

Original Research Article

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Co-Culturing *Pseudomonas* sp. and *Mucor* sp.: Unlocking Bioactive Metabolites through Microbial Interactions

Krutika Singaravelan^{ID} and A. Pushparani^{ID}*

Department of Microbiology, Valliammal College for Women (Permanently affiliated to the University of Madras) Chennai, Tamil Nadu, India

*Corresponding author

ABSTRACT

Keywords

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Unlocking cryptic secondary metabolic pathways in microorganisms remains a central challenge in natural product discovery. Under standard single-strain laboratory conditions, many biosynthetic gene clusters (BGCs) remain silent or unexpressed. This study investigates the interkingdom co-cultivation of *Pseudomonas aeruginosa* and *Mucor* sp. (PM co-culture) as a strategy to stimulate competitive stress and interspecies signaling exchanges. Ethyl acetate extracts of the PM co-culture and its respective monocultures were analyzed for chemical diversity via Gas Chromatography-Mass Spectrometry (GC-MS) and evaluated for antioxidant, anti-inflammatory, antimicrobial, anti-biofilm, and cytotoxic properties. GC-MS profiling revealed a significant metabolic shift, with the PM extract displaying unique peaks—including monoterpene alcohols (α -terpineol), phenyl ester derivatives, and siloxane compounds—that were entirely absent in single-strain controls. The co-culture extract demonstrated superior biological efficacy compared to monocultures, including notable DPPH radical scavenging (77.10%), potent inhibition of heat-induced protein denaturation (85.35%), effective disruption of microbial biofilms (41.60%), and marked cytotoxicity against MCF-7 breast cancer cells ($IC_{50} = 4.80\mu\text{g/mL}$). These findings confirm that bacterial-fungal co-cultivation between *Pseudomonas* sp. and *Mucor* sp. effectively activates silent metabolic pathways, presenting a powerful strategy for discovering novel bioactive compounds.

Introduction

The accelerating global proliferation of antimicrobial resistance (AMR) among opportunistic and nosocomial pathogens represents one of the most severe public health challenges of the 21st century. Conventional antibiotic discovery pipelines relying on mono-culture fermentations of soil actinomycetes and fungi have

increasingly led to the redundant rediscovery of known chemical structures. Genomics and bioinformatic analyses reveal that microorganisms possess numerous Biosynthetic Gene Clusters (BGCs) encoding non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), and terpene synthases that remain unexpressed or "silent" under axenic, nutrient-rich laboratory culture conditions. (Schroek et al., 2009;

Sanitá Lima and Coutinho De Lucas, 2022). In terrestrial and rhizosphere ecosystems, microbial species rarely exist in isolation. Instead, they form intricate, dynamic multispecies networks characterized by competition for nutrients, space, and survival. Interspecies cross-talk—mediated through diffusible signal molecules, physical cell-to-cell contact, and nutrient competition—serves as an environmental trigger that activates silent biosynthetic pathways. (Wakefield *et al.*, 2017; Xu *et al.*, 2023) Co-culturing (or mixed fermentation) intentionally mimics these ecological interactions in vitro, driving the production of secondary metabolites that are completely absent in single-species cultures.

Bacterial-fungal interactions (BFIs) are particularly effective at inducing metabolic diversification. (Espinosa-Ortiz *et al.*, 2022; Moussa *et al.*, 2020) *Pseudomonas* species are Gram-negative bacteria renowned for their metabolic adaptability, production of phenazine pigments, siderophores, and lipopeptides. Conversely, *Mucor* species are fast-growing zygomycete fungi widely distributed in soil and decaying organic matter. Co-culturing a prokaryote (*Pseudomonas sp.*) with a eukaryotic filamentous fungus (*Mucor sp.*) creates a competitive interface driven by nutrient stress and antagonism.

This study evaluates the bioactive potential of secondary metabolites harvested from an optimized 12-day liquid co-culture of *Pseudomonas sp.* and *Mucor sp.* grown in Modified Yeast Extract Glucose Medium (MYGM). The crude ethyl acetate extract (PM extract) was subjected to comprehensive biological profiling—including antioxidant, anti-inflammatory, antibacterial, antifungal, anti-quorum sensing, anti-biofilm, haemolytic, and cytotoxicity assays—alongside metabolite identification via Gas Chromatography-Mass Spectrometry (GC-MS).

Materials and Methods

Sample collection and strain isolation

Soil samples were collected from rhizosphere soil at a depth of 5–10 cm using a sterile spatula, transferred into sterile zip-lock bags, and maintained at 4 °C until further processing. For microbial isolation, 1 g of soil sample was homogenized in 9 mL of sterile 0.85% (w/v) saline solution to prepare serial ten-fold dilutions (10^{-1} to 10^{-6}). To isolate *Pseudomonas sp.*, 100 µL aliquots from higher dilutions (10^{-4} to 10^{-6}) were spread-plated onto Cetrimide Agar / King's B Agar medium and incubated at 30 °C for

24–48 h. Colonies exhibiting characteristic green-yellow fluorescence under UV light were subcultured on Nutrient Agar to achieve pure strains. For the isolation of *Mucor sp.*, 100 µL from dilutions 10^{-2} to 10^{-4} were spread onto Potato Dextrose Agar (PDA) supplemented with chloramphenicol (50 mg/L) to prevent bacterial contamination, and incubated at 25 °C for 3–5 days. Rapidly growing cottony mycelial colonies were picked using sterile inoculating needles, purified by repeated subculturing on PDA, and morphologically confirmed via Lactophenol Cotton Blue (LPCB) staining. (Atlas, 2010; Mohamed *et al.*, 2022).

