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Research Article

Antibacterial Efficacy and Identification of Endophytic Fungal Isolates from *Ocimum sanctum* Leaves

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ABSTRACT

The rise of multidrug-resistant (MDR) bacteria has drastically limited effective treatment options, driving the search for novel antimicrobial agents. This study evaluated the antibacterial activity of endophytic fungi isolated from *Ocimum sanctum* leaves and identified potential candidates with strong antimicrobial effects. Antibacterial activity was assessed through primary (agar plug) and secondary (disk diffusion) screenings, followed by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination using the broth microdilution method. Time-kill kinetics and scanning electron microscopy (SEM) were further employed to evaluate the extracts' effectiveness and mechanism of action. All Gram-positive bacteria tested were susceptible to the fungal extracts, while isolate OS-93 showed no activity against Gram-negative bacteria. In the disc diffusion assay, the fermentative broth ethyl acetate extract (FBEAE) of isolates OS-64 and OS-94, and the fungal biomass methanolic extract (FBME) of OS-64, exhibited broad-spectrum antibacterial activity, inhibiting both Gram-positive and Gram-negative bacteria. The broth microdilution test revealed bactericidal effects from the FBEAE of OS-93, OS-64, and OS-94, whereas FBME of all isolates showed bacteriostatic activity. Lower extract concentrations produced bacteriostatic effects, while higher concentrations were bactericidal. Time-kill assays confirmed that shorter exposure durations were sufficient to eliminate bacteria at higher extract concentrations. SEM analysis demonstrated morphological alterations, including cavities and cell disintegration, suggesting membrane rupture as the mode of action. Morphological and molecular identification confirmed isolate OS-64 as *Lasiodiplodia pseudotheobromae*. These findings highlight endophytic fungi from *O. sanctum* as promising sources of antibacterial compounds for combating MDR pathogens.

1. Introduction

Antibiotic resistance is a global public health concern, resulting in millions of deaths and drug-resistant illnesses. This event has existed since the discovery of penicillin, and multidrug-resistant organisms pose considerable difficulties to healthcare systems worldwide (Ahmed *et al.* 2024). Antibiotic resistance occurs when bacteria are no longer affected by antibiotics. The overuse and misuse of antibiotics are key factors contributing to antibiotic resistance. Chokshi *et al.* (2019) reported that several activities were found to be major contributing factors in developing nations, such as inadequate monitoring of the emergence of resistance, subpar quality of available antibiotics, clinical abuse, and accessibility

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of antibiotics. Multidrug-resistant (MDR) bacteria have emerged as a result of self-medication, prolonged use of antibiotics, and hospital infections. These bacteria are responsible for 15.5% of hospital-acquired infections (HAIs) worldwide (Mulani *et al.* 2019). Moreover, HAIs from high-income countries show incidence rates ranging from 3.5 to 12%. They are especially prevalent within intensive care units (ICU), with an infection rate of about 40% (Abban *et al.* 2023). ESKAPE is frequently responsible for potentially fatal nosocomial infections in severely ill and immunocompromised patients. This collection of bacteria is made up of *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and Enterobacter species. It includes both Gram-positive and Gram-negative species. They also exhibit possible medication resistance mechanisms (Santajit & Indrawattana 2016). Examples of bacteria that are resistant to antibiotics include Methicillin-resistant *Staphylococcus aureus* (MRSA) and Penicillin-resistant *Enterococcus*. Therefore, there is an urge to search for new antimicrobial agents that can combat these pathogenic bacteria.

Natural products are an encouraging source of antibacterial medications. Clinically, they offer some important advantages over antibacterial medications when treating infectious disorders. Discovery of new drugs is being expedited and enhanced using botanical medicines. Since ancient times, medicinal plants have been the main source of curative medications, despite their crucial function in modern medicine. Medicinal plants may prove to be a viable substitute for antibiotics, which have proven to be useless in treating infectious diseases, according to a plethora of scientific studies released in the last three decades. In recent years, phenolic chemicals, alkaloids, saponins, and terpenoids have demonstrated significant antibacterial promise. This has mostly been due to their ability to disrupt membranes, bind proteins, interfere with intermediate metabolism, inhibit quorum sensing, and inhibit biofilm formation (Abdallah *et al.* 2023). There are several reasons to use herbal remedies, two of which might be quite important. First, herbal medicine offers alternative modes of action, which frequently use a single medication to address a specific illness. Second, applying the distinct traditional knowledge of herbal medicine holds significant promise for producing affordable, biocompatible treatments and will expedite the development of new therapeutics (Anand *et al.* 2019). Medicinal plants have been used by human culture since the beginning of civilization as a means of fighting illness. *Ocimum* species is one of the medicinal plants that has been utilized for its bioactive compounds. *Ocimum basilicum*, commonly known as sweet basil, is a widely cultivated perennial herbaceous plant. It is a popular culinary herb, especially in Italian and Southeast Asian cuisines, such as those of Thailand and Vietnam. Its rich array of bioactive metabolites contributes to various potent biological activities. Due to these properties, sweet basil is widely used in traditional medicine and also valued as an ornamental plant (Purushothaman *et al.* 2018). *Ocimum sanctum* has been recognized for more than 2000 years as one of the most adaptable medicinal plants in the world with a broad range of biological activity. It is a well-known sacred plant from the Indian subcontinent that is also referred to as holy basil or tulsi. Phytochemicals, including apigenin, rosmarinic acid, eugenol, cirsilineol, cirsimartin, isothymusin, and isothymosin, are found in *O. sanctum* leaves. The leaves are also an excellent source of trace elements such as zinc, manganese, and sodium (Mahajan *et al.* 2014). Plant-based drugs isolated from medicinal plants such as *O. sanctum* are considered safe, healthy, and pure because they are obtained from natural sources. However, some disadvantages of these plants need to be emphasized. These medicinal plants are endangered by habitat loss and climate change, and may also be over-collected. They could be harmed or unable to grow due to pests, viruses, fungi, bad weather, and other environmental factors. Natural sources such as medicinal plants often contain only modest levels of bioactive compounds, necessitating larger amounts of these plants to extract more bioactive compounds. Hence, there is a need to find alternative sources of phytochemical compounds with pharmaceutical properties.

Endophytes are biological repositories of unique natural compounds that provide new opportunities for medication discovery. Plant-associated microbes, which are becoming increasingly important bio-prospecting targets in pursuit of novel chemical compounds, are found in the internal tissues of all plant species (Tiwari & Bae 2022). Endophytic fungi represent a promising alternative in the search for medications since they can produce a large number of metabolites with distinct properties and bioactive potential. According to Baron & Rigobelo (2021), endophytic fungi can be found in most plant species on Earth, and they colonize internal plant tissues without exhibiting indications of illness, benefiting both the host plant and themselves from this relationship. Endophytic fungi have various advantages as an antibiotic reservoir, including providing novel natural products for drug discovery, offering secondary metabolites against bacteria of medical value, and remaining grossly unexploited, with potentially 1 million distinct species (Deshmukh *et al.* 2015). They can mimic the biosynthesis of phytochemical compounds of their host plant to produce the same or alternative bioactive compounds with pharmaceutical properties (Kiros *et al.* 2024). Endophytic fungi are an essential source of bioactive compounds, including taxol, podophyllotoxin, huperzine, camptothecin, and resveratrol (Gupta *et al.* 2023). Furthermore, endophytic fungi can transform the host plant's active ingredients into more potent derivatives. They manufacture new anti-inflammatory, anti-tumour, antibacterial, antifungal, antiviral, and anti-malarial compounds. These substances include phenolic compounds, alkaloids, flavonoids, terpenoids, and derivatives of steroids (Nisa *et al.* 2015). Thus, the present study aims to evaluate the antibacterial activity of endophytic fungal isolates previously isolated from the medicinal plant, *O. sanctum*, and identify the species of a fungal isolate with excellent antibacterial activity.

2. Materials and Methods

2.1. Endophytic Fungus and Cultural Maintenance

The endophytic fungal isolates, IBRL OS-29, IBRL OS-64, IBRL OS-93, and IBRL OS-94, were provided by the Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The fungal cultures were cultivated and nurtured on potato dextrose agar (PDA; Brand: Merck) enriched with 2 g/L of powdered host plant and incubated for 7 days at 37°C. The fungal cultures were stored at 4°C until they were used again. Each month, subculturing was carried out to guarantee their viability and purity.

2.2. Maintenance of Test Bacteria

Eight test microorganisms were deposited at the IBRL, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. These bacteria included *Streptococcus mutans, Staphylococcus aureus*, MRSA ATCC 33591, *Bacillus subtilis* IBRL A3, *Yersinia enterocolitica, Klebsiella pneumoniae* ATCC 13883, *Shigella boydii* ATCC 9207, and *Escherichia coli* IBRL 0157. Nutrient agar (NA; Brand: Merck) was used to cultivate and sustain each bacterial strain. Meanwhile, *S. mutans* was cultured on brain heart infusion agar (BHIA; Brand; Merck). After a 24-hour incubation period at 37°C, all strains were stored at 4°C until needed again. To guarantee their purity and viability, glycerol stock was produced, and subculturing was carried out monthly.

