OPTIMIZATION OF BIOFILM FORMATION IN FUNGI ISOLATED FROM CRUDE OIL REFINERY SLUDGE FOR ENHANCED BIOREMEDIATION

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ABSTRACT

Biofilm formation enhances the survival of filamentous fungi in hydrocarbon contaminated environments. Optimization of biofilm formation is significant in selecting fungal species with biodegradation potential. In this study, fungal isolates including *Talaromyces verruculosus* SFAJ2, *Rhizopus nigricans*, *Aspergillus aculeatus* SFO13 and *Fusarium chlamydosporum* were assessed for biofilm forming ability under varying culture conditions. The effect of carbon sources (glucose, sucrose, crude oil), pH, temperature and incubation period on biofilm production were evaluated in a microtiter plate assay using one-factor-at-a-time (OFAT) method. The biofilm mass was quantified spectrophotometrically (OD_{560} nm). The results showed that optimal biofilm (OD_{560} = 0.92) formation occurred at 28 °C after 24 h of incubation at pH 5-7, when glucose or sucrose was used as carbon source. Biofilm development in the presence of crude oil as a sole carbon source, was achieved when incubation period was extended, giving biofilm density of OD560 = 0.22. *Aspergillus aculeatus* SFO13 and *Talaromyces verruculosus* SFAJ2 showed higher biofilm-forming capacity at OD_{560} nm 0.22 and 0.15, respectively, in oil-supplemented medium. These findings indicate that optimizing growth conditions can enhance formation of biofilms and crude oil utilization by fungi, thereby supporting the use of these fungi for sustainable bioremediation technologies.

Keywords: Crude oil pollution, fungal biofilm, fungal species, hydrocarbon biodegradation

1. | Introduction

Crude oil refining activities generate substantial volumes of sludge containing complex and highly persistent mixtures of saturated and aromatic hydrocarbons, heavy metals, and surfactants. (Teng *et al*., 2021; Jerez *et al*., 2021). Owing to their toxicity, recalcitrant nature and tendency to accumulate in living organisms, these wastes constitute long-term ecological risks, affecting both soil and fluvial ecosystems (Ali *et al*., 2019; Barik *et al*., 2025; Paramanik and Bal, 2025). Despite these limitations, oil-refinery sludge hosts communities of microorganism with specialized physiological adaptation and stress-tolerant biochemical pathways, which enable them to tolerate, transform, and breakdown hydrocarbons. However, fungi biofilms constitute a promising yet comparatively underexplored group in hydrocarbon biodegradation study. Fungi possess extensive hyphal networks, broad enzyme systems, and ability to colonize hydrophobic surfaces. These potentials give them distinct ecological and functional advantage in accessing and breaking down petroleum compounds (Bokade and Bajaj, 2023). Another important feature is their capacity to form biofilms; structured, communities of microorganisms attached to surfaces and embedded in a self-produced matrix of polysaccharides, proteins, lipids, and other biopolymers (Flemming *et al*., 2016; Das and Kungwani, 2022). Biofilms formed in hydrocarbon-rich environments create protective microenvironments that enhance nutrient retention, concentrate extracellular enzyme, and increase resistance to oxidative and chemical stress (Balan *et al*., 2021). These biofilms also improve substrate accessibility by promoting cell–substrate attachment and accelerate petroleum hydrocarbon biodegradation (Abbasnezhad *et al*., 2011; Zhu *et al*., 2025). Despite their ecological relevance and growing biotechnological significance, the factors that determine biofilm formation in hydrocarbon-adapted fungi remain insufficiently understood. While bacterial biofilms in petroleum contaminated systems have been extensively characterized, fungal biofilms, particularly those isolated from refinery sludge, have not received equivalent scientific attention. Consequently, the cultivation of high-performing fungal biofilms for bioremediation is often constrained by insufficient knowledge of how environmental and nutritional conditions drive their development. Optimizing growth conditions that support biofilm development is therefore essential towards improving biofilm stability and biodegradative competence of these isolates. This study was aimed at examining the key growth parameters that modulate biofilm formation in fungi isolated from crude oil-refinery sludge for potential application in bioremediation of hydrocarbon-polluted environment.