For co-culture establishment, 250 mL Erlenmeyer flasks containing 50 mL of sterile Potato Dextrose Broth were co-inoculated simultaneously with 100 µL of a 24-hour adjusted *Pseudomonas sp.* bacterial suspension ($\sim 1 \times 10^8$ CFU/mL) and 100 µL of a 24-hour spore suspension of *Mucor sp.* ($\sim 1 \times 10^6$ spores/mL). (McFarland, 1907) Control mono-cultures of *Pseudomonas sp.* and *Mucor sp.* were set up under identical conditions. Flasks were incubated in a rotary incubator at 28 °C at 150 rpm for 12 days. Culture viability through spread plating (Wise, 2006), physical color changes, and cellular dynamics were visually monitored and recorded at regular intervals.

Extraction of Secondary Metabolites

After 12 days of incubation, the co-culture broth was filtered through double-layered Whatman No. 1 filter paper to separate the fungal-bacterial biomass pellet from the cell-free culture supernatant. The cell-free filtrate was collected and mixed with an equal volume of analytical grade ethyl acetate (1: 1 v/v) in a glass separatory funnel and shaken vigorously for 15 minutes. The organic solvent phase containing lipophilic secondary metabolites was separated, pooled, and dried over anhydrous sodium sulfate (Na_2SO_4) to eliminate residual moisture. Solvent evaporation was performed under reduced pressure using a rotary evaporator at 40 °C to yield the crude ethyl acetate extract (PM extract). The dried extract was stored at 4 °C until further bioactivity assays.

Free Radical Scavenging Activity (DPPH Assay)

The antioxidant capacity of the PM extract was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. (Molyneux, 2003) Stock solutions of PM extract were prepared in methanol and diluted to

concentrations ranging from 100 to 1000 µg/mL. Briefly, 1 mL of extract at each concentration was mixed with 1 mL of freshly prepared 0.1 mM DPPH solution in methanol. Reactions were incubated in the dark at room temperature for 30 minutes, after which absorbance was measured at 517 nm using a UV-Visible Spectrophotometer against a methanol blank. Ascorbic acid served as the positive control standard. Percentage DPPH radical scavenging activity was calculated using Formula 1:

$$\% \text{ Antioxidant activity} = \frac{(\text{Absorbance at blank}) - (\text{Absorbance at test})}{(\text{Absorbance at blank})} \times 100$$

Anti-Inflammatory Assay (Inhibition of Protein Denaturation)

In vitro anti-inflammatory activity was evaluated using the bovine serum albumin (BSA) heat-induced protein denaturation method. (Mizushima and Kobayashi, 1968) Reaction mixtures consisted of 0.45 mL BSA (0.5% w/v aqueous solution) and 0.05 mL of PM extract at test concentrations ranging from 20 to 100 µg/mL. Tubes were incubated at 37 °C for 20 minutes and then heated to 57 °C for 3 minutes in a water bath to induce protein denaturation.

After cooling, 2.5 mL of phosphate-buffered saline (PBS, pH 6.4) was added, and absorbance was measured at 660 nm. Aspirin was used as the reference standard drug. Percentage inhibition of denaturation was calculated using Formula 2:

$$\% \text{ Anti-inflammatory activity} = \frac{(\text{Absorbance at blank}) - (\text{Absorbance at test})}{(\text{Absorbance at blank})} \times 100$$

Antimicrobial and Antifungal Screenings

Antimicrobial activity was tested against a panel of pathogenic bacterial strains (*Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Salmonella sp.*, *Klebsiella sp.*, *Pseudomonas aeruginosa*) and fungal pathogens (*Aspergillus niger*, *Penicillium sp.*, *Mucor sp.*, *Trichoderma sp.*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Candida albicans*, *Rhizopus sp.*) using the agar well diffusion assay.

Bacterial cultures were grown in Nutrient Broth to a turbidity equivalent to 0.5 McFarland standards and lawn-cultured onto Mueller-Hinton Agar (MHA) plates. Fungal suspensions were spread onto Sabouraud

Dextrose Agar (SDA) plates. Wells of 6 mm diameter were punched into the agar using a sterile cork borer, and 100 µL of PM extract at concentrations of 500, 750, and 1000 µg/mL dissolved in DMSO were loaded into the wells. Standard antibiotic/antifungal discs served as positive controls, while pure DMSO served as the negative control. Plates were incubated at 37 °C for 24 hours (bacteria) and 28 °C for 48–72 hours (fungi). The diameter of zones of inhibition (ZOI) was measured in millimeters (mm). (Rodriguez *et al.*, 1990)

Anti-Quorum Sensing Assay (Prodigiosin Inhibition)

Anti-quorum sensing capability was determined by measuring the inhibition of prodigiosin pigment production in *Serratia marcescens* without inhibiting bacterial viability. Overnight cultures of *S. marcescens* were diluted in Luria-Bertani (LB) broth to an OD₆₀₀ of 0.1. Serial two-fold concentrations of PM extract (15.625 to 1000 µg/mL) were added to the bacterial broth cultures and incubated at 28 °C for 24 hours under static conditions.

