Bioprospecting oil contaminated habitats for efficient lipase producing microorganisms

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Abstract:

Microbial lipases are versatile, high demand enzymes that catalyze hydrolysis, esterification, and transesterification of fats and oils. It catalyzes the hydrolysis of triacylglycerols into free fatty acids and glycerol. In this present study soil samples were collected from oil contaminated sites of garage and food stalls and domestic wastewater in Navsari, Gujarat. From the present study, soil and wastewater samples were collected from oil-contaminated sites including garages, food stalls, and domestic wastewater sources in Navsari, Gujarat. A total of seven bacterial isolates (LB1–LB7) and ten fungal isolates (LF1–LF10) were obtained. All ten fungal isolates were subjected to primary screening for lipase production, among which LF3, LF5, and LF9 were selected for further study based on their lipolytic potential. Among these, isolate LF9 exhibited the highest lipase activity (12.25±0.006 U/mL). Further, various nutritional factors were studied for lipase production by isolate LF9 was studied. Maximum lipase activity was achieved using lactose as the carbon source (13.25±0.005 U/ml), tryptone as the nitrogen source (14.30±0.003 U/ml), and sunflower oil as the substrate (14.33±0.005 U/ml), with a 7% inoculum size (14.58±0.008 U/ml), at pH 5 (11.00±0.005 U/ml). It may be further explored for various applications such as in food processing, bioremediation, and industrial biocatalysis etc.

1. INTRODUCTION

Lipases (triacylglycerol acyl-hydrolases, EC 3.1.1.3) are enzymes that naturally function to break down Triacylglycerol (TAG) into glycerol and free fatty acids, and they also facilitate esterification and transesterification reactions. Lipases are a subclass of esterases and have long chain triacyl-glycerols, which is very low soluble in water and the reaction is catalyzed at lipid-water interface (Aulakh & Prakash, 2010). Lipases play a critical role in the food, detergent, chemical, and pharmaceutical industries (Chandra et al., 2020). Fungus lipases have drawn a lot of interest from the industry due to their stability and substrate selectivity under a range of chemical and physical conditions. Numerous microorganisms, such as fungus, yeast, and bacteria, release lipases. Lipase-producing microorganisms have been discovered in a variety of environment, including industrial waste, plant oil processing plants, dairy farms, and oil-contaminated soil (Kumar et al., 2023).The purpose of the present study is to isolate lipase producing microorganisms from various oil contaminated sites soil and water samples and increase their lipase producing efficiency by characterizing the production medium.

2. MATERIALS AND METHODS

2.1 Sample collection

Samples were collected from three different locations around Navsari, Gujrat. Oil – contaminated sites were selected as potential sources, including garage soil (S1), Domestic wastewater (S2) and street food stall soil (S3) samples.

2.2 Isolation of lipolytic microorganisms

1 gm/5ml of Samples like garage soil, domestic wastewater and street food soil were added to 100ml of Luria Bertani Broth and Potato Dextrose Broth. The mixture was shaken for 24 hours at 37 ºC. After Incubation, dilutions in the range of 10-1 to 10-8 were made. Then, the spread plate method was used to distribute the diluted samples on Luria Bertani agar plates and Potato dextrose agar plates. After being inverted, the plates were incubated for 24 to 48 hours at 37 ºC and PDA at room temperature **(Ilesanmi et al., 2020).**

2.3 Screening of Lipolytic Fungi

2.3.1 Primary screening

The primary screening of lipase producing microorganism was carried out using a plate assay method to detect extracellular lipase activity. The isolated fungal cultures were screened using cup borer method and were inoculated with 0.1 ml culture in the tributyrin agar medium (Peptone-5gm, Yeast extract-3gm, Sodium chloride-5gm, Tributyrin (glycerol tributyrate)-10ml, Agar-15gm, pH (7.0±0.2), Distilled water- (1000ml). The plate was incubated at 28-30ºC for 48-72 hours for fungal isolates **(Veerapagu et al., 2013).**

2.3.2 Secondary screening

The secondary screening of lipolytic fungi was done by using p-nitrophenyl acetic acid (pNPA) assay by measuring the release of fatty acids or p-nitrophenol. Secondary screening of the potential lipase-producing isolates obtained from primary screening was carried out to confirm and compare their lipase-producing efficiency under submerged fermentation conditions. Selected isolates were inoculated into lipase production broth ((NH4)2SO4-1gm, KH2PO4- 5gm, MgSO4-1gm, CaCl2-1gm, Soya peptone-10gm, sucrose-10gm, Groundnut oil-20ml, distilled water-1000ml, pH-7.2) supplemented with lipid substrate (such as groundnut oil) and incubated at room temperature for 48-72 hours **(Hombalimath, 2021).**