2. | Materials and Methods

2.1 | Sample Collection from Oil Refinery Sludge

Sludge samples were collected as previously reported by Nebo *et al*. (2025) from an oil refinery sludge site in Umutu, Delta State during the rainy and dry seasons.

2.2 | Isolation and Identification of Fungi From Crude Oil Sludge

Fungal populations in the sludge samples were determined according to the method of Obire and Wemedo (2002). A 0.5 mL of 10^{-3} dilution was pour-plated in triplicates onto sterilized potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA). Incubation was carried out at 28 °C for 72 h and the colonies formed were counted and recorded. The fungal isolates were identified based on their morphological features after the spores were stained with lactophenol blue dye and observed under an electron microscope (Cheesbrough, 2006).

2.3 | Cultivation of Fungal Isolates for Biofilm Optimization

Pure cultures of *Talaromyces verruculosus* SFAJ2, *Rhizopus nigricans*, *Aspergillus fumigatus Aspergillus aculeatus* SFO13 and *Fusarium chlamydosporum* were inoculated on PDA slant in a sterile bijou bottle and incubated at 28 °C for 5 days. Conidia were recovered by adding 5 mL of sterile Tween-20 solution (0.1% v/v) to each culture, gently agitating the surface and carefully loosening the spores with a sterile spatula. The spore suspensions were transferred into sterile universal bottle, by sieving through a sterile cheesecloth to eliminate hyphal debris. Conidial densities were subsequently adjusted to desired concentration and preserved in SDA broth at 4 °C until use (Ogundijo and Adetunji, 2017).

2.4 | Optimization of Biofilm Production in Fungal Isolates

Optimization was conducted according to the modified method of Stepanovic *et al.* (2007) and McFall *et al.* (2024). Sterile test tubes containing Brain Heart Infusion (BHI) broth (37 g/L) supplemented with 2% (w/v) glucose were inoculated with spore suspensions. The influence of incubation temperatures (28, 37 and 50 °C), incubation period (24, 48, 72, and 120 h), pH (5, 7, and 9), and carbon source (glucose, sucrose, and crude oil) on fungal biofilm formation was investigated using the one-factor-at-a-time (OFAT) method. Following incubation, the culture medium was carefully removed, and the test-tubes were rinsed thrice to eliminate unbound cells. Two milliliters of crystal violet dye (0.1%) were added to each test tube and subsequently washed three times with distilled water to get rid of the excess dye. Subsequently, acetic acid (30% v/v) was added to each test tube and incubated at 25 °C for 15 minutes. Thereafter, 250 µL from the content of each test tube was transferred to a flat bottom microtiter polystyrene plate (Jabuk and Jarallah, 2021). The optical density of the biofilm mass was measured with Labtech Auto Elisa Plate Reader at OD = 560 nm, while 30% acetic acid served as the blank.

3. | Statistical Analysis

Data was statistically analyzed using SPSS 22 version, and data obtained was analyzed with two-way analysis of variance (ANOVA). Means was compared by Duncan's new multiple range test and considered statistically significant when $p \leq 0.05$.

4. | Results and Discussions

4.1 | Total Heterotrophic Fungal Count in Crude Oil Refinery Sludge Samples

The total heterotrophic population of fungi isolated from crude oil sludge sample during dry and rainy seasons are presented in Tables 1a and 1b. The results indicated a statistically significant effect of season on fungal load ($p < 0.049$). The total fungal counts were significantly higher in dry season (5.33×10^3 SFU/mL) compared to rainy season 3.67×10^3 SFU/mL on PDA while on SDA, the total heterotrophic fungi were 3.6×10^4 SFU/mL and 9.0×10^3 SFU/mL in dry and rainy seasons, respectively. This result is similar with the findings of Nebo *et al.* (2025) where the authors observed higher loads of heterotrophic bacteria in crude oil contaminated sludge in dry season compared to rainy season. The significant increase in fungal load during dry season may be attributed to availability of nutrients and reduced dilution effects. In contrast, increased rainfall dilute or wash away essential nutrients required for fungal proliferation. This observation is consistent with the report of Hawkes *et al.* (2011) and Martínez-García *et al.* (2017) who noted that dilution of contaminants or nutrients may occur as a result of increased rainfall, resulting in nutrient inaccessibility.