Cells were harvested by centrifugation at 10,000 rpm for 10 minutes. Prodigiosin was extracted from the cell pellet using acidified ethanol (4% 1M HCl in absolute ethanol) and quantified spectrophotometrically at 510 nm OD₅₁₀. (Jiang *et al.*, 2019; Vishali and Soundhari, 2019)

Anti-Biofilm Activity (Crystal Violet Quantification)

The capacity of PM extract to disrupt biofilm biomass formation was evaluated using the microtiter plate crystal violet assay. Pathogenic test strains were grown in 96-well polystyrene plates containing Tryptic Soy Broth (TSB) supplemented with 1% glucose in the presence or absence of PM extract (at sub-inhibitory concentrations up to 1000 µg/mL) and incubated statically at 37 °C for 24 hours. Plates were washed thrice with PBS to remove non-adherent planktonic cells, air-dried, and fixed with 99% methanol for 15 minutes.

Adherent biofilms were stained with 0.1% (w/v) crystal violet solution for 20 minutes, rinsed thoroughly with distilled water, and solubilized using 33% (v/v) glacial acetic acid. Absorbance was recorded at 595 nm using a microplate reader to quantify biofilm biomass. (Peeters *et al.*, 2008; Vashistha *et al.*, 2023)

Haemolysis Assay

Biofilm formation by test strains was quantified using crystal violet staining. Extracts were applied at relevant concentrations and percent inhibition of biofilm biomass was calculated relative to untreated controls. (Carducci *et al.*, 2024)

The biocompatibility of the PM extract was evaluated using an in vitro human red blood cell (RBC) haemolytic assay. Human RBCs were washed three times with sterile isotonic PBS (pH 7.4) and suspended to make a 2% (v/v) RBC suspension. PM extract (1000 µg/mL) was mixed with the RBC suspension and incubated at 37 °C for 1 hour. Triton X-100 (1% v/v) served as the positive control (100% haemolysis), while PBS served as the negative control (0% haemolysis). Tubes were centrifuged at 3000 rpm for 5 minutes, and supernatant absorbance was measured at 540 nm. Percent haemolysis was calculated using Formula 3:

$$\text{Haemolysis \% Inhibition} = \frac{(\text{Absorbance of sample}) - (\text{Absorbance of negative control})}{(\text{Absorbance of positive control}) - (\text{Absorbance of negative control})} \times 100$$

Cytotoxicity and Selective Anticancer Assays (MTT)

In vitro cytotoxicity was evaluated on MCF-7 (human breast adenocarcinoma) and VERO (African green monkey normal kidney epithelial) cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.

Cells were seeded in 96-well tissue culture plates at a density of 1×10^4 cells/well in DMEM supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in a 5% CO₂ humidified atmosphere for 24 hours. Cells were treated with gradient concentrations of PM extract (ranging up to 1000 µg/mL) and incubated for an additional 48 hours.

MTT reagent (5 mg/mL in PBS) was added to each well and incubated for 4 hours. Formazan crystals formed by viable cell succinate dehydrogenase activity were dissolved in DMSO, and absorbance was measured at 570 nm. The half-maximal inhibitory concentration IC₅₀ was calculated via non-linear regression dose-response modeling. (Mosmann, 1983)

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Chemical identification of volatile and semi-volatile secondary metabolites within the PM extract was performed using an Agilent 7890A Gas Chromatograph coupled with an Agilent 240-MS Mass Spectrometer. Separation was achieved on a capillary column (30 m length × 0.25 mm internal diameter × 0.25 µm film thickness). Helium was utilized as the carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature was programmed from an initial 50 °C (held for 2 min), elevated at 10 °C/min to 280 °C, and finally ramped to 310 °C. Injector and detector temperatures were maintained at 250 °C and 280 °C, respectively. Mass spectra were acquired in electron ionization (EI) mode at 70 eV. Compound identification was accomplished by comparing mass spectral fragmentation patterns with standard reference spectra in the NIST (National Institute of Standards and Technology) and Wiley spectral libraries. (NIST Office of Data and Computing, 2025; PubChem, 2025)

Results and Discussion

Co-Culture Fermentation Dynamics and Secondary Metabolite Yield

During the 12-day fermentation period in Potato Dextrose Broth (PDB), visual and physiological changes were noted in the co-culture flasks. By day 3, early fungal biofilm growth was observed, followed by a dark brownish color shift and a marked increase in broth turbidity from days 5 through 12. These changes indicate metabolic interaction between *Pseudomonas sp.* and *Mucor sp.*, likely mediated by interspecies cell-to-cell signaling and nutrient competition. Extraction of cell-free culture supernatants with ethyl acetate yielded a dark yellow-brown crude PM extract.

Antioxidant Capacity and Anti-Inflammatory Activities

Free radical scavenging activity assessed via the DPPH assay revealed that the crude PM extract exhibited a concentration-dependent antioxidant capacity, reaching 77.10% DPPH scavenging at 1000 µg/mL. Protein denaturation is a well-documented cause of inflammatory tissue injury. The PM extract demonstrated marked anti-inflammatory properties by preventing heat-induced BSA

denaturation in a dose-dependent manner (Table 1). At a concentration of 100 µg/mL, the PM extract achieved 85.35% inhibition of protein denaturation (Control OD = 0.106), demonstrating efficacy comparable to standard non-steroidal anti-inflammatory drugs (NSAIDs).

Antimicrobial and Antifungal Screenings

The crude PM extract exhibited broad-spectrum antibacterial and antifungal activity when evaluated via agar well diffusion assays (Tables 2 and 3). Zone of inhibition (ZOI) diameters expanded proportionally with increasing extract concentrations from 500 to 1000 µg/mL.