2.4 Lipase assay

Lipase enzyme activity was determined by screening isolates with a higher zone for substrate consumption and inoculating them into lipase fermentation medium, as described by Winker and Stuckman (1979). After 24 hours of incubation, 5 ml culture was withdrawn and was centrifuged at 6000 rpm for 20 minutes. Supernatant was used further for determination of

extracellular enzyme. Enzyme determination was carried using p-nitrophenyl acetate (pNPA). 0.1ml supernatant was considered as an extracellular enzyme. 0.8ml Tris buffer was added to the reaction mixture, 0.1ml pNPA (p-nitrophenyl acetate) as a substrate and was kept in water bath at 60 ºC for 20 minutes. After incubation 0.25ml of Na2CO3 was added in the reaction mixture to stop the reaction between the enzyme and substrate. Further this reaction mixture was centrifuged at 6000 rpm for 10 minutes. Yellow obtained colour product was used to determine lipase colorimetrically at 410 nm **(Intwala & Barot, 2022).**

2.5 Characterization of isolate (LF9)

The fungal isolate (LF9) was physically characterized by looking at its microscopic characteristics using picric acid and colony appearance.

2.6 OFAT (One Factor at a Time) analysis of Lipase

2.6.1 Effect of Different Carbon Source on Enzyme Production

Lipase production was performed with different carbon sources in the production system. The production medium received 48hr cultures of and LF9. The following carbon sources (1%) were utilized: sucrose, lactose, dextrose and maltose. The effects of each type of carbon source on the lipase production were monitored at 24-hour intervals for five days and the cultures from each time point were analyzed for lipase production **(Ghaima et al., 2014).**

2.6.2 Effect of Different Nitrogen Source on Enzyme Production

Enzyme production was carried out using different nitrogen sources based on the literature. Peptone, tryptone, beef extract & casein were used individually at a concentration of 1% (w/v) in the production medium. The medium was inoculated with a 48-hour old culture of isolate LF9 incubated under suitable conditions. Lipase production was checked every 24 hours for up to 5 days by measuring enzyme activity **(Sharma et al., 2001).**

2.6.3 Effect of Different pH on Enzyme Production

The effect of pH of the production medium for lipase production was performed by varying pH of the medium from 5 to 8 whereas the other parameters were unaltered. The medium was inoculated with a 48-hour old culture of isolate LF9 incubated under suitable conditions. Lipase production was checked every 24 hours for up to 5 days by measuring enzyme activity **(Bharathi et al., 2019).**

2.6.4 Effect of Different Substrate on Enzyme Production

Enzyme production was carried out using different substrate based on literature. Olive oil, Peanut oil, Coconut oil & Sunflower oil were used in the production medium. The medium was inoculated with a 48-hour old culture of isolate LF9 incubated under suitable conditions.

Lipase production was checked every 24 hours for up to 5 days by measuring enzyme activity **(Khatape et al., 2015).**

2.6.5 Effect of Different Inoculum Size on Enzyme Production

Enzyme production was carried out using different inoculum size based on literature. 1%, 3%, 5% & 7% inoculum size were used in the production medium. The medium was inoculated with a 48-hour old culture of isolate LF9 incubated under suitable conditions. Lipase production was checked every 24 hours for up to 5 days by measuring enzyme activity **(Veerapagu et al., 2013).**

3. RESULTS AND DISCUSSION

3.1 Sample collection

During study, oil-contaminated soil and water samples were collected from various garages, street food stalls and domestic wastewater located in Navsari district. A total of 3 samples were collected in clean containers from various sites. soil samples were collected from a depth of approximately 5-6 cm in clean zip lock bags, while wastewater samples were collected directly from drainage (**Alhamdani *et al*., 2016) (Chaturvedi *et al*.,2010)**. Total of 17 isolates were obtained from the samples by using serial dilution technique on Luria Bertani agar plates and Potato dextrose agar plates. Out of that 7 were bacterial isolates and 10 were fungal isolates. Primary screening of these fungal isolates was performed on Tributyrin agar plates by cup borer technique **(Patel *et al*.,2018)**.