Table 1a | Fungal load in crude oil contaminated sludge

Sources of Variation	sum_sq	df	F	P-value
Season	616.3333	1	6.8365	0.0309
Media	972.0011	1	10.7800	0.0111
Season: Media	481.3333	1	5.3383	0.0497
Residual (Error)	721.3333	8		

Table 1b | Mean, standard deviation, and Tukey HSD grouping for fungal load in sludge

Season	Media	Mean±SD	Tukey Group
Dry Season	PDA	5.33±1.53	a
Dry Season	SDA	36.00±18.36	b
Rainy Season	PDA	3.67±1.53	a
Rainy Season	SDA	9.00±4.36	b

Groups with the same alphabetic label are not significantly different, while those with different labels are significantly different.

4.2 | Effect of Incubation Temperature on Biofilm Production by Fungal Isolates from Crude Oil Refinery Sludge

The effect of incubation temperature on biofilm formation by fungal isolates is shown in Figure 1. Most isolates exhibited optimal biofilm production at 28 °C with corresponding biofilm densities (OD_{560}) of 0.92 nm, 0.22 nm, 0.5 nm and 0.61 nm, respectively. This temperature range was consistent with the optimal growth condition of many filamentous fungi and supports maximal metabolic activity (Andrade-Linares *et al*., 2016; Shay *et al*., 2022). Notably, strong biofilms formation was predominantly observed among *Aspergillus* species. This finding agrees with previous report indicating that *Aspergillus* species are known biofilm formers owing to their rapid hyphal extension and efficient surface colonization (Arora *et al*., 2024). Furthermore, significant biofilm formation was observed in *Talaromyces verruculosus* SFAJ2, *Aspergillus* species and *Fusarium chlamydosporum* at elevated temperatures of 37 °C and 50°C. This suggests their ability to tolerate thermal stress, possibly due to prolonged exposure to heavily contaminated harsh environment (Arora *et al*., 2024; Hernández-Benítez *et al*., 2025). In contrast, *Rhizopus nigricans* produced minimal biofilm density across all temperature conditions under study, indicating a weak biofilm-forming ability. However, previous studies by Singh *et al*. (2011) and Harris *et al*. (2018) have reported that *Rhizopus* species can form biofilms under certain conditions.

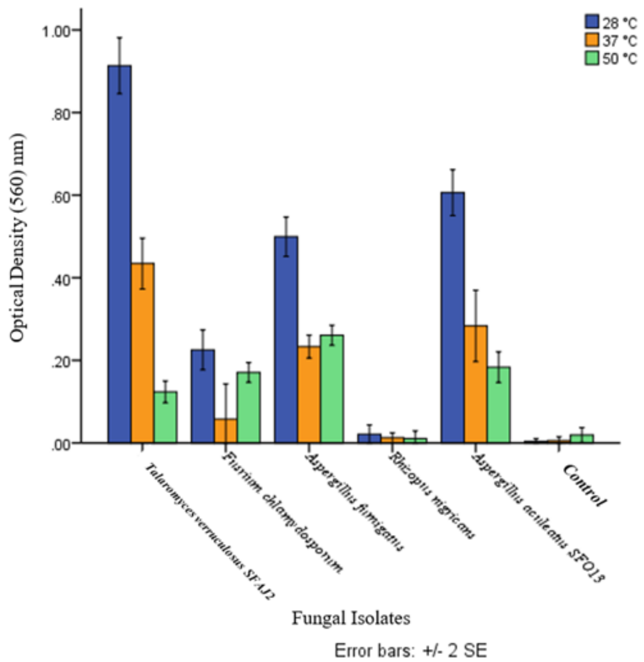

Figure 1 | Effect of temperature on biofilm production by fungal isolates from crude oil refinery sludge

