanced on a large scale for bioremediation by conducting detailed research. It is suggested that untapped oil-contaminated soil sites can play an important role in investigating the new diversity of beneficial bacteria with many unique hidden industrial and environmental potentials. Furthermore, this study can be an encouraging step for other researchers to explore their indigenous untapped areas in terms of bioprospecting, because it is a deep realization during current study that need of comparative study between or among different areas through different researchers can leads this type of basic but powerful research towards solutions at higher industrial scale in terms of bioremediation.

**Acknowledgment**

The authors declare that they have no acknowledgments to disclose.

**Ethical Statement**

Not Applicable

**Conflict of Interest**

Authors declare no conflict of interests.

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