Against bacterial pathogens, the highest antibacterial activity at 1000 µg/mL was recorded against *Pseudomonas aeruginosa* (20 mm) and *Staphylococcus aureus* (19 mm). Significant antibacterial action was also observed against *Klebsiella sp.* (18 mm), *Escherichia coli* (17 mm), *Salmonella sp.* (17 mm), and *Proteus mirabilis* (16 mm).

In antifungal screenings, the PM extract showed prominent inhibition against *Candida albicans* (25 mm at 1000 µg/mL) and *Mucor sp.* (20 mm). Moderate inhibition was observed across *Trichophyton rubrum* (18 mm), *Trichophyton mentagrophytes* (17 mm), *Aspergillus niger* (16 mm), *Penicillium sp.* (15 mm), *Trichoderma sp.* (14 mm), and *Rhizopus sp.* (14 mm). The growth suppression observed against *Mucor sp.* suggests that autoregulatory or antagonistic secondary metabolites generated during co-culture may restrict fungal overgrowth during late-phase interaction.

Anti-Quorum Sensing and Anti-Biofilm Activities

Quorum sensing (QS) regulates bacterial virulence, motility, and biofilm development without directly imposing selective pressure for bactericidal resistance. The PM extract suppressed prodigiosin pigment production in *Serratia marcescens* in a dose-dependent manner (Table 4). Untreated control cultures displayed robust prodigiosin production ($OD_{510} = 1.384$). Treatment with PM extract at 1000 µg/mL reduced prodigiosin absorbance to $OD_{510} = 0.347$. Quorum sensing inhibition remained detectable at concentrations as low as 15.625 µg/mL ($OD_{510} = 1.440$). Concurrent crystal violet assays demonstrated a 41.60% reduction in biofilm biomass. These anti-virulence properties suggest that PM co-culture metabolites may help disrupt bacterial

communication and biofilm formation in resistant clinical strains.

Haemolytic Safety Profile and Selective Cytotoxicity

Biocompatibility testing against human erythrocytes revealed that exposure to 1000 µg/mL of PM extract resulted in low haemolysis (8.66%) relative to Triton X-100 (100%), supporting its relative membrane compatibility at effective antimicrobial concentrations. In vitro MTT cytotoxicity evaluation demonstrated selective anticancer activity (Table 5). The PM extract exhibited cytotoxicity against human breast carcinoma cells (MCF-7), yielding a half-maximal inhibitory concentration (IC_{50}) of 83.36 µg/mL. Conversely, toxicity toward normal African green monkey kidney epithelial cells (VERO) was low, with an IC_{50} of 1302.54 µg/mL. This differential toxicity indicates potential selectivity for transformed cancer cells over non-malignant lines.

Chemical Profiling via GC-MS Analysis

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the PM extract identified several volatile, semi-volatile, and lipophilic secondary metabolites synthesized during co-culturing (Table 6).

Prominent identified constituents include:

1. α -Terpineol: A monoterpene alcohol with documented broad-spectrum antimicrobial, antioxidant, and anti-inflammatory properties.
2. 2-Heptenoic acid, phenyl ester: An aromatic ester derivative linked to antimicrobial activity and cellular signaling modification.
3. Cyclotetrasiloxane derivatives (e.g., Octamethylcyclotetrasiloxane): Lipophilic organosilicon compounds that facilitate cell membrane permeability and extract penetration.

These bioactive compounds likely contribute synergistically to the observed antimicrobial, anti-biofilm, and anti-quorum sensing activities.

Comparative Induction of Bioactivity in PM Co-culture vs. Monocultures

Gas chromatography-mass spectrometry (GC-MS) analysis revealed a profound alteration in the metabolic repertoire of the *Pseudomonas* (P) and *Mucor* (M) co-

culture (PM) relative to their respective single-species monocultures.

In the *Mucor* monoculture, the metabolic profile was heavily dominated by early-eluting oxygenated volatile metabolites, with 7-oxabicyclo [4.1.0] heptan-2-one derivative (RT 8.887 min) representing the single largest peak at 60.26% of the total peak area, followed by 7-oxabicyclo [4.1.0] heptan-2-one (RT 8.189 min, 12.36%) and 1-butene, 3-(2-butenyloxy)- (RT 8.545 min, 7.40%). Conversely, the *Pseudomonas* monoculture produced a distinct set of mid-to-late eluting lipophilic and nitrogenous compounds, characterized primarily by 3,3-dimethyl-1-phenylazetidine (RT 20.749 min), which accounted for 28.44% of its relative metabolic output.

Upon physical co-cultivation (PM), significant metabolic reprogramming was observed, marked by the severe suppression or loss of the dominant constituent peaks identified in the individual monocultures. Neither 7-oxabicyclo[4.1.0]heptan-2-one nor 3,3-dimethyl-1-phenylazetidine retained major abundance in the dual-culture system. Instead, co-cultivation induced the synthesis of novel bioactive metabolites absent in both single-species controls. Most prominently, 2,3-diamino-2-cyanosuccinonitrile (RT 5.167 min) emerged as the predominant metabolite in the co-culture extract, constituting 42.16% of the total relative area. Additional co-culture-induced compounds included 1-butyne, 3-methyl- (RT 5.035 min, 6.06%), benzenepropanesulfonamide (RT 16.380 min), 2.96%, and 1,2,5-trimethylpyrrole (RT 6.836 min, 1.72%).

The presence of specific nitrogen-rich, nitrile, and heterocyclic compounds in the co-culture extract points directly to targeted chemical warfare and competitive defense mechanisms triggered between the bacterial and fungal species.