4.3 | Effect of incubation time on biofilm production by fungal isolates from crude oil refinery sludge

The effect of incubation time on biofilm formation by fungal isolates is presented in Figure 2. *Talaromyces verruculosus* SFAJ2, *Aspergillus fumigatus* and *Aspergillus aculeatus* SFO13 reached optimal biofilm formation at 24 h, after which a gradual decline was observed overtime. This pattern suggests that the fungi quickly utilized the available nutrients to produce biofilm early, while subsequent reduction was likely due to nutrient depletion or the aging and weakening of biofilms (Simões *et al*., 2015). These observations corroborate earlier reports (Quresh *et al*., 2005; Wang and Chen, 2009; Siqueira and Lima, 2013). Among the isolates, *Talaromyces verruculosus* SFAJ2 demonstrated the highest biofilm yield (1.26 nm), followed by *Aspergillus aculeatus* SFO13 (1.06 nm). In contrast, *Fusarium chlamydosporum*

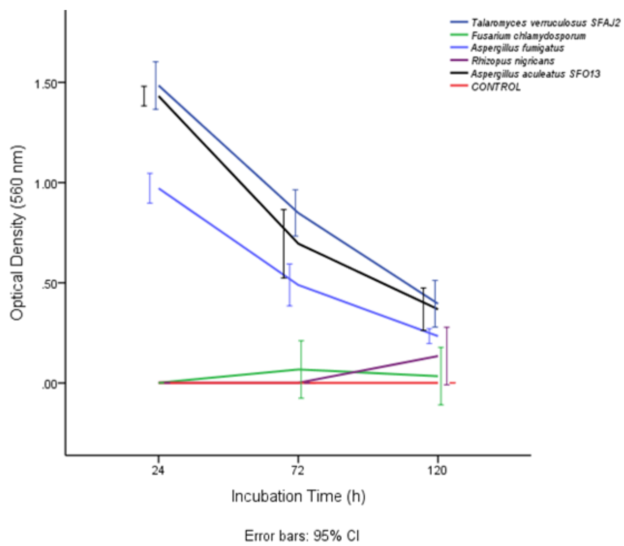

Figure 2 | Effect of Incubation time on biofilm production by fungal isolates from crude oil refinery sludge

and *Rhizopus nigricans* produced minimal biofilm at 72 h and 120 h, respectively (Figure 2). This extended incubation period may reflect physiological adaptation to new environmental condition and growth factors (Alotaibi *et al*., 2021). Similarly, previous report by Luo *et al*. (2021) revealed that biofilm biomass reaches a maximum between 24–72 h before declining as biofilm stability decreases.

4.4 | Effect of pH on Production of Biofilm by Fungal Isolates from Crude Oil Refinery Sludge

The effect of pH on biofilm formation by the isolated fungi is presented in Figure 3. The results demonstrate that biofilm formation was optimal under slightly acidic to neutral conditions (pH 5-7) whereas alkaline condition (pH 9) markedly reduced biofilm yield across all isolates. Among the isolates, *Talaromyces verruculosus* SFAJ2, *Aspergillus fumigatus* and *Aspergillus aculeatus* SFO13 exhibited the highest biofilm production at pH 5 and pH 7, respectively, achieving optical densities (OD_{560}) of 0.78 nm and 0.83 nm. This observation is consistent with the previous reports indicating that pH 5.5 was the optimal pH for biofilm formation in *Candida* species (de Vasconcellos *et al*., 2014; Gonçalves *et al*., 2020) while Siqueira and Lima (2013) reported pH 7 as optimal for filamentous fungi. Remarkably, *Fusarium chlamydosporum* formed optimal biofilm mass (0.46 nm) at pH 5 while *Rhizopus nigricans* formed weak biofilm across all pH conditions tested. Biofilm formation was generally inhibited by alkaline condition (pH 9).