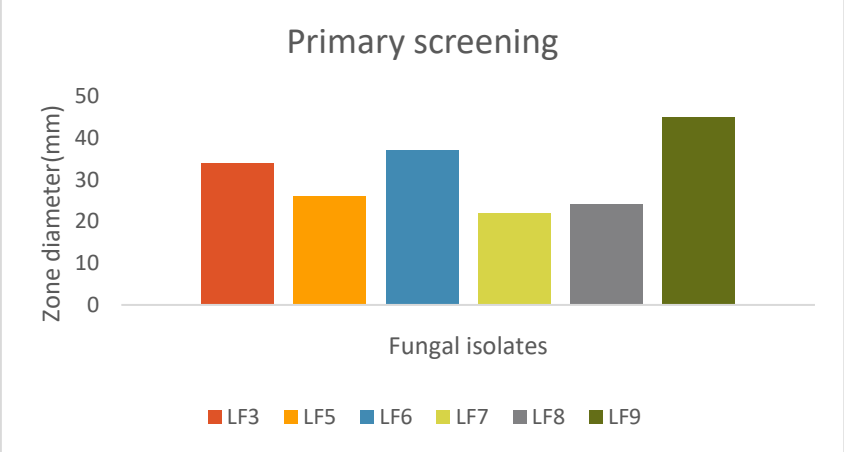

Fig. 1 Zone Diameter on Tributyrin agar by Fungal Isolates

Upon primary screening of these 10 fungal isolates, 6 isolates showed clear zone. Among 10 isolates the highest clear zone was found in isolate LF9-45mm, LF6-37mm, LF3-34mm (Fig.1).

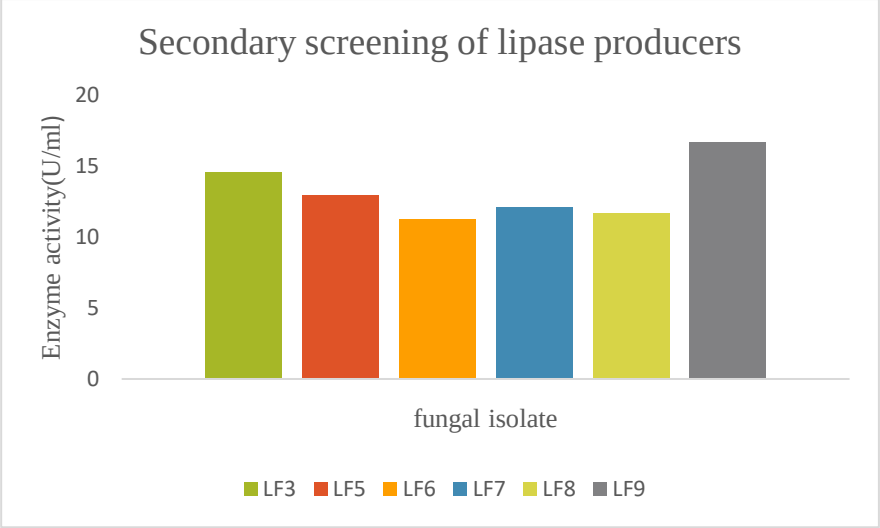

Fig. 2 Secondary screening of lipase producers

And these 6 isolates were further proceeded for the secondary screening. The activity of lipase in the production medium was assayed colorimetrically following the method of **Winkler and Stuckmann (1979).** It is based on the hydrolysis of p-Nitrophenyl acetate(p-NPA) **(Intwala & Barot, 2022).** Selected isolates from primary screening were subjected to this assay. Among the tested strains, LF9 exhibited the highest lipase activity (≈16.7 U/ml), followed by LF3 (≈14.6 U/ml), LF5 (≈12.9 U/ml), LF7 (≈12.1 U/ml), LF8 (≈11.66 U/ml) and LF6 (≈11.25U/ml) **(Fig.2).**

3.2 Result of Morphological study

Table no.1 About here Morphological study

Fungi	**Growth characteristics on PDA**	**Microscopic observation (Fungal mounting)**
LF9	cottony to woolly colonies, initially white, turning greyish brown to blackish brown with age	

The microscopic characteristics of the fungi LF9 were examined using picric acid staining under a light microscope **(Table no. 1).** The isolate showed broad, aseptate hyphae with irregular branching. Sporangiophores bearing globose sporangia filled with sporangiospores were clearly observed. LF9 on Potato Dextrose Agar (PDA) appeared fluffy, white to greyish-brown colony with a cotton-like texture. The presence of these characteristic structures, and morphology suggested that the isolate belongs to the genus ***Rhizopus.***

3.3 OFAT of Lipase using Rhizopus spp:

3.3.1 Effect of Carbon sources

The influence of different carbon sources (1%) (glucose, lactose, sucrose and maltose) On lipase production was investigated using the OFAT (one factor at a time) method over a period of five days.

