OPTIMIZATION OF FERMENTATION PARAMETERS FOR ENHANCED PRODUCTION OF ANTIFUNGAL METABOLITES FROM *ASPERGILLUS TERREUS* AGAINST MUCORALEAN FUNGI

Tamanna Tandona and Pranay Jain*a

a-Department of Biotechnology, University Institute of Engineering and Technology,

Kurukshetra University Kurukshetra, 136119, Haryana, India.

* For Correspondence: E-mail: drpranayjain@gmail.com

Highlights of the Study

Organism Studied: Endophytic fungus *Aspergillus terreus* was used for antifungal metabolite production.

Target Pathogen Group: The metabolites produced were active against Mucorales, a group of fungi responsible for mucormycosis.

Fermentation Optimization:

Carbon Source: 2% glucose found to be optimal.

Nitrogen Source: 2% ammonium sulphate supported maximum yield.

Growth Medium: Potato Dextrose Broth (PDB) provided the best environment.

Physical Parameters: Incubation at 35 °C and pH 6 yielded the highest metabolite production.

Outcome: Optimized conditions significantly improved the production of antifungal metabolites with potential therapeutic value.

What's new about this Study?

Novel Host-Associated Strain: Utilization of an endophytic strain of *A. terreus* is relatively less explored for anti-Mucorales activity.

Target-Specific Metabolite Production: Focused optimization to produce metabolites specifically effective against Mucorales, which are challenging pathogens due to their resistance and severity in immunocompromised patients.

Tailored Fermentation Conditions: The study provides a detailed optimization profile, establishing a reproducible method for enhancing metabolite yield under solid-state fermentation.

Potential Therapeutic Application: Offers an eco-friendly and scalable biotechnological approach for developing natural antifungal agents as alternatives to synthetic drugs.

Article Highlights

Organism Studied: Endophytic fungus *Aspergillus terreus* was used for antifungal metabolite production.

Target Pathogen Group: The metabolites produced were active against order Mucorales, a group of fungi responsible for mucormycosis.

Fermentation Optimization:

Carbon Source: 2% glucose found to be optimal.

Nitrogen Source: 2% ammonium sulphate supported maximum yield.

Growth Medium: Potato Dextrose Broth (PDB) provided the best medium.

Physical Parameters: Incubation at 35 °C and pH 6 yielded the highest metabolite production.

Outcome: Optimized conditions significantly improved the production of antifungal metabolites with potential therapeutic value.

ABSTRACT

The parameter to be employed for optimization is one of the core components of the microbial production system. The right physiological conditions are provided by an optimal growth environment, which enhances a chosen strain's capacity to produce antimicrobial compounds. Numerous physical elements, such as temperature, pH, inoculum size as well as nutritional variables, such as growth medium and sources of carbon and nitrogen, affect the generation and efficacy of bioactive chemicals. Various fermentation parameters, including the fermentation medium, incubation temperature, pH, and carbon and nitrogen sources, were optimized in order to achieve the best possible anti-mucorales metabolite production through fermentation by the endophytic fungi *Aspergillus terreus*. The maximum production of the antifungal metabolites by *A. terreus* strain was achieved in Potato dextrose broth medium supplemented with glucose (2%) as carbon and ammonium sulphate (2%) as nitrogen source. Further process parameters, like incubation temperature at 35 ℃, pH-6, found to be optimum for the maximal production of anti Mucorales metabolites.

Key words- Optimization**,** *Aspergillus terreus*, Fermentation, Antifungal metabolite.

1. INTRODUCTION

The most urgent issue facing humanity right now is the emergence and spread of new infectious illnesses. Drug resistance in pathogenic microorganisms has raised significant risks to human health and well-being in recent decades, and the rise of new and deadly viral diseases has further escalated the problem. The continuing COVID-19 pandemic, which is the result of a new corona virus outbreak that has killed over 6.5 million people worldwide, is perhaps the clearest example of this (1). The overuse of pesticides, herbicides, rodenticides, and fungicides in farming and agriculture has also led to disastrous environmental issues. These harmful chemical compounds unavoidably build up in the environment as a result, which causes biological diversity to decline, bacteria to become more resistant, and land and aquatic habitat to become contaminated (2). Antimicrobial resistance in the microbial community is a result of excessive antibiotic use. There is a constant need to find completely new and incredibly powerful biologically active chemicals that can relieve various burden auxiliary without using harmful, artificial, and ineffective chemical agents in order to address some of these issues. Compounds derived from natural sources are common and often show significant and all-encompassing biological activity. They also have less harmful and more environmentally friendly properties.

Traditionally, plants, animals, fungi, and microbes are the sources of natural products. Recent developments in technology have caused the emphasis on finding new compounds to change from these natural sources to screening compound libraries made up of molecules that have been chemically created. Such high-throughput regimen did not show much promise, despite their seeming potential expectations.

As a result, interest in natural chemicals as an alternative source of new compounds with therapeutic qualities is once again growing (3). A wide range of novel therapeutic natural chemicals, especially those with anticancer and antibacterial capabilities, have been found through research on a variety of plants, bacteria, fungi, and animals (4). Because protein-protein links are formed during interaction, leading to the rare development of microbe resistance, the use of naturally occurring antimicrobial compounds is recommended. Endophytes are an unstudied group of microorganisms that have recently come to light as a possible source of these naturally occurring chemicals. Within the robust plant's internal tissue structure, endophytic microbes flourish without causing any harmful symptoms. It has been shown that they produce a wide range of biologically active compounds that support the host plant's inclusive, healthy growth while coexisting in a mutually advantageous arrangement. These compounds were found to have bioactivities that could have potential uses in industries, agriculture, and medicine (5). Although it is widely accepted that at least one endophyte is present in the three million plant species that currently exist, just a few of these endophytic microorganisms have been investigated and their associated microbial diversity evaluated. However, the variety of compounds associated with endophytes is astounding, comprising a vast array of biological characteristics and structures.