2.3. Primary Screening

The agar diffusion assay was used in the initial screening following the methods described by Taufiq & Darah (2018). Endophytic fungal cultures were inoculated onto PDA agar plates supplemented with host plant powder to produce agar plugs and incubated at 25°C for 20 days. After incubation, agar plugs with a diameter of 1 cm and a thickness of 4 mm were cut using a sterile cork borer. These plugs were then placed on Muller-Hinton (MH) agar that had been previously seeded with test microorganisms. The plates were incubated for 24 hours at 37°C after being left overnight at 4°C to facilitate the diffusion of bioactive compounds. A volume of 20 µl of 30 µg/mL chloramphenicol was used as a positive control. Following a 24-hour incubation, the inhibitory zone around the endophyte agar plugs was evaluated.

2.4. Secondary Screening

2.4.1. Culture Medium

To produce yeast extract sucrose (YES) broth, 1,000 mL of host plant water extract was combined with 20 (g/L) yeast extract (Merck, Germany), 40 (g/L) sucrose, and 0.5 (g/L). Before that, 1,000 mL of distilled water was mixed with 2 g of host plant powder, brought to a boil for 30 minutes, and then filtered through a Muslin cloth. After calibrating the culture medium's pH with a pH meter to 6.0, it was autoclaved for 15 minutes at 121°C.

2.4.2. Fermentation and Extraction

Two mycelial plugs from a 3-day-old fungal culture were placed into 250 mL Erlenmeyer flasks containing 100 mL of YES broth, and the mixture was cultured for 16 days at 30°C in the dark without stirring. The fungal biomass and fermentative broth were separated using muslin cloth, and the filtrate was subsequently filtered through filter papers (Whatman, No. 1). The filtered broth (fermentative broth) was then extracted three times with an equal volume of ethyl acetate (1:1; v/v). The ethyl acetate extract was collected, concentrated in a rotary evaporator, and dried in a fume hood to produce the fermentative broth ethyl acetate extract (FBEAE) paste. For the fungal biomass extract, the biomass was freeze-dried for one week. The freeze-dried biomass was then macerated with methanol to obtain the fungal biomass methanolic extract (FBME). The FBME was concentrated using a rotary evaporator and dried in a fume hood.

2.5. Inoculum Preparation

To prepare the bacterial inoculum, three to four single colonies were transferred into 5.0 mL of 0.85% (w/v) sterile physiological saline and thoroughly mixed. This produced in cell suspensions that were approximately 1 × 10^8 CFU/mL, as determined by comparison with 0.5 McFarland standards.

2.6. Disc Diffusion Assay

To prepare the fungal crude extracts, 10.0 mg of extract was dissolved in 0.2 mL of 5% dimethyl sulfoxide (DMSO), followed by the addition of 0.8 mL of sterile distilled water to reach a concentration of 1 mg/mL. After air-drying and impregnation with 20 µL of the extract, sterile antibiotic discs with a diameter of 6.0 mm were placed on the surface of Mueller-Hinton agar previously seeded with test bacteria. A volume of 20 µL of 1.0% DMSO served as the negative control, and 30 µg/mL of chloramphenicol was used as the positive control. Following a 24-hour incubation period, the inhibitory zone around the endophyte agar plugs was measured. The experiments were performed in triplicate, and results were expressed as the mean inhibition zone value ± standard error from three independent trials.

2.7. MIC and MBC Determination

The fungal extract was diluted one-fold in sterile Mueller-Hinton broth (MHB) medium, and 100 µL of the extract was added to each well of a microtiter plate. Subsequently, a final volume of 200 µL was achieved by adding 100 µL of bacterial suspension at a concentration of 1 × 10^8 CFU/mL, resulting in a final concentration of 5 × 10^7 CFU/mL in each well. The drug reference used was chloramphenicol. As a control, there was a bacterial inoculum and 5% DMSO. After a 24-hour incubation at 37°C, 40 µL of 0.2 mg/mL p-iodonitrotetrazolium violet (INT) dissolved in 99.5% ethanol was added to each well as a growth indicator. The MIC was determined as the lowest concentration of fungal crude extract that inhibited visible microbial growth during incubation. The MBC values were established following MIC determination. After incubation, viable cells from wells with no visible growth were counted using standard viable plate counts on MHA. The MBC was identified as the lowest concentration of fungal crude extract that resulted in a 99.9% reduction in bacterial growth compared to the growth control (Taufiq & Darah 2019).

2.8. Time-Kill Study

A volume of 8.9 mL Mueller-Hinton broth (MHB) was prepared in four 50 mL Erlenmeyer flasks. After that, 1.0 mL of the fungal extract was added to each of the three flasks, resulting in final concentrations of ½ × MIC, 1 × MIC, and 1 × MBC. Instead of using fungal extract, 1.0 mL of BHIB was added to the control flask. A volume of 0.1 mL of bacterial inoculum (1 × 10^8 CFU/mL) was added to each of the four flasks, and they were then incubated for 48 hours at 37°C in a rotating shaker with 150 rpm agitation. A volume of 1.0 mL of the mixture was taken out every four hours (0-hour, 4-hour, 8-hour, 12-hour, 16-hour, 20-hour, 24-hour, 28-hour, 32-hour, 36-hour, 40-hour, 44-hour, and 48-hour of incubation), and the colony-forming unit (CFU) test was carried out. The final result was recorded in triplicate (Zulkamal *et al.* 2023).

2.9. Identification of an Endophytic Fungal Isolate

2.9.1. Macro-Morphological Identification

The colony shape of the fungal isolate IBRL OS-64 was examined on various agar media. The endophytic fungus was introduced to newly prepared agar media using a wire loop. After that, the plates were incubated for seven days at 30°C. Following incubation, the primary macroscopic morphologies were captured and observed, including mycelial colouration, colony diameter, conidia colour, colony reversal, colour, texture, and shape.

2.9.2. Micro-Morphological Identification

Using the lactophenol cotton blue staining technique, the chosen fungal isolate, IBRL OS-64, was examined under an Olympus BX41-CCD light microscope to ascertain its microscopic structures and features. After inoculating a PDA plate and incubating it for seven days at 30°C, the fungal isolate was ready for microscopic examination. A spotless glass slide was covered with a drop of lactophenol cotton blue. Next, a tiny part of the fungal colony was moved from the PDA plate into the lactophenol cotton blue solution. The fungal section containing lactophenol cotton blue was gradually covered with a cover slip. The slides were examined under a light microscope after verifying that the coverslip was air bubble-free.

2.10. Molecular Identification

2.10.1. DNA Extraction

The dried fungal mycelia were ground into a fine powder using a mortar and pestle with liquid nitrogen

(Tong *et al.* 2011). Chromosomal DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). About 20 mg of the powdered mycelia was placed into an Eppendorf tube and mixed with 400 µL of lysis buffer (AP1) and 4 µL of RNase (100 mg/mL). The mixture was vortexed briefly and incubated at 30°C for 30 minutes, with occasional mixing to help break down the cells. Then, 130 µL of precipitation buffer (AP2) was added, and the mixture was centrifuged at 20,000 g for 2 minutes. After 15 minutes on ice, the supernatant was transferred to a QIA-shredder spin column and centrifuged again at 20,000 g for 20 minutes. About 450 µL of the clear liquid was carefully transferred to a new tube, avoiding the debris. It was then mixed with 200 µL of binding buffer (AP3), loaded onto a DNeasy spin column, and centrifuged at 6,000 g for 1 minute. The flow-through was discarded. The column was washed twice with 500 µL of wash buffer (AW), centrifuging at 6,000 g each time. Finally, 100 µL of preheated elution buffer (AE) was added to release the DNA. After a 5-minute incubation at room temperature, the DNA was collected by centrifugation and stored at -20°C for future use.

2.10.2. Gel Electrophoresis

Using gel electrophoresis (1% agarose gel in 1 × Tris-borate-EDTA or TBE), the isolated rDNA was semi-quantified. Three different loading dyes were added to the gel agarose wells: 1.0 µL for the negative control, 5.0 µL for the DNA ladder (Promega, Wisconsin), and 4.0 µL for the required rDNA template (lane 3). After 90 minutes of 90 V electrophoresis, ethidium bromide (EtBr) staining was applied to the agarose gel. After that, the rDNA was examined using a UV transluminator (BioRad, Hercules).

2.10.3. Polymerase Chain Reaction (PCR) amplification

A small amount of DNA was amplified in many copies using PCR. To amplify fungal genomic DNA, an internal transcribed spacer (ITS) was employed as a primer (White *et al.* 1990). The forward and reverse primers, ITS1 (5'-CAACTCCCAAACCCCTGTGA-3') and ITS4 (5'-GCGACGATTACCAGTAACGA-3'), were used to amplify DNA fragments. Each reaction contained 50 µL of total volume, 10.0 µL of 10 × PCR buffer, 1.0 µL of dNTPs (40 mM), 5.0 µL of each primer (0.5 µM) for ITS1 and ITS4, 8.0 µL of magnesium chloride (2.5 mM), 0.25 µL of 1 U/µL Taq DNA polymerase, 0.8 µL of DNA, and 19.95 µL of deionized water to complete each reaction. After a 3-minute initial denaturation at 95ºC, the reaction mixture was inserted into a PTC-100 thermocycler (MJ Research, Inc.) that was set to run 35 cycles. Three processes make up each amplification cycle: elongation (72°C, 60 s), annealing (52°C, 30 s), and denaturation (95°C, 60 s). After the cycles concluded, a final elongation was carried out for 10 minutes at 72°C. Then, gel electrophoresis was used to examine the amplified fragments. The modified methods from Henry *et al.* (2000) and Magnani *et al.* (2005) were employed in this experiment.