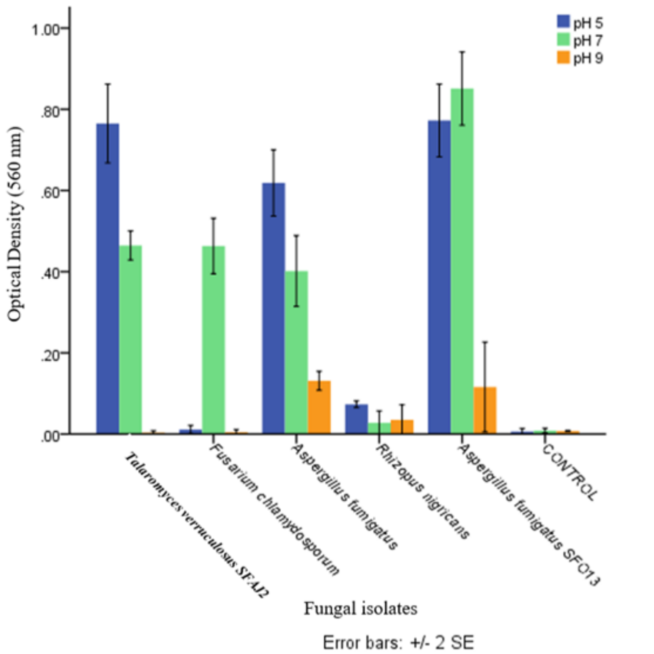

Figure 3 | Effect of pH of biofilm production by fungal isolates from crude oil refinery sludge

4.5 | Influence of carbon source on biofilm production by fungal isolates from crude oil refinery sludge

Influence of different carbon sources including glucose, sucrose and crude oil on biofilm production by fungal isolates are illustrated in Figure 4. The *Talaromyces verruculosus* SFAJ2 and *Aspergillus fumigatus* strains effectively utilized glucose and sucrose as carbon sources for biofilm formation, whereas *Aspergillus aculeatus* SFO13 exhibited preference for glucose. The highest biofilm production (0.86 OD) was observed in *A. fumigatus* in the presence of sucrose, followed by biofilm yield of OD_{560} = 0.72, when glucose was used as sole source of carbon. In contrast, *Aspergillus aculeatus* SFO13 produced comparatively lower biofilm level (0.46 OD) with glucose. Generally, fungi are sugar fermenters; however, preferences for specific carbon sources vary with some preferring sucrose

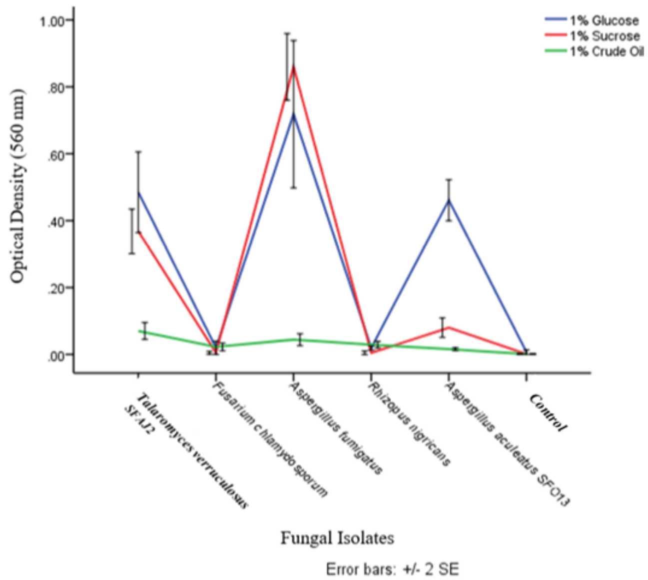

Figure 4 | Effect of carbon source on biofilm production by fungal isolates from crude oil refinery sludge

(Pemmaraju *et al*., 2016) others glucose (Martinez, *et al*., 2007) or a blend of sugars (Stepanovic *et al*., 2007). Notably, none of the isolates exhibited significant biofilm formation when crude oil was provided sole source of carbon after 24 h of incubation, although *T. verruculosus* and *A. fumigatus* showed slight biofilm formation.