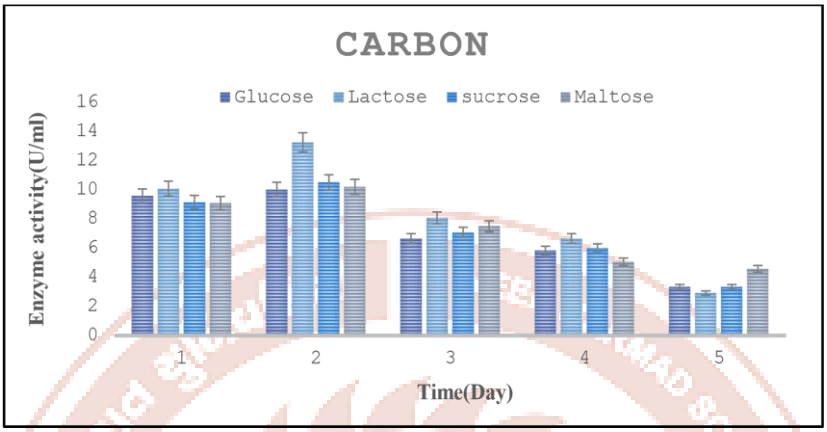

Fig.3 Effect of carbon sources on Lipase activity

The graph **(figure. 2)** shows how different carbon sources (1%) (glucose, lactose, sucrose, maltose) affect enzyme activity (U/ml) over 5 days for Rhizopus. From this graph Rhizopus spp. Giving maximum Lipase activity (13 U/ml) with 1% Lactose as carbon source supplement. In contrast to **Riyadi et al., (2017)** who reported ethanol as an effective carbon source for high lipase production in Rhizopus. This difference suggests that carbon-source preference varies depending on the microorganism and enzyme system, highlighting the need for strain-specific optimization. Similarly, **Bhattacharya et al., (2019)** found mustard oil to be the most suitable carbon source for lipase production by fungi strain from agricultural soil. In another study, **Fatima et al., (2021)** reported amalgam of olive oil cake and sugar cane bagasse as effective carbon source for lipase production in fungal strains. Likewise, **Issa QM (2025)** demonstrated that 2% glucose enhances lipase activity (10.96 U/ml) of Aspergillus niger. Comparison with literature **(Alabdalall et al., 2020)** shows 1% fructose giving high lipase activity by Aspergillus niger. This reinforces the idea that carbon-source efficiency is highly organism-dependent and must be optimized individually for each strain.

3.3.2 Effect of Nitrogen sources:

Lipase production by fungi generally requires slightly higher levels of nitrogen sources compared to those needed to produce other microbial enzymes (Riyadi et al., 2017). The influence of different Nitrogen sources (1%) on lipase production was investigated using Casein, peptone, tryptone and urea as different nitrogen sources by OFAT.