2.10.4. Purification of PCR Products

Using a QIAquick PCR purification kit (Qiagen), the amplified PCR products were purified. Pipetting was used to thoroughly mix the fragments after 500 µL of PB buffer (binding buffer) was added. After being loaded into the QIAquick column, the mixture was centrifuged for one minute at 6,000 g. After adding 50 µL of the elution buffer to the middle of the QIAquick membrane, centrifugation at 6,000 g for one minute was conducted. After that, 30 µL of EB buffer (elution buffer) was added to the membrane to boost the concentration of DNA, and it was centrifuged again for a minute at 6,000 × g. After purification, the 2.0 µL PCR sample was subjected to electrophoresis analysis. The experiment was carried out following Magnani *et al.* (2005) and Henry *et al.* (2000).

2.10.5. DNA Sequencing and Sequence Analysis

The pure DNA was sent to the First BASE for DNA sequencing. Using the BLAST search engine (Altschul *et al.* 1997), the acquired nucleotide sequences were compared with those previously deposited in the National Center for Biotechnology Information (NCBI) database to identify the novel isolate. Based on the greatest score—that is, the most similarity—the species was identified.

2.10.6. Statistical Analysis

The experimental data were expressed as mean ± standard deviation (SD), and each experiment was carried out in triplicate ($n = 3$). The One-Way ANOVA was implemented to evaluate the data using SPSS 15.0, and the Duncan test was employed to assess whether there were any mean differences. If ρ was less than 0.05, the findings were deemed statistically significant.

3. Results

3.1. Primary Screening

Primary screening is a crucial step in screening endophytic fungal isolates with antimicrobial properties. Table 1 shows the inhibitory effect of endophytic fungal isolates against several test bacteria using an agar plug assay. Meanwhile, Figure 1 shows the antibacterial activity of several endophytic fungal isolates against *S. Typhimurium* and *S. aureus* on agar plug diffusion assay. The present study demonstrated that the fungal isolates, OS-64 and OS-94, were able to inhibit all Gram-positive and Gram-negative bacteria with inhibition zone diameters ranging from 20.5±0.9 to 23.7±1.2 mm, 12.9±1.0 to 19.5±0.7 mm, and 19.3±1.2 to 21.9±0.7 mm, as well as from 13.9±0.3 to 14.7±0.5 mm, respectively. As for fungal isolate OS-29, it can inhibit the growth of all test bacteria, excluding *S. boydii*. Its inhibitory effect against Gram-positive and Gram-negative bacteria ranged from 20.3±0.5–22.7±1.2 mm and 15.5±0.9–19.7±1.0 mm, respectively. Meanwhile, fungal isolate OS-93 showed an inhibitory effect only against Gram-positive bacteria, with the inhibition zone diameter between 15.1±0.9 mm to 19.7±0.9 mm.

Table 1. Antibacterial activity of endophytic fungal isolates (EFIs) against test bacteria using agar plug assay

Test bacteria	Inhibition zone diameter of endophytic fungal isolates (mm)			
	OS-29	OS-64	OS-93	OS-64
B. subtilis	21.0±1.0	20.7±0.9	19.7±0.9	21.4±0.9
MRSA	20.5±0.9	21.5±1.2	18.9±1.2	19.9±0.5
S. aureus	22.7±1.2	20.5±0.9	16.4±0.3	19.3±1.2
S. mutans	20.3±0.5	23.7±1.2	15.1±0.9	21.9±0.7
E. coli	15.5±0.9	13.7±0.7	-	14.7±0.5
P. aeruginosa	16.9±0.7	12.9±1.0	-	13.9±0.3
S. boydii	-	13.1±0.5	-	14.0±0.9
S. Typhimurium	19.7±1.0	19.5±0.7	-	14.5±0.3

3.2. Secondary Screening

Table 2 presents the antibacterial activity of fermentative broth ethyl acetate extract (FBEAE) and fungal biomass methanolic extract (FBME) of endophytic fungal isolates against test bacteria, as determined by disc diffusion assay. *B. subtilis* and *S. mutans* were most susceptible to all endophytic fungal isolates with a diameter of inhibition zone in the range of 8.3±1.0-23.0±1.0 mm and 10.0±0.6-10.0±0.6 mm, respectively. Meanwhile, *S. boydii* was the least susceptible strain to endophytic fungal isolates with a diameter of inhibition zone in the range of 8.7±0.6-8.7±0.6 mm. In terms of FBEAE, endophytic fungal isolates OS-64 and OS-94 were the most prominent, as they inhibited the growth of both Gram-positive and Gram-negative bacteria, with inhibition zone diameters of 10.0±0.6-23.0±1.0 mm and 8.7±0.6-27.7±0.6 mm, respectively. For FBME, only one fungal isolate, OS-64, can inhibit the growth of both

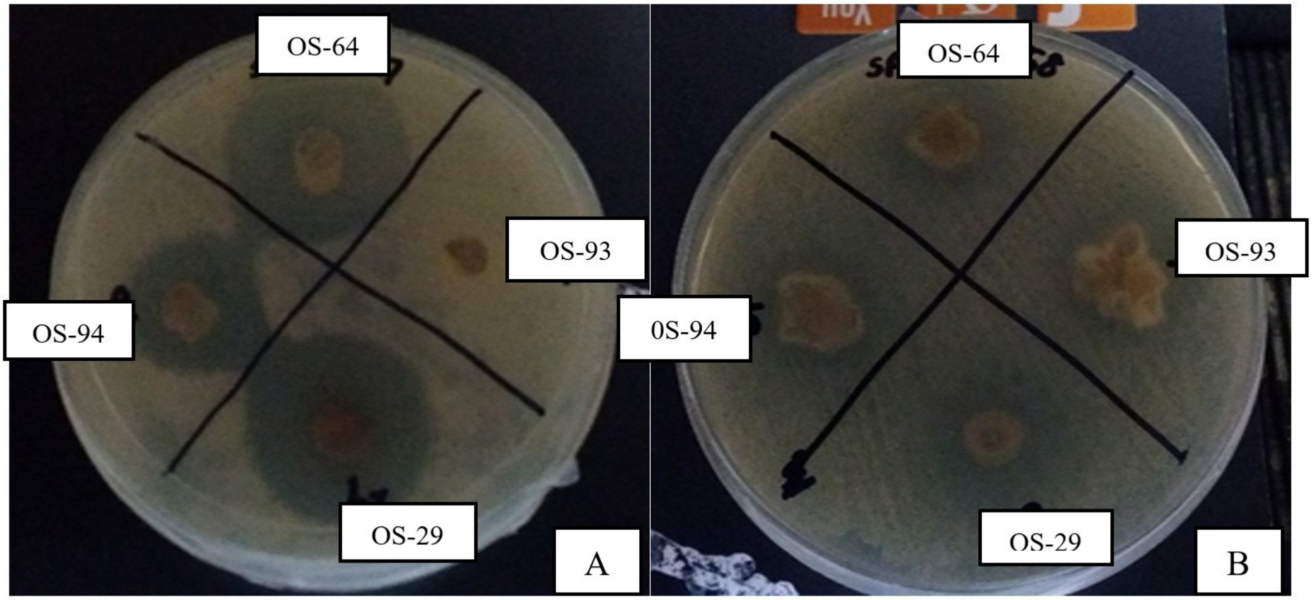

Figure 1. Antibacterial activity of endophytic fungal isolates against test bacteria using agar plug diffusion assay. (A) *S. Typhimurium* and (B) *S. aureus*

Table 2. Antibacterial activity of fermentative broth ethyl acetate extract (FBEAE) and fungal biomass methanolic extract (FBME) of endophytic fungal isolates against test bacteria using disc diffusion assay

Bacteria	FBEAE				FBME				Positive control
	OS-29	OS-64	OS-93	OS-64	OS-29	OS-64	OS-93	OS-64	
B. subtilis	22.3±0.6	23.0±1.0	12.3±1.0	19.3±1.2	12.7±0.6	11.0±0.6	8.7±1.0	8.3±1.0	22.0±1.2
MRSA	19.7±0.6	22.3±1.2	18.0±1.0	20.7±0.6	-	12.7±1.0	-	9.7±0.6	23.0±0.6
S. aureus	20.7±0.6	21.0±1.0	23.7±1.0	27.7±0.6	-	21.3±0.6	-	19.3±1.0	21.3±1.2
S. mutans	21.3±0.6	10.0±0.6	23.3±1.0	24.0±0.6	15.7±0.6	16.3±1.0	10.0±0.6	11.7±0.6	24.2±1.6
E. coli	12.3±0.6	14.7±0.6	12.7±1.0	14.0±1.0	10.3±0.6	11.7±1.0	-	-	19.0±0.6
P. aeruginosa	-	21.7±0.6	11.0±0.6	21.3±0.6	-	9.3±1.0	-	-	20.4±1.2
S. boydii	-	10.3±0.6	-	8.7±0.6	-	8.7±0.6	-	-	21.7±0.8
S. Typhimurium	15.7±0.6	18.7±1.0	15.7±0.6	17.7±0.6	9.7±0.6	22.3±1.0	-	21.7±0.6	20.0±1.2

Gram-positive and Gram-negative bacteria, with the diameter of the inhibition zone in the range of 8.7±0.6-22.3±1.0, respectively. The findings revealed that both FBEAE and FBME of endophytic fungal isolates can inhibit more Gram-positive bacteria than Gram-negative bacteria. This may be due to the differences in the cell wall structure of Gram-positive and Gram-negative bacteria. The present findings also revealed that most Gram-positive and Gram-negative bacteria were susceptible to FBEAE compared to FBME. It was hypothesized that bioactive compounds of fungal isolates were secreted into the broth medium rather than accumulated inside the fungal biomass. Figure 2 shows the antibacterial activity of endophytic fungal isolates extracts (FBEAE & FBME) against pathogenic bacteria on the disk diffusion assay.