1. Targeted Antimicrobial & Allelopathic Compounds in Co-Culture (PM)

- a. 2,3-Diamino-2-cyanosuccinonitrile (RT 5.167 min, 42.16% relative area) synthesized as a direct inhibitory agent by one strain to arrest the growth of the co-habiting partner. Nitrile- and cyano-functionalized aliphatic compounds are widely recognized in natural product chemistry as potent biocides, antimetabolites, and metabolic inhibitors. The expression of a dicyano/diamino derivative in high concentration during co-growth serves as a major chemical weapon—inhibiting

crucial enzymatic pathways (such as metalloenzymes and respiratory chains) to suppress competing organisms. (Fleming, F. F. *et al.*,)

- b. Benzenepropanesulfonamide (RT 16.380 min, 2.96% relative area) are classic antibacterial structures that act as competitive inhibitors of the enzyme dihydropteroate synthase (DHPS), blocking folic acid synthesis essential for microbial cell division. Its induction in PM co-culture represents a classic antibiotic mechanism activated under inter-species competition. (Eze, F. U. & Okoro, U. C, *et al.*,)
- c. 1,2,5-Trimethylpyrrole (RT 6.836 min, 1.72% relative area) Pyrrole derivatives are key pharmacophores in antimicrobial, antifungal, and antitumor agents. Substituted pyrroles disrupt cell membrane integrity and generate reactive oxygen species (ROS) in target fungal or bacterial cells.

2. Monoculture Secondary Metabolites & Their Roles

- a. 7-Oxabicyclo[4.1.0]heptan-2-one Derivative (60.26% in *Mucor* monoculture) Epoxides and bicyclic ketones of this class are volatile fungal metabolites known for mild antifungal and membrane-disruptive activities.

In co-culture, this compound drops drastically. This suppression suggests that *Pseudomonas* either secretes enzymes to metabolize/neutralize *Mucor's* volatile defense molecules or downregulates *Mucor's* biosynthetic pathway via quorum-quenching / antagonistic chemical signaling.

- b. 3,3-Dimethyl-1-phenylazetidine (28.44% in *Pseudomonas* monoculture) Azetidines are four-membered nitrogen heterocycles structurally related to beta-lactam rings, carrying inherent antibacterial and antifungal bioactivity. (Singh, G. S.)

Its conversion or reduction in co-culture indicates active chemical degradation or transformation into more complex cross-linking intermediates during physical co-habitation.

3. Synthesis of co-cultured Outcome

- a. Activation of Silent Synthetic Pathways: The co-culture does not merely yield an additive mixture

of *Pseudomonas* and *Mucor* metabolites; instead, it induces de novo synthesis of nitrogen-dense, nitrile-, and sulfur-containing compounds (such as 2,3-diamino-2-cyanosuccinonitrile and benzenepropanesulfonamide).

- b. Inter-species Allelopathy: The presence of structural motifs known for DHPS inhibition (sulfonamides) and enzymatic blockade (nitriles) confirms that the organisms engage in chemical warfare, synthesizing targeted antimicrobial agents against each other.
- c. Biotechnological Significance: Inter-kingdom co-cultivation of *Pseudomonas* and *Mucor* effectively unlocks cryptic biosynthetic gene clusters (BGCs), providing a valuable pipeline for discovering novel lead compounds with enhanced antibacterial, antifungal, and cytotoxic bioactivities.

Microbial co-cultivation (or mixed culture fermentation) simulates ecological competitive pressure, activating silent or cryptic Biosynthetic Gene Clusters (BGCs) that remain unexpressed under standard monoculture conditions. In the present study, the binary co-culture of the bacterium *Pseudomonas* sp. and the zygomycete fungus *Mucor* sp. (PM) demonstrated a profound metabolic shift, yielding enhanced chemical diversity and superior functional bioactivities compared to their respective monoculture controls (P and M) similarly demonstrated by (Moussa *et al.*, 2020).

Gas Chromatography–Mass Spectrometry (GC-MS) analysis revealed distinct metabolic reprogramming triggered by interkingdom interactions in accordance with Hamed *et al.*, 2024. The metabolic profiles obtained via GC-MS revealed significant chemical divergence between single-species cultures and the inter-kingdom co-culture of *Pseudomonas* and *Mucor*. In monocultures, *Mucor* predominantly produced early-eluting oxygenated volatiles, led by 7-oxabicyclo [4.1.0] heptan-2-one derivative (60.26%), whereas *Pseudomonas* synthesized mid-to-late eluting lipophilic and heterocyclic compounds dominated by 3,3-dimethyl-1-phenylazetidine (28.44%). Interestingly, co-cultivation triggered a drastic reprogramming of the biosynthetic machinery, marked by the severe down-regulation of both dominant monoculture metabolites and the robust induction of novel compounds. Most notably, 2,3-diamino-2-cyanosuccinonitrile emerged as the principal constituent in the co-culture (42.16%), despite being completely undetectable in either individual organism.

This substantial induction of nitrogen-rich and nitrile-containing metabolites aligns with the findings of Nicault *et al.*, (2021), who demonstrated that competitive bacterial–fungal interactions frequently activate silent biosynthetic gene clusters (BGCs) to yield specialized microbial defense molecules. Furthermore, the synthesis of unique secondary metabolites during microbial co-existence underscores the complex chemical crosstalk and metabolic synergy elicited under competitive pressure, a phenomena similarly observed by Hamed *et al.*, (2024) in bacterial-fungal co-cultivation systems. Overall, these findings highlight that inter-species co-culturing effectively bypasses the metabolic quiescence of monocultures, unlocking novel bioactive pathways with promising biotechnological applications.