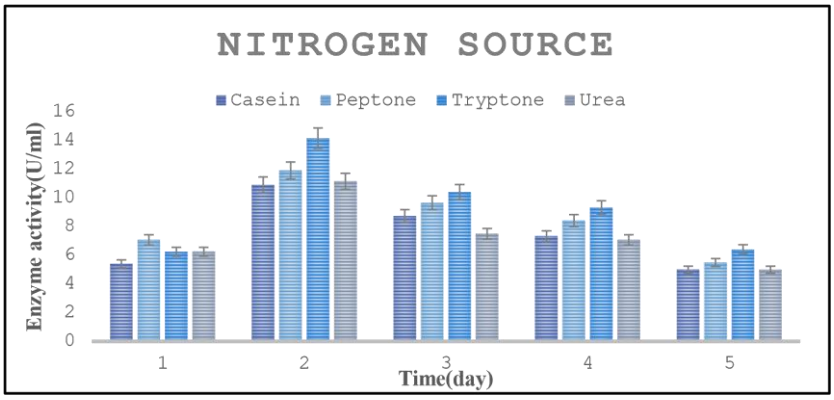

Fig.4 Effect of Nitrogen sources on Lipase activity

Among all nitrogen sources this fungus isolate Rhizopus shows maximum enzyme activity **(14.30±0. 002 U/ml)** on day 2 with Tryptone (1%) as the nitrogen source (Fig.4). Tryptone produces the highest activity on Day 2 and sustains better performance than casein, peptone, or urea, indicating it is the optimal nitrogen supplement for maximizing enzyme production with Rhizopus. While higher lipolytic activity was demonstrated by *Rhizopus spp*. when urea was added as a nitrogen source **(Rodríguez et al., 2006; Mehta et al., 2017).** Compared with other literature by **Salihu et al. (2011)** that shows peptone giving high lipase production by Candida cylindracea. Peptone as an organic nitrogen source also gave high lipase activity **(Issa QM, 2025).** Ammonium sulfate promoted high lipase activity in Penicillium aurantiogriseum **(Lima et al., 2003).** Comparing these references with current study result, tryptone yielded the highest lipase activity (14.30±0.002 U/ml on Day 2) for *Rhizopus*, outperforming the nitrogen sources reported in the cited studies. This may vary with strain or medium composition.

3.3.3 Effect of substrates

The influence of different lipid substrates on lipase production was investigated using sunflower oil (1%), olive oil (1%), peanut oil (1%), and coconut oil (1%) as a substrate in lipase production medium.

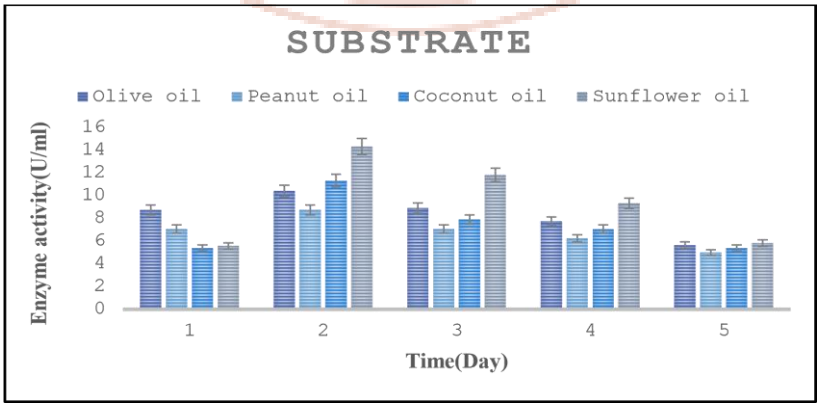

Fig.5 Effect of different substrates on Lipase activity

Among these substrates, sunflower oil showed the highest lipase activity, indicating that it effectively induced lipase production by fungal isolate **(14.33 ±0. 005U/ml)** Rhizopus spp. (Fig.5). Comparing our findings with the literature, **Das et al., (2016)** reported that an Aspergillus tamarii isolate exhibited better lipase activity when coconut oil was used as the substrate, implying that coconut oil can be an effective inducer for certain fungal species. In contrast, **Bhattacharya et al., (2019)** found mustard oil to give maximum lipase activity by fungal strain of agricultural soil, indicating substrate-specific responses among different microorganisms. From this it can be concluded that for this Rhizopus spp. isolate sunflower oil is best for high lipase production.

3.3.4 Effect of inoculum size (%)

Inoculum size is important in determining the productivity of the enzyme. The influence of inoculum size on lipase production was evaluated using 1%, 3%, 5% and 7% inoculum levels for this fungal isolate.