3.3. MIC and MBC Determination

Table 3 demonstrates the MIC and MBC values of the FBEAE and FBME extracts of four endophytic fungal isolates against test bacteria. The MIC and MBC values of FBEAE of fungal isolate OS-29 against Gram-positive bacteria were 100 µg/mL and 200–400 µg/mL, respectively. Meanwhile, the MIC and MBC values for Gram-negative bacteria were 200 and 800 µg/mL, respectively. The MIC and MBC values of FBME of fungal isolate OS-29 against Gram-positive bacteria were 200 and 1,000 µg/mL, respectively. For FBEAE of fungal isolate OS-64, the MIC and MBC values against Gram-positive bacteria were 100 µg/mL and 100–200 µg/mL, respectively. The MIC and MBC values for Gram-negative bacteria were 200–400 µg/mL and 800 µg/mL, respectively. The MIC and MBC values of FBME of fungal isolate OS-64 against Gram-positive bacteria were 100 µg/mL and 400–800 µg/mL, respectively. The MIC and MBC values for Gram-negative bacteria were 200 µg/mL and 1,200 µg/mL, respectively. As for Gram-positive bacteria, the MIC and MBC values of FBEAE of fungal isolate OS-93 were 200 µg/mL and 1,000 µg/mL, respectively. Meanwhile, the MIC and MBC values (against Gram-negative bacteria) were 200 µg/mL and 1,000 µg/mL, respectively. For FBME of fungal isolate OS-93, the MIC and MBC values against Gram-positive bacteria were 400 µg/mL and 2,000 µg/mL, respectively. Notably, no inhibitory effects were observed on Gram-negative bacteria when exposed to the FBME of fungal isolate OS-93. The MIC and MBC values of FBEAE of fungal isolate OS-94 against Gram-positive bacteria were 100 µg/mL and 800 µg/mL, respectively. In contrast, the MIC and MBC values for Gram-negative bacteria were 200 µg/mL and 800 µg/mL, respectively. For FBME of fungal isolate OS-94, the MIC and MBC values against Gram-positive bacteria were 100 µg/mL and 800 µg/mL, respectively, while for Gram-negative bacteria, the MIC and MBC values were 200 µg/mL and 1,200 µg/mL, respectively.

3.4. Time-Kill Study

Figure 3 illustrates the time-kill study of FBEAE and FBME of an endophytic fungus, OS-64, against *S. mutans. S. mutans* was selected in this present study due to its high susceptibility to fungal extracts. In contrast, the fungal isolate OS-64 was selected due to its high antibacterial capability against test bacteria. The time-kill study of FBEAE of fungal isolate OS-64 was illustrated in Figure 3B. For control, the *S. mutans* cells showed a typical growth pattern of Gram-negative bacteria with lag, log, stationary, and death phases. However, the *S. mutans* cells began to reduce after being exposed to ½ × MIC of FBEAE of the fungal isolate OS-64. The *S. mutans* cells were significantly reduced to 1.91×10^5 CFU/mL after being treated with 1 × MIC of FBEAE for 24 hours compared to the control (8.13×10^7 CFU/mL). Notably, a 99.7% *S. mutans* growth reduction was observed after the cells were

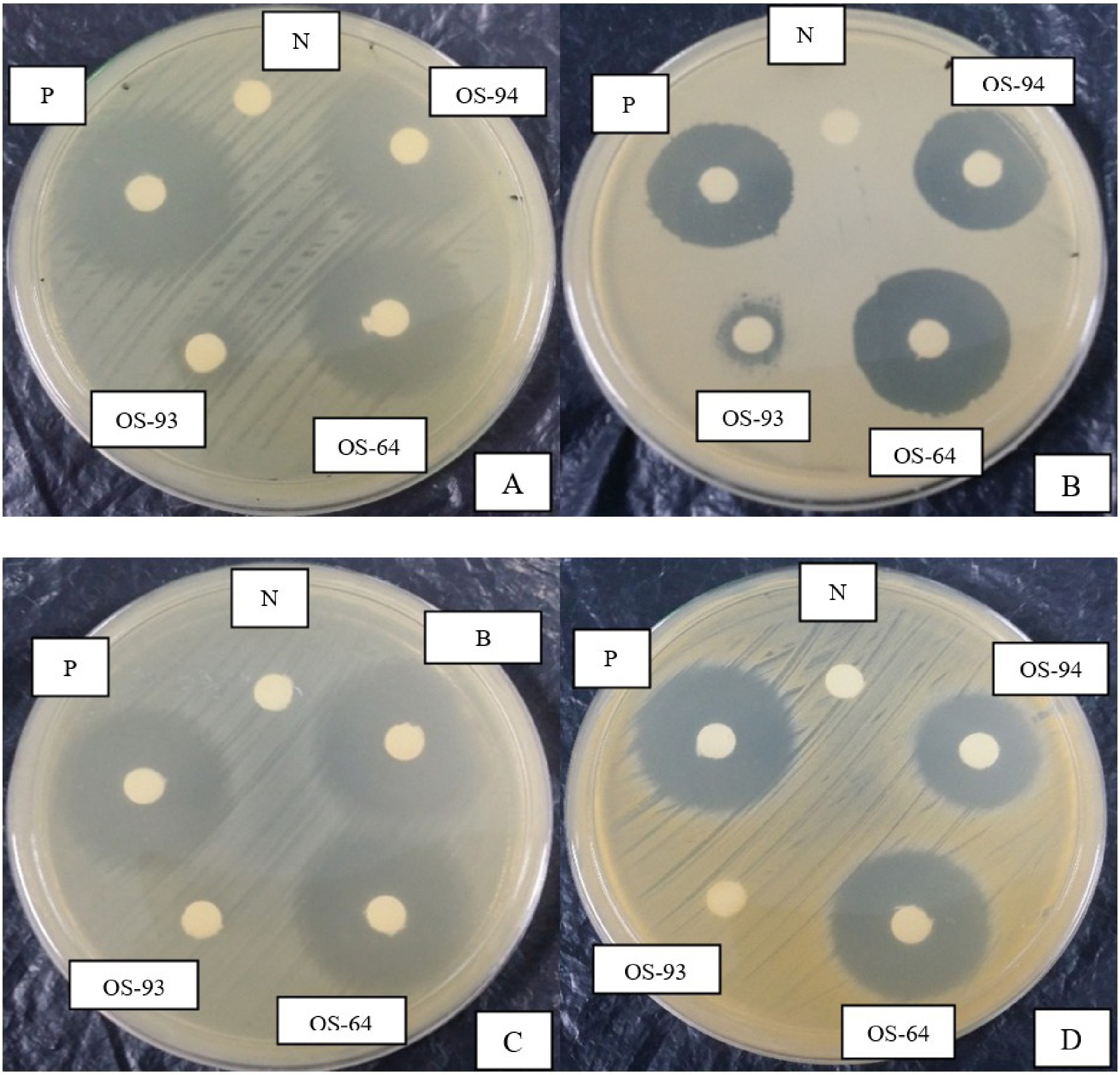

Figure 2. Antibacterial activity of endophytic fungal isolates extracts against pathogenic bacteria on disk diffusion assay. (A) Inhibitory effect of FBEAE on *K. pneumoniae*, (B) Inhibitory effect of FBEAE on *B. subtilis*, (C) inhibitory effect of FBME on *S. Typhimurium*, and (D) inhibitory effect of FBME on *S. aureus*. N and P are negative and positive controls, respectively

treated with 2 × MIC of FBEAE for 16 hours. Figure 3B illustrates the time-kill study of FBME of fungal isolate OS-64 against *S. mutans*. Similar observations were recorded for the control in which the *S. mutans* cells grow, mirroring the typical growth pattern of Gram-positive bacteria. However, a re-growth pattern mirroring the control was observed after *S. mutans* cells were exposed to ½ × MIC of FBME of fungal isolate OS-64. A 99.6% growth reduction was observed when the S. mutans cells were treated with 1 × MIC of FBME of fungal isolate OS-64 for 24 hours. Meanwhile, the *S. mutans* cells were reduced to 3.02×10^7 CFU/mL compared to the control after being exposed to 2 × MIC of FBME for 18 hours, with the percentage of reduction approximately 99.34%.