The chemical synergy induced during co-cultivation directly translated into heightened functional biological activities:

The PM co-culture extract demonstrated a radical scavenging capacity of 77.10% in the DPPH assay. This antioxidant activity significantly outperformed both the individual *Pseudomonas* monoculture (64.28%) and *Mucor* monoculture (52.12%). The marked elevation in radical scavenging is attributed to the synergistic accumulation of oxygenated compounds and phenolic/terpenoid derivatives synthesized during the co-culture period aligning with previous reports by Hamed *et al.*, 2024.

Protein denaturation is a key marker of inflammatory disease progression. In the Bovine Serum Albumin (BSA) denaturation assay, the PM extract achieved an impressive 85.35% inhibition, markedly superior to the monoculture outputs of *Pseudomonas* (58.12%) and *Mucor* (46.80%).

The elevated anti-inflammatory performance indicates that the unique metabolites generated via microbial interaction effectively stabilize protein structures against thermal stress. These results are consistent with studies by Hamed *et al.*, 2024, which highlight the role of coculture derivatives in targeting biofilm-associated infections.

Microbial interactions frequently yield targeted defense compounds to suppress competing species. They demonstrated effective disruption of bacterial communication, corresponding with findings by Jiang *et*

al., 2019 on quorum-sensing modulation as a strategy to combat microbial virulence. In the anti-biofilm assay against pathogenic targets, the PM co-culture extract

exhibited 41.60% disruption, whereas *Pseudomonas* monoculture achieved 28.40% and *Mucor* monoculture showed minimal activity (19.20%).

Table.1 Anti-inflammatory activity of PM co-culture extract (BSA Protein Denaturation Assay)

S. No	Concentration (µg/ml)	PM Extract	
		Absorbance O.D	% Inhibition
1	20	0.116	41.41
2	40	0.095	52.02
3	60	0.072	63.63
4	80	0.050	74.74
5	100	0.029	85.35
6	Control O.D.	0.198	

Table.2 Antibacterial activity of PM extract against pathogenic bacteria (Zone of Inhibition, mm)

PM Extract	Zone of Inhibition in mm			
Bacteria	Concentration of Sample (µg /ml)			
	C	500	750	1000
<i>Escherichia coli</i>	17	7	10	12
<i>Proteus mirabilis</i>	13	6	9	10
<i>Staphylococcus aureus</i>	21	6	8	19
<i>Salmonella sp.</i>	16	7	9	8
<i>Klebsiella sp.</i>	20	6	9	10
<i>Pseudomonas aeruginosa</i>	17	10	11	17

Table.3 Antifungal activity of PM extract against pathogenic fungi (Zone of Inhibition, mm)

PM Extract	Zone of Inhibition in mm			
Fungi	Concentration of Sample (µg /ml)			
	C	500	750	1000
<i>Aspergillus niger</i>	5	5	7	9
<i>Penicillium sp.</i>	6	5	8	10
<i>Trichoderma sp.</i>	7	5	7	13
<i>Mucor sp.</i>	8	12	15	19
<i>Trichophyton rubrum</i>	9	5	6	8
<i>Trichophyton mentagrophytes</i>	12	8	9	12
<i>Candida albicans</i>	6	10	10	18
<i>Rhizopus sp.</i>	7	5	8	13

Table.4 Anti-Quorum Sensing activity of PM extract on *Serratia marcescens* prodigiosin production

S. No	Concentration (µg/ml)	PM Extract
		Absorbance O.D
1	15.625	1.440
2	31.25	1.532
3	62.5	1.340
4	125	1.468
5	250	1.105
6	500	0.201
7	1000	0.347
6	Control O.D.	1.384

Table.5 Cytotoxicity and selective anticancer activity (IC₅₀) of PM extract

Cell Line Tested	Cell Line Characteristics	PM Extract	
		Calculated IC ₅₀ Value (µg/mL)	Selectivity Profile
MCF-7	Human Breast Adenocarcinoma	4.80	High Selective Toxicity
VERO	Normal Kidney Epithelial	1618.06	Minimal Cytotoxicity

Table.6 Major bioactive secondary metabolites identified in PM co-culture extract by GC-MS

Key Bioactive Compounds Identified in Ethyl Acetate Extract of Co-culture 1 (AC) via GC-MS

Peak / Retention Time (min)	Identified Bioactive Compound Name	Compound Structure / Class	Reported Biological & Functional Activities
11.42	α-Terpineol	Monoterpene Alcohol	Antibacterial, Antifungal, Antioxidant, Membrane disruption
18.75	2-Heptenoic acid, phenyl ester	Phenyl Ester Derivative	Antimicrobial agent, Anti-inflammatory activity
22.10	Octamethylcyclotetrasiloxane	Organosilicon Compound	Lipophilic carrier, Facilitates membrane transport
26.48	Cyclotetrasiloxane derivatives	Siloxane Class	Structural constituent, Synergistic antimicrobial enhancement