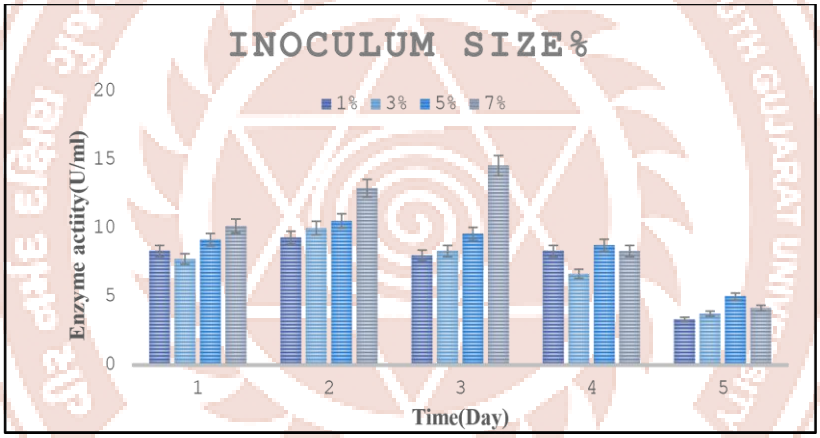

Fig.6 Effect of inoculum size on Lipase activity

The experimental results (Fig.6) shows that the fungal isolate produces the highest lipase activity (14.58 U/ml) with a 7 % inoculum, whereas lower activities are observed at 1 %, 3 % and 5 %, and the peak activity shifts from Day 5 to Day 3 with larger inoculum. In comparison with recent literature, **Ali et al., (2023)** reports a maximum lipase activity at 3 % inoculum, suggesting a narrower optimal range for their strain. **Riyadi et al., (2017)** finds that a smaller 1.5 % inoculum gives high lipase activity in a thermotolerant Rhizopus sp., indicating species-specific inoculum needs. **Bhattacharya et al., (2019)** also note 3 % inoculum of fungal strain from agricultural soil for maximum lipase production, aligning with **Ali et al., 2023** while **Issa QM (2025)** observes high activity with a 4 % inoculum by Aspergillus niger. The variation in optimal inoculum size among these studies (1.5 %–7 %) can be attributed to differences in microbial growth rates, biomass development, and nutrient utilization patterns of the specific fungal strains. Consequently, the 7 % optimum in this isolate (Rhizopus) suggests a

requirement for higher initial biomass to achieve peak enzyme expression, unlike the lower optima reported in the referenced works.

3.3.5 Effect of pH

Each microorganism has its own optimal pH and a specific pH range in which it grows and functions most effectively (Riyadi et al., 2017). The fungal isolate Rhizopus displays maximum lipase activity at pH 5, reaching about 11±0.005 U/ml on day 2.

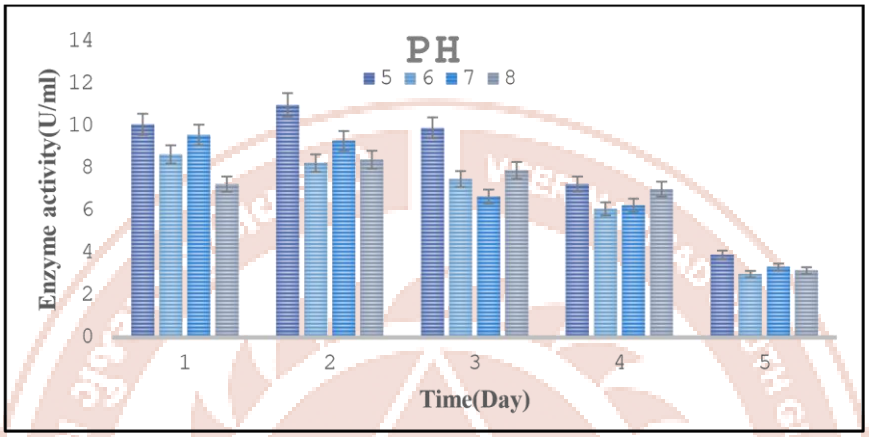

Fig.7 Effect of pH on Lipase activity

The fungal isolate Rhizopus exhibits maximum lipase activity of **11±0.005U/ml** at pH 5, demonstrating that slightly acidic conditions are most suitable for lipase production by this isolate (Fig.7). This finding aligns with previous studies that have reported acidic pH optima for lipase synthesis in various fungal species. Specifically, **Ayinla et al., (2017)** identified pH 5 as optimal for Rhizopus oryzae, **Issa QM (2025)** observed peak activity at pH 6 by Aspergillus niger, **Bindiya & Ramana (2013)** reported pH 5–6 for Aspergillus sydowii, and **Helal et al., (2021)** noted pH 6 as favorable for Rhizopus oryzae. The consistency of these results suggests that maintaining an acidic environment (pH 5–6) is a key factor in maximizing lipase yield for Rhizopus and related fungal isolates, supporting the selection of acidic conditions in fermentation processes for enhanced enzyme production.

4. Conclusion

From the above study it can be concluded that the isolated organism Rhizopus spp Can produce lipase enzyme. Further studies are recommended to enhance the enzyme production using biotechnological approach and statistical approach.

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