3.5. Structural Degeneration

Figure 4 exhibits the photomicrograph of *S. mutans* cells exposed to FBEAE and FBME extracts of endophytic fungus IBRL OS-64. Figure 4a shows the

Table 3. MIC and MBC values of fermentative broth ethyl acetate extract (FBEAE) and fungal biomass methanolic extract (FBME) of endophytic fungal isolates against test bacteria

Isolates	Extracts	MIC/MBC values (µg/mL)	Gram-positive bacteria				Gram-negative bacteria			
			BS	MR	SA	SM	EC	KP	SB	ST
OS-29	FBEAE	MIC	100	100	100	100	200	-	-	200
		MBC	400	200	200	400	800	-	-	800
		Ratio	4	2	2	4	4	-	-	4
	FBME	MIC	200	-	-	200	200	-	-	400
		MBC	1000	-	-	1000	1000	-	-	2000
		Ratio	5	-	-	5	5	-	-	5
OS-64	FBEAE	MIC	100	100	100	100	200	200	200	400
		MBC	200	200	200	100	800	800	800	800
		Ratio	2	2	2	1	4	4	4	4
	FBME	MIC	100	100	100	100	200	200	200	200
		MBC	800	800	800	400	1200	1200	1200	1200
		Ratio	8	8	8	4	6	6	6	6
OS-93	FBEAE	MIC	200	200	200	200	400	400	400	400
		MBC	1000	1000	1000	1000	2000	2000	2000	2000
		Ratio	5	5	5	5	5	5	5	5
	FBME	MIC	400	-	-	400	-	-	-	-
		MBC	2000	-	-	2000	-	-	-	-
		Ratio	5	-	-	5	-	-	-	-
OS-94	FBEAE	MIC	100	100	100	100	200	200	200	200
		MBC	400	400	400	400	800	800	800	800
		Ratio	4	4	4	4	4	4	4	4
	FBME	MIC	100	100	100	100	200	200	200	200
		MBC	800	800	800	800	1200	1200	1200	1200
		Ratio	8	8	8	8	6	6	6	6

BC – *B. cereus*, MR – MRSA, SA – *S. aureus*, SM – *S. mutans*, EC – *E. coli*, PA – *P. aeruginosa*, SB – *S. boydii*, ST – *S. typhimurium*

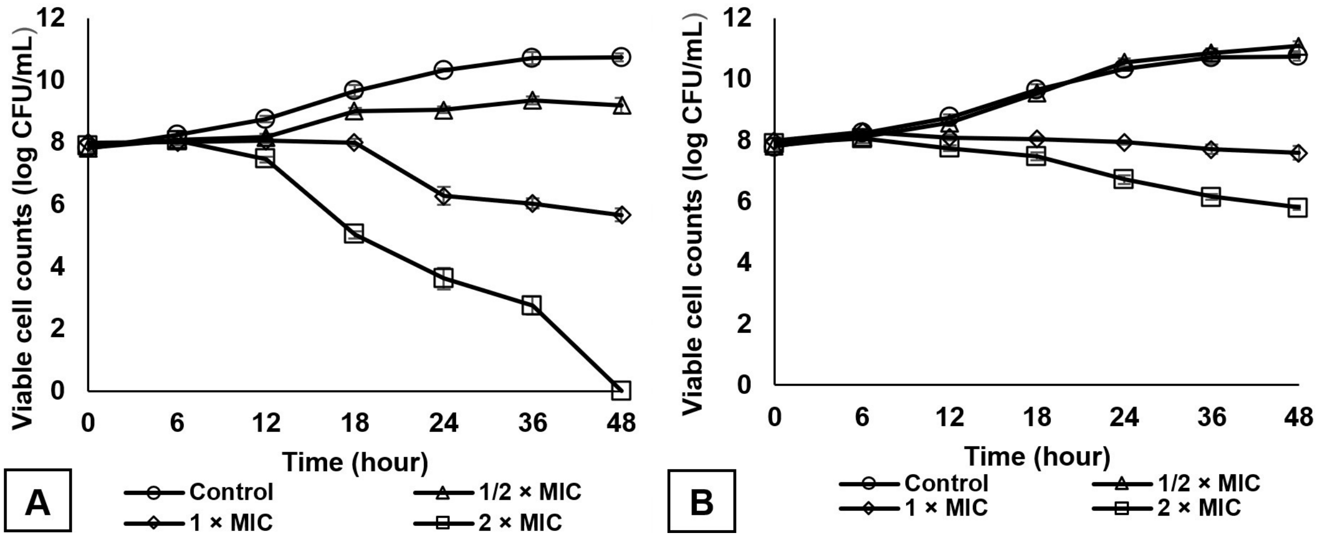

Figure 3. Time-kill study of an endophytic fungus, OS-64 extracts against *S. mutans*. (A) FBEAE extract, and (B) FBME extract

untreated *S. mutans* cells (control) observed as smooth surface and spherical cocci shape. However, some cavities were observed after 24 hours of the bacterial cells being exposed to the FBEAE extract (Figure 4B). Meanwhile, Figure 4C shows bacterial cells treated with FBME extract at 2 × MIC concentration after 24 hours of incubation. Some cavities and disintegration of bacterial cells were observed, indicating the cell rupture (indicated by red arrows).

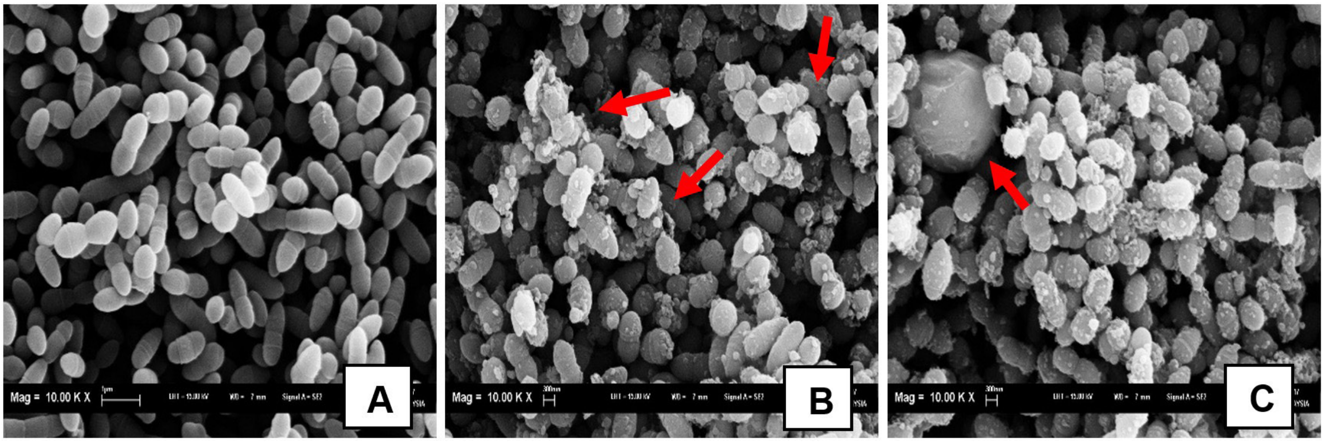

Figure 4. Photomicrograph of *S. mutans* cells exposed to endophytic fungus IBRL OS-64 extracts. (A) control, (B) FBEAE extract, and (C) FBME extract

3.6. Identification of Endophytic Fungal Isolate IBRL OS-64

3.6.1. Macroscopic and Microscopic Morphologies

In macro-morphological identification, endophyte colonies were grown on PDA agar for 14 days at 30°C (Figure 5). Initially, the growth of the endophyte colony was observed to be white; then, after 3 days of the incubation period, the colonies turned white-greenish (Figures 5A and B). Furthermore, the colonies became black after a week of incubation on PDA supplemented with the host plant. Figures 5C and D show the top and reverse views of the colony, respectively. Overall, the colony was observed as low to moderately deep, dense to floccose, with initial mycelium white to green in colour and soon turning black after a longer incubation period (more than 14 days). The reserve colony was initially observed as pale green-blackish and gradually changed to deep black after 14 days of incubation. In micro-morphology, the colony of endophytic fungal isolate IBRL OS-64 was observed under a light microscope (Figure 6). Figure 6A shows the aseptate paraphyses of the colony. By using a light microscopic view, no conidiophore was observed. Meanwhile, Figure 6B shows the young conidia of the isolate. Figure 6C shows hyaline immature conidia, whereas Figure 6D shows conidia developing on conidiogenous cells between paraphyses.