4.6 | Effect of Incubation Time on Biofilm Production by Fungal Isolates Using Crude Oil (1%) as Carbon Source

The influence of incubation time on biofilm production by fungal isolates using crude oil (1%) as carbon source is presented in Figure 5. A progressive increase in biofilm formation was observed with extended incubation time across all the isolates. Among the assessed species, *Aspergillus aculeatus* SFO13 exhibited the highest biofilm production (0.22 nm) after 120 h incubation, followed by *Talaromyces verruculosus* SFAJ2 swhich attained a biofilm density of 0.15 nm under the same conditions. Compared to growth in nutrient-rich media, biofilm formation in the presence of petroleum hydrocarbon appeared to be slower at early stage but became sustained over time. This delay likely reflects the period required for physiological adaptation before the fungi can effectively utilize crude oil as a source of carbon. A similar trend was reported by Nebo *et al*. (2025), who observed that low concentrations of crude oil support

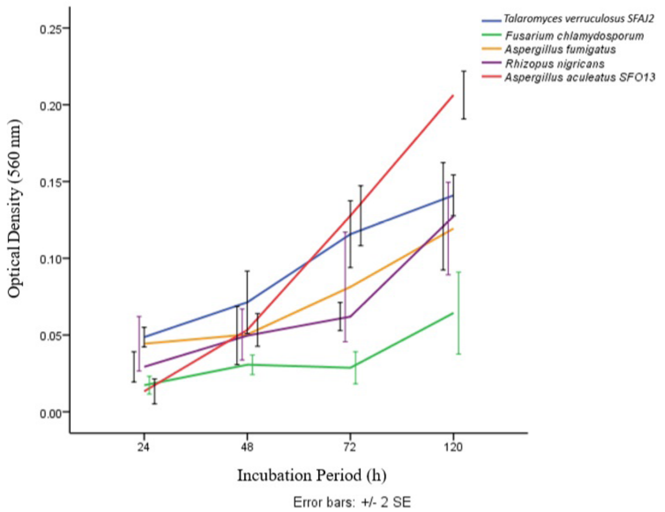

Figure 5 | Effect of incubation period on biofilm production by fungal isolates using crude oil as a source of carbon

microbial growth following an initial lag phase of 2–4 days and increased metabolic activity between day 4 and 8. In contrast, *Fusarium chlamydosporum* showed very weak biofilm formation (0.06 OD) after 72 h (Figure 5). Generally, prolonged incubation improved biofilm development when crude oil was provided as the sole carbon source (Figure 5). This suggests the capability of some the fungal species to colonize oil-polluted environments, thereby supporting their potential role in biodegradation of petroleum hydrocarbons (Zahari *et al*., 2022).

5. | Conclusion

This study showed that both nutritional and physicochemical factors play important role formation of biofilms among of the isolated fungi. Biofilm development was optimum at 28 °C after a period of 24 hours of incubation, particularly when glucose or sucrose was used as a carbon source. Most isolates formed stronger biofilms under slightly acidic condition (pH 5). However, *Aspergillus aculeatus* SFO13 exhibited optimal biofilm production at neutral pH (pH 7). Longer incubation periods improved the capability of the isolates to utilize crude oil as sole source of carbon, suggesting metabolic adaptation and possible use in cleaning up oil-contaminated environments. Additionally, *Talaromyces verruculosus* SFAJ2 and *Aspergillus* species showed strong biofilm forming ability, supporting their suitability for biofilm-based environmental and biotechnological applications.

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