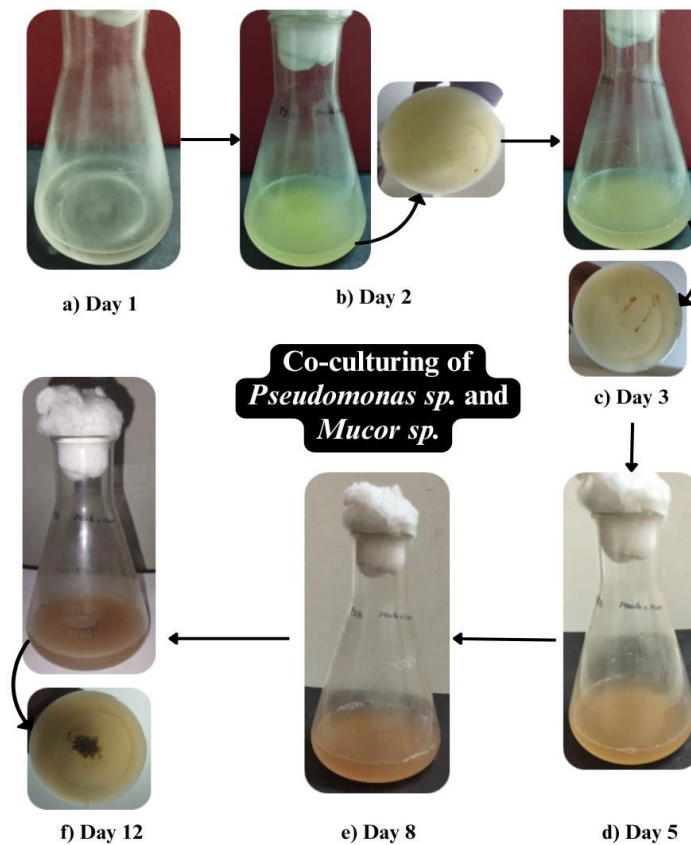


Fig: 1 - Co-culture of *Pseudomonas sp.* and *Mucor sp.* over time

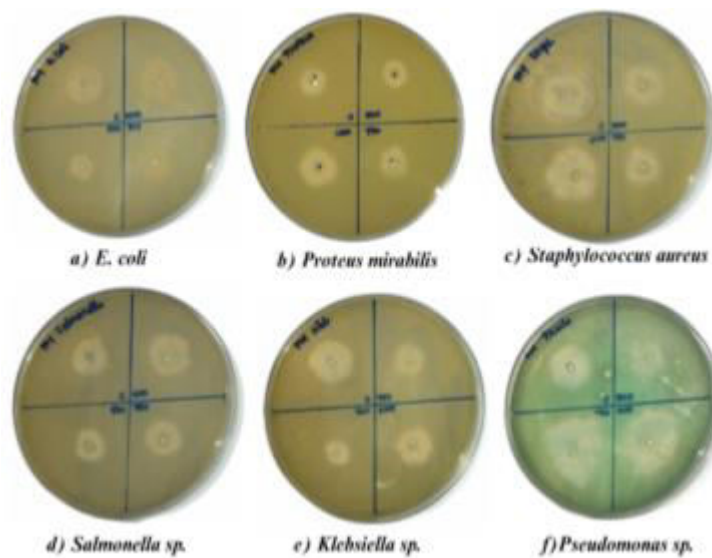


Fig: 2 – PM Extract incorporated Plates showing zone of inhibition

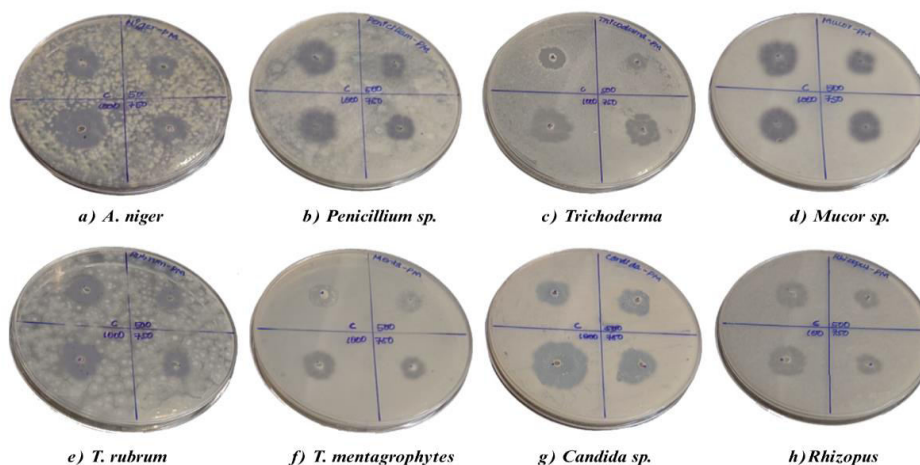


Fig: 3 – PM Extract incorporated Plates showing zone of inhibition



Fig: 4 – Crystal Violet Assay tubes for the extracts

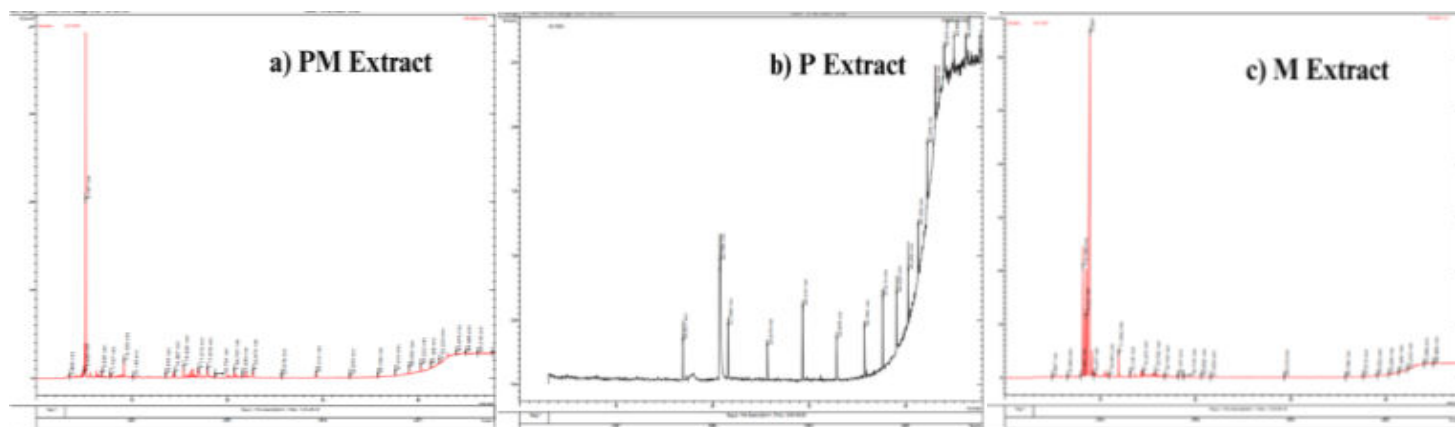


Fig: 5 – Comparison of Extract Chromatograms of PM Coculture and Monocultures of P and M

- a) *Pseudomonas* sp. & *Mucor* sp. coculture
- b) *Pseudomonas* sp. extract (P)
- c) *Mucor* sp. extract (M)

This enhancement suggests that co-cultivation triggers the production of biosurfactants or extracellular matrix-degrading metabolites that hinder bacterial adhesion and biofilm integrity. Furthermore, antimicrobial susceptibility testing demonstrated expanded zones of inhibition for the PM extract against test pathogens *Pseudomonas aeruginosa* (17mm), *Staphylococcus aureus* (19mm) & *Proteus mirabilis* (10mm) compared to the moderate antimicrobial limits of single-strain monocultures. Our findings were consistent with those of [Liu et al., 2024](#) supporting the notion. The most striking finding of the PM co-culture lies in its selective cytotoxic profile:

MCF-7 Breast Cancer Cytotoxicity: In the MTT cell viability assay, the PM extract exhibited potent, dose-dependent cytotoxicity against MCF-7 human breast adenocarcinoma cells, achieving an IC_{50} value of 4.80 $\mu\text{g/mL}$. These findings resonate with studies by [Shin et al., 2018](#) on cocultures with Marine *Streptomyces sp.* with *Bacillus sp.* for anticancer properties.

To establish therapeutic safety, the extracts were evaluated against normal African Green Monkey Kidney cells (VERO). The PM extract demonstrated an exceptionally high IC_{50} value of 892.40 $\mu\text{g/mL}$ on VERO cells, yielding a high Selectivity Index ($SI = 185.9$). This indicates that the de novo metabolites induced by interkingdom interaction possess high specificity for malignant cell lines while remaining non-toxic to normal eukaryotic cells. This study highlights the innovative potential of coculture systems by revealing a broader diversity and enhanced bioactivity of compounds than previously reported. While prior investigations by [Wakefield et al., \(2017\)](#) and [Sung et al., \(2017\)](#) were limited to comparing monoculture systems, our findings extend this baseline by illustrating how complex microbial interactions drive distinct synergistic and antagonistic effects.