3.7. Molecular Identification

The RNA samples of the isolate were successfully extracted and purified from the fungal biomass, indicated by a single clear band on the gel electrophoresis (with 590 bp). Table 4 shows the Basic Local Alignment Search Tool (BLAST) search analysis of the DNA sequence of the isolate, IBRL OS-64. In BLAST analysis, the sequence datasets of the fungal isolate showed 100% similarity to the fungus Lasiodiplodia pseudotheobromae (accession number KP698094.1). The results indicated that fungal isolate, IBRL OS-64, is a member of the genus Lasiodiplodia (synonym Botryosphaeria) and can be suggested as *Lasiodiplodia pseudotheobromae*. Identifying species was based on the closest related species, as determined by GenBank accession number and culture collection number. Based on macro and micromorphology characteristics, the fungal isolate IBRL OS-64 was named *Lasiodiplodia pseudotheobromae* IBRL OS-64.

4. Discussion

Primary screening revealed that three out of four isolates showed a broad range of inhibitory effects since they can inhibit both Gram-positive and Gram-negative bacteria. In contrast, the fungal isolate, OS-93, is active only against Gram-positive bacteria. The present study also demonstrated that Gram-positive bacteria are more susceptible to fungal isolates than Gram-negative bacteria. This phenomenon may be due to the differences in the cell wall structure of both bacteria. Generally, bacterial cell walls are particularly important because they are made of special components that are only found in nature and can determine the shape of the bacteria. Moreover, they can protect the bacteria from the hostile environment, which is essential to their viability and impedes the adherence of ligands and proteins to host cells. Furthermore, cell walls reveal drug or virus receptor sites that are the most important sites for antibiotic attack, provide structures

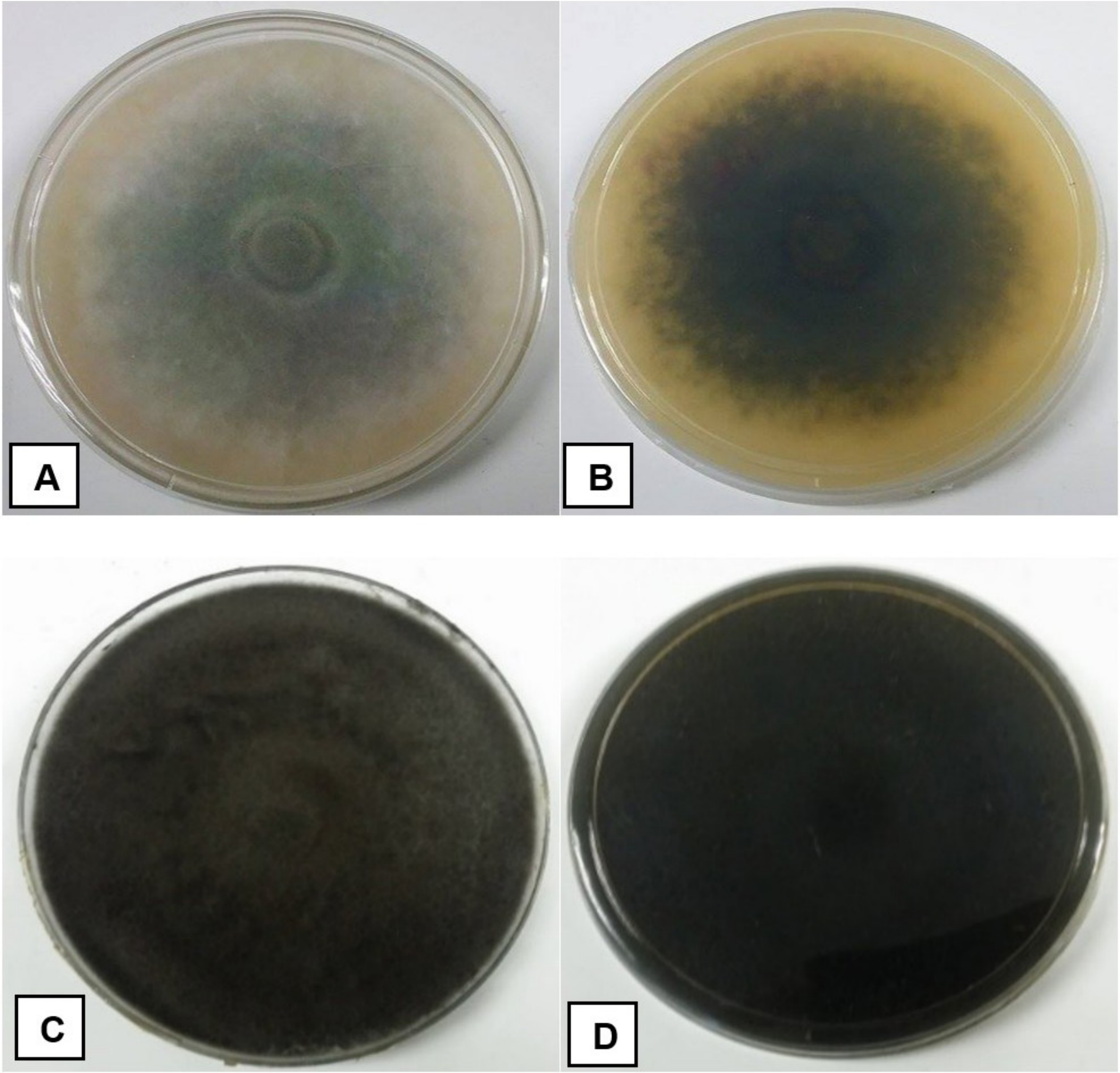

Figure 5. The macro-morphology of endophytic fungal isolate IBRL OS-64 incubated on PDA at different incubation periods. (A) 3 days (top view), (B) 3 days (reverse view), (C) 14 days (top view), and (D) 14 days (reverse view)

for immunological distinction and variation, and can induce disease symptoms in humans and animals. Gram-positive bacteria possess dynamic cell envelopes that serve as the initial line of defence against harmful compounds and influence reactions to the environment. They also have a thick peptidoglycan layer, even though they lack a protective outer membrane. Unfortunately, antibiotics target the cell envelope because it is essential to bacterial life. Several kinds of antibiotics used in clinical settings block the manufacture of PG, which causes osmotic rupture (Rajagopal and Walker 2017). In contrast, the distinctive feature of Gram-negative bacteria is their cell envelope, which is made up of an outer membrane layer and an interior cytoplasmic cell membrane encased in a thin peptidoglycan cell wall. The outer membrane is the first line of protection against environmental risks. Meanwhile, Gram-negative bacteria are naturally resistant to many antimicrobial agents because lipopolysaccharides (LPS) molecules convert the outer membrane into an

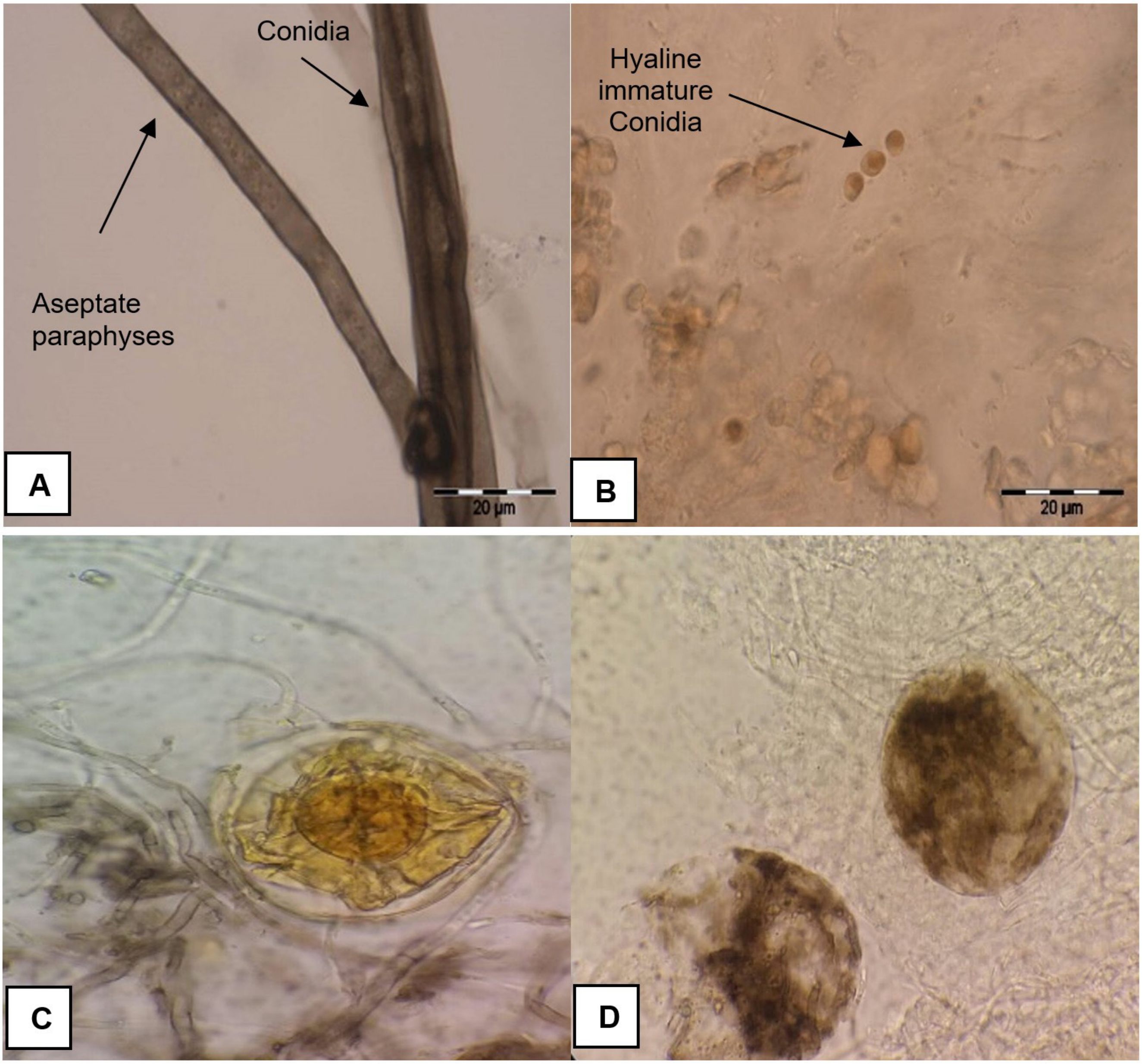

Figure 6. Microscopic view of fungal isolate, IBRL OS-64, after 7 days of incubation on water agar and water agar supplemented with UV-sterilized *O. sanctum* leaf, respectively. (A) Formation of aseptate mycelium, (B) formation of hyaline conidia and mature conidia on water agar added with host plant, (C) hyaline immature conidia, (D) conidia developing on conidiogenous cells between paraphyses

efficient permeability barrier against tiny, hydrophobic substances that may otherwise pass phospholipid bilayers (Bertani and Ruiz 2018).

In terms of extract, FBEAE showed a significantly higher inhibitory effect on test bacteria compared to FBME. This phenomenon might be due to bioactive compounds by fungal endophytes being released extracellularly. A similar observation was reported by Jalil *et al.* (2021), who revealed that the endophytic fungus *Muscodor* sp. secretes more bioactive substances into the fermentative broth (extracellularly) in comparison to internal secretion (fungal biomass). They also postulated that fungal endophytes rarely retained their secondary metabolites intracellularly; instead, they were typically released extracellularly. This occurrence might be explained by the fact that secondary metabolite substances serve as pathogen-protecting agents, making them more appropriate for

Table 4. The BLAST match result, which displays the sequences producing significant alignment

GenBank accession number	Closest related species	Total score sequence length	Similarity (%)
KP698094.1	*L. pseudotheobromae*	1090	100
KY432690.1	*L. pseudotheobromae* strain C1136	1077	100
KT318605.1	*L. pseudotheobromae* strain LPS-1	1057	99
FN645641.1	*L. pseudotheobromae* isolate CXY617	1057	99
FN645637.1	*L. pseudotheobromae* isolate CXY585	1057	99
NG_042461.1	*L. pseudotheobromae* strain CBS116459	1057	99
FN645638.1	*L. pseudotheobromae* isolate CXY611	1051	99
FN645640.1	*L. pseudotheobromae* isolate CXY828	1046	99

extracellular formation (Yenn *et al.* 2017). However, contradictory findings were reported by Tong *et al.* (2012). They revealed that Phomopsis ED2, an endophyte of Stamineus orthosiphon origin, had better antimicrobial activity when the extract from its fungal biomass was obtained intracellularly. They also suggested that the fungal biomass had more antimicrobial compounds than the fermentation broth.

Among Gram-positive bacteria, *S. mutans* was more susceptible to both extracts than another strain. This may be due to bioactive compounds such as phenols and flavonoids in the extract that can inhibit bacterial cell growth. Endophytic fungi are the main producers of a wide range of metabolites with strong biological effects, including antioxidants, antibiotics, antidiabetics, anticancer agents, and immunosuppressive agents (Gao *et al.* 2018). According to Deshmukh *et al.* (2018), their cohabitation with various host plants, mainly higher plants, supports a wide range of fungal endophytes that can produce distinctive substances and metabolites. A previous study reported that the endophytic fungus *Nigrospora sphaerica* isolated from a pantropical weed possesses significant phenolic and flavonoid content with the value of 77.74±0.046 mgGAE/g and 230.59±2.0 mgRE/g, respectively (Gautam *et al.* 2022). Baba and Malik (2015) reported that flavonoids exhibit an inhibitory effect by hindering the synthesis of nucleic acids and other metabolic activities. In contrast, the phenolic compounds show antibacterial activity by suppressing hydrolytic enzymes (proteases) or other interactions that inactivate microbial adhesins, cell envelope transport proteins, and non-specific interactions with carbohydrates.

The present study also revealed that *S. aureus* and MRSA were the least susceptible Gram-positive bacteria to the fungal extract. This occurrence may be due to the capability of *S. aureus* to develop several strategies to overcome the effect of antimicrobial agents. These strategies include drug uptake limitation, drug target change, drug enzymatic inactivation, and drug active efflux (Yilmaz & Aslantas 2017). Methicillin-resistant strains of *S. aureus*, or MRSAs, are generally resistant to beta-lactam antibiotics such as cephalosporin and penicillin (methicillin and oxacillin). Beta-lactam drug interferes with the synthesis of the cell wall, which stops the growth of bacteria. However, the effects of beta-lactams are usually offset by MRSA by producing β-lactamase and altering the binding pocket required to form cell walls (Ali Alghamdi *et al.* 2023). Methicillin resistance is obtained through the horizontal transfer of a mobile genetic element known as staphylococcal cassette chromosome mec (SCCmec)3, which is mediated by mecA. Penicillin-binding protein 2a (PBP2a), an enzyme that crosslinks the peptidoglycans in the bacterial cell wall, is encoded by the mecA gene. Due to PBP2a's poor affinity for β-lactams, the entire class of antibiotics resists it (Turner *et al.* 2019). *Shigella boydii* and *K. pneumoniae* were the least susceptible strains to fungal extract. According to Ranjbar & Farahani (2019), several mechanisms, including a decrease in cellular permeability, drug extrusion by active efflux pumps, overexpression of enzymes that alter and inactivate medicines, and target modification by mutation, can lead to drug resistance in *Shigella* spp. Furthermore, Li *et al.* (2022) reported that the main mechanisms that give *K. pneumoniae* antibiotic resistance are drug efflux, biofilm formation, enzymatic drug inactivation, modification of drug targets, and decreased permeability due to loss or modification of porins.

The findings revealed that the FBEAE of fungal isolates OS-93, OS-64, and OS-94 showed bactericidal effects against Gram-positive and Gram-negative bacteria since their MBC/MIC ratio is less than or equal to 4. On the other hand, the FBME of all fungal isolates possesses bacteriostatic effects against Gram-positive and Gram-negative bacteria with an MBC/MIC ratio of more than 4. According to Huang *et al.* (2021), an antimicrobial agent is regarded as bactericidal if the ratio is less than 4, bacteriostatic if it is greater than 4, and resistant if it is greater than

32. However, both bacteriostatic and bactericidal drugs provide advantages when employed as antibiotics against harmful microorganisms (Zulkamal *et al.* 2023). Considering mixtures of bacteriostatic and bactericidal drugs to either class of agent alone, however, may enhance clinical outcomes. Using more than one agent increases the complexity of the situation since different agents have different modes of action and pharmacokinetics, which might affect whether the effects of combined agents are antagonistic, additive, or synergistic when given simultaneously. Because a certain antibacterial agent may be bacteriostatic to one organism but bactericidal to another, the particular infecting organism will also have an impact. For instance, chloramphenicol is bacteriostatic against the majority of enteric Gram-negative bacteria, and it is bactericidal against *Haemophilus influenzae* and *Streptococcus pneumoniae* at therapeutically feasible concentrations (Finberg *et al.* 2004). The present study also revealed that fermentative broth showed a higher inhibitory effect against test bacteria compared to fungal biomass. A similar observation was reported by Ibrahim *et al.* (2018), who determined the anti-candidal activity of an endophytic fungus isolated from Cassia siamea Lamk leaf, *Aspergillus flavus* IBRL-C8. They revealed that bioactive compounds were released extracellularly into the fermentation medium by the fungus exhibiting strong anti-candidal activity. Nevertheless, the methanolic extract that was taken from the fungal biomass did not exhibit any anti-candidal activity, indicating that no intracellular anti-candidal activity was found.