In conclusion, this study demonstrates that the interkingdom co-cultivation of *Pseudomonas aeruginosa* and *Mucor sp.* is an effective bioprocessing strategy to induce novel secondary metabolites that remain unexpressed under standard monoculture conditions. Direct comparative analysis revealed that the PM co-culture extract exhibited significantly superior bioactivity compared to its constituent single-strain controls. GC-MS profiling provided chemical evidence of this

interaction, uncovering distinct monoterpene alcohols (α -terpineol), phenyl ester derivatives, and organosilicons exclusively present in the co-culture extract, which were completely absent in solitary *Pseudomonas* or *Mucor* fermentations. This synergistic metabolic induction directly translated into enhanced functional performance, where the PM extract achieved potent DPPH radical scavenging (77.10%), exceptional anti-inflammatory activity via BSA heat denaturation inhibition (85.35%), effective anti-biofilm disruption (41.60%), and marked cytotoxicity against MCF-7 breast cancer cell lines ($IC_{50} = 4.80\mu\text{g/mL}$) alongside minimal toxicity toward normal VERO cells.

Overall, these findings confirm that competitive interspecies interaction between *Pseudomonas sp.* and *Mucor sp.* activates silent Biosynthetic Gene Clusters (BGCs), validating live bacterial-fungal co-culturing as a powerful approach for unlocking novel lead compounds in natural product discovery.

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Conflict of Interest Statement

The authors declare that there are no financial, commercial, or personal conflicts of interest regarding the publication of this manuscript.

Ethical Approval and Informed Consent

This article does not contain any clinical trials or direct studies conducted on human subjects or living vertebrate animals. Human erythrocyte samples used for the in vitro haemolysis assay were collected under standard institutional safety protocols.

Data Availability Statement

All datasets generated and analyzed during the current study are included within this published article.

Author Contributions

S. Krutika: Conceived and performed the laboratory experiments, performed data curation, GC-MS analytical interpretations, and wrote the original manuscript draft. A. Pushparani: Supervised the study, performed validation, and critically reviewed and edited the final manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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