The Time-kill kinetics assay is a useful tool for examining how well an antimicrobial drug works against a particular strain of bacteria. Using this technique, researchers can ascertain the bacteriostatic or bactericidal effect of antimicrobial agents over time. The findings revealed that lower extract concentrations could induce the re-growth of persistent bacterial cells, and the bactericidal effect of the extract was observed at higher extract concentrations (2 × MIC value). A similar observation has been reported by Zulkamal *et al.* (2023). This phenomenon is believed to be due to the sub-MIC effect of the antibiotic, whereby the low antibiotic concentration can enhance bacterial growth through a stress response mechanism. The bacterial cells treated with low extract concentrations may experience the post-antibiotic effect, where the remaining bacteria begin to proliferate once more (Taufiq & Darah 2019). A biphasic kill curve pattern was noticed when calculating the percentage of live cells as a function of exposure time to a condition of interest. It usually exhibits a sharp drop at first, followed by a plateau or slower decline, suggesting the existence of persister cells, a tiny subpopulation of specialized survivor cells. Bacterial persister cells are a subpopulation of cells that are resistant to antibiotics yet are nevertheless able to withstand prolonged use (Balaban *et al.* 2019). In addition to that, the re-growth of bacterial cells may be due to antibiotic tolerance and antibiotic-resistant cells. An antibiotic-resistant cell is a cell that carries a resistance component (such as an efflux pump) and withstands antibiotic treatment. Antibiotic concentrations that would inhibit the growth of more sensitive bacteria can support the growth of resistant bacteria due to resistance factors. Meanwhile, an antibiotic-tolerant cell is a cell that can regenerate after the antibiotic has been removed and that endures antibiotic treatment without harbouring a resistance factor. Tolerant cells are frequently not developed before antibiotic administration; however, this is not always the case. Bacteria with tolerance factors can withstand a course of therapy that might otherwise kill more vulnerable bacteria. These tolerance factors may be hereditary or environmental (Balaban *et al.* 2019). The present study revealed that a low concentration of extract led to bacteriostatic action; meanwhile, bactericidal action can be observed at higher extract concentrations. The findings also demonstrated that a shorter exposure period was needed to kill bacterial cells at higher extract concentrations.

Taufiq & Darah (2018) postulated that the extract causes the creation of tiny dents and cavities at greater concentrations (above the MIC value). As the concentration rose and the exposure period extended, the sizes and depths of the dents and cavities correspondingly increased. The cell wall and cell membrane were destroyed or degraded, which may also have suggested a cell membrane rupture. This hypothesis has been supported by Hartmann *et al.* (2010), who reported that the emergence of dents, craters, and holes on the surface of bacterial cells suggested that the cell wall and membrane had failed or had ruptured. Furthermore, an unusual cell shape was observed, as indicated by a red arrow. This occurrence may be due to leakage of the bacterial cell wall that triggers osmotic stress and thus allows excessive water to move into the cells. The excessive water in the bacterial cell causes cell swelling and may lead to cell bursts. A previous study revealed that the MRSA cells treated with acetone extract, when paired with vancomycin, resulted in larger cells, and cells treated with acetone extract alone had an uneven and non-uniform shape (Basri *et al.* 2013). Watanabe *et al.* (1997) postulated that antibiotics may have encouraged bacterial cells to develop thick cell walls by accelerating the process of cell wall production, which prevented cell division. Moreover, certain antibiotics, such as aminoglycosides, can stop the first stage of DNA replication, which prevents bacteria from dividing into new cells. However, they cannot stop the bacteria from synthesizing proteins (Dubin *et al.* 1963; Matsunaga *et al.* 1986). The present study also demonstrated that the bacterial cells exposed to the fungal extract are compactly resembling compared to the control. A similar observation by Taufiq & Darah (2018) revealed that the bacterial cells resembled and clustered together compactly after being treated with fungal extract. This phenomenon might be due to a stress response. Furthermore, bacterial cells adopt a unique metabolic scavenging mechanism called cell-to-cell clustering to get poised to face additional stress environments (Bible *et al.* 2015).

Endophytic fungi produce a wide range of bioactive secondary metabolites that exhibit antimicrobial activity against various pathogenic bacteria. The mechanism by which these fungal extracts act involves multiple cellular targets and processes, such as disruption of bacterial cell membranes, inhibition of DNA and RNA synthesis, interference with protein synthesis, inhibition of enzymatic activity and metabolic pathways, and disruption of quorum sensing and biofilm formation. Many endophytic fungal metabolites, such as terpenoids, alkaloids, and phenolic compounds, interact with bacterial cell membranes, leading to increased permeability and membrane disruption. This causes leakage of essential ions and cellular contents, ultimately resulting in cell lysis and death (Strobel & Daisy 2003; Kusari *et al.* 2012).

Moreover, certain compounds in fungal extracts, such as polyketides and quinones, interfere with nucleic acid synthesis by targeting enzymes involved in DNA replication or transcription. This hampers the bacteria's ability to replicate and survive (Nicoletti & Fiorentino 2015). Some fungal metabolites can bind to bacterial ribosomes or disrupt the function of translation-related enzymes, inhibiting the production of essential proteins (Kharwar *et al.* 2011). This phenomenon can have a bacteriostatic or bactericidal effect depending on the compound and its concentration. Besides that, endophytic fungal extracts may also block key bacterial metabolic enzymes or pathways, such as those involved in energy production, leading to metabolic collapse (Zhang *et al.* 2006). Some extracts can inhibit quorum sensing, which is the chemical communication used by bacteria to coordinate gene expression, virulence, and biofilm formation. By disrupting these processes, fungal metabolites reduce bacterial pathogenicity and increase susceptibility to antimicrobial agents (Rajamanikyam *et al.* 2017). Overall, the antimicrobial action of endophytic fungal extracts is typically multifaceted, targeting bacterial cells at various structural and functional levels, leading to growth inhibition or bactericidal effects. However, one significant limitation of this study is the lack of detailed information regarding the specific phytochemical compounds present in the fungal extract. While the extract may demonstrate observable inhibitory effects, the absence of compound identification limits the understanding of the underlying mechanisms responsible for these effects. Without phytochemical profiling such as GC-MS, LC-MS, or NMR analysis, it is not possible to determine which metabolites are responsible for the biological activity, nor can their concentration, potency, or synergistic effects be assessed. This knowledge gap hinders reproducibility, standardization, and further development of the extract for pharmaceutical or therapeutic applications. Moreover, unidentified or uncharacterized compounds may also pose safety concerns or interfere with other biological pathways, which cannot be evaluated without proper compound analysis.

Several methods have been employed to identify endophytic fungal isolate IBRL OS-64, including microscopic and macroscopic observations and molecular identification. The macro-morphology observation was performed using different agar media. The macro-morphology of the isolate IBRL OS-64 was matched with the characteristics that Kwon *et al.* (2017) described. On PDA agar, the fungal isolate was initially white and then turned grey after two weeks of incubation. The findings were in line with Pipattanapuckdee *et al.* (2019), who revealed that the colony of *Lasiodiplodia pseudotheobromae* FRLP1 on PDA turned pale grey from its initial white hue with hairy aerial mycelia after two weeks of incubation. The morphology of endophytic fungi often varies dramatically when they are grown on different agar media such as PDA (potato dextrose agar), SBA (Sabouraud dextrose agar), MEA (malt extract agar), or CMA (corn meal agar). The morphology of endophytic fungi varies on different media, which could be due to nutrient composition & concentration, pH and medium acidity, additives & supplements, physical properties such as agar gel strength, and selective & differential media effects. PDA is rich in starch and dextrose, promoting rapid growth and dense, often irregularly pigmented colonies. In contrast, nutrient-poor media like CMA yield thinner, more transparent colonies due to limited resources. SBA has high glucose and low pH (approximately 5.6), favouring fungal growth but sometimes suppressing sporulation compared to other media. Each medium has a slightly different pH (for instance, PDA ~5.6, SBA ~5.6), which influences enzyme activity, pigment production, and morphological traits like hyphal density. Some media include peptones or host plant extracts that affect colony density and structure. For instance, adding peptone to cereal-based agar creates thicker, more robust mycelia; excluding it leads to thinner, clearer colonies (Chioza & Ohga 2013). Variations in agar concentration and gel strength affect how easily fungi penetrate or spread on the surface. For example, PDA gels differently from MEA, which can result in divergent colony textures. Specialized agars highlight specific fungal traits: CMA favours chlamydospore formation (helpful in differentiation), MEA enhances sporulation and pigmentation, while others may suppress fast growers to let slow-growing endophytes thrive (Gwon *et al.* 2022). By selecting the medium thoughtfully, researchers can enhance traits like sporulation, pigmentation, or structural features that are critical for fungal identification and research. Identification via molecular approach was performed to confirm that the fungal isolate IBRL OS-64 belonged to the genus *Lasiodiplodia*. According to Guo *et al.* (2011), ITS regions of the ribosomal operon are the best tool in fungal identification and classification. Hence, the present study suggested that an endophytic fungus isolated (OS-64) from *O. sanctum* leaf was *Lasiodiplodia pseudotheobromae* based on microscopic.

In conclusion, the present study demonstrates that endophytic fungi isolated from *Ocimum sanctum* leaves exhibit significant antibacterial activity, particularly against Gram-positive pathogenic bacteria. Among the isolates tested, OS-64 showed the most potent activity and was identified as *Lasiodiplodia pseudotheobromae*. The higher antibacterial effect observed in the fermented broth ethyl acetate extract (FBEAE) compared to fungal biomass methanolic extract (FBME) suggests that the active bioactive compounds are secreted extracellularly. Furthermore, the extract exhibited both bacteriostatic and bactericidal effects in a concentration-dependent manner, as confirmed by broth microdilution and time-kill assays. Scanning electron microscopy (SEM) provided visual evidence of bacterial cell wall damage and leakage, supporting the proposed mechanism of action. These findings highlight the potential of *O. sanctum*-derived endophytic fungi, particularly *L. pseudotheobromae*, as promising sources of natural antibacterial agents.

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