Furthermore, the vast majority of the endophytic fungal species are currently unknown, with estimates of up to 10 lakh different species being possible (6). Endophytic microorganisms are a very potential resource for investigating new bioactive chemicals because of the previously described factor.

Endophytic fungi have a strong association with their host and rely on the nutrients in plants to exist. They assist plants in numerous ways, such as producing metabolic products that have biological activity like growth hormone and protecting them against microbes that cause illness. The endophyte's secretions contain antioxidant, antifungal, and antibacterial properties, among others (7). Some fungi, including *Aspergillus*, *Penicillium*, and *Fusarium*, produce a variety of metabolites that have biological effects, including antiviral, antibacterial, and anticancer properties. Wherein *A. terreus* produces certain antibiotic and antifungal compounds, including terremid A, terremid B, and terrein. When grown in submersible fermentation with barley flour black as a carbon source, *A. terreus* also generates enzymes like invertase and β-glucosidase; it also produces β-xylanase and cis-aconitic decarboxylase (CAD), an essential enzyme that makes itaconic acid, beta-glucosidase, etc.(8).

Neem is a traditional medicinal plant in India that has antibacterial, antifungal, anti-cancer, antimenorrhea, antioxidant, and anti-inflammatory qualities. Ayurvedic medicine has long utilized the plant because nearly all of its constituents have medicinal benefits. Neem is an ideal host for endophytic fungal isolation due to its unique geographic distribution and therapeutic qualities. The aim of the present research was to

bioprospect the variety of fungi linked to neem for its antifungal compounds and properties. A novel line of inquiry with potential for future use is the effect of environmental factors on endogenous fungus that can produce highly active metabolites using the agar well diffusion method.

2. Materials and Methods

2.1 Isolation and screening of endophytic fungi for anti Mucorales activity from medicinal plant-Neem

A medicinally valued neem plant native to the Karnal district in the Indian state of Haryana yielded fresh, healthy plant specimens (leaf, bark, and stem) free from any obvious mechanical damage. To reduce the chance of contamination, plant parts were gathered and sent straight to the research facility in sterile zip bags, where they were processed right away. (9). Healthy explant segments (root, stem, and leaf) had their outermost layer of particle matter removed by two to three washings with regular tap water and then distilled water. Plant samples were carefully extracted, then immersed in 70% ethanol for 60–70 seconds, steeped in 2% NaOCl solution for 120 seconds, and then dipped in 70% ethanol once more for 60 seconds. This was followed by two or three thorough washings in autoclaved distilled water. One hundred microliters of distilled water from the most recent washing was applied to Mueller Hinton Agar (MHA) at 28°C and 37°C to test the sterilization procedure' effectiveness (10). Segments (5 × 5 mm) that had been surface sterilized were aseptically placed on Potato Dextrose Agar (PDA) plates and cultured at 28±2°C for five to seven days. Streptomycin, a broad spectrum antibiotic, was added to PDA medium to inhibit the growth of the bacterial population (11). Fungal growth was regularly monitored on the plates.

2.2 Identification and purification of Endophytic fungi

Fungi were selected based on different physiological characteristics and they then underwent purification by transferring them onto new potato dextrose agar medium plates. For the initial characterization, macroscopic characteristics such as aerial mycelium, colony colour, mycelium pattern, and pigmentation were employed. Lacto phenol cotton blue staining was used to examine further microscopic characteristics such as conidiophores, hyphae structure, conidia, etc. (12). The most promising isolated endophyic fungi was subjected to CSIR MTCC, IMTECH Chandigarh for characterization and molecular identification based on ITS sequencing data (18s sequencing) (Figure 1 and 2).

2.3 Solvent extraction

Fungal discs with a diameter of 6 mm and freshly developed (4-5 days) were placed in 500 ml of potato dextrose broth and kept at 28±2°C for 10–14 days. Whatman filter sheets were used to separate the fermentation broth from the mycelium following completion of the incubation time. By mixing fermentation broth with ethyl acetate (1:1 ratio), the bioactive secondary metabolites that were generated were extracted. To create layers of ethyl acetate minutes and then allowed to rest overnight. To create crude metabolic extracts, ethyl acetate was evaporated at 32°C using a vacuum evaporator.

Fungal metabolites extracted in ethyl acetate were evaluated for their anti-Mucorales properties using the Agar Well Diffusion technique. On MHA, 120 µl suspensions of standardized test microorganisms were evenly distributed using sterilized loop.

Agar plates were pierced with 6 mm-diameter wells using a sterile cork borer. 200 µl of the crude extract solution was put into punctured wells after it had been mixed with 10% DMSO. Agar plates were incubated at 30±2°C for 24 to 48 hours after being left undisturbed for two hours to allow the extracts to diffuse. The Hi Antibiotic Zone Scale was used to assess the establishment of discrete zones of inhibition against the test organism surrounding the agar wells in milli-meters.

2.4 Procurement and maintenance of test microorganisms

Two pure lyophilized test organisms were procured from MTCC-CSIR- Institute of Microbial Technology, Chandigarh, India; (MTCC No. 262) *Rhizopus oryzae* and (MTCC No. 148) *Absidia blakesleeana.* By aseptically transferring lyophilized cultures into sterile potato dextrose broth to revive the test microbes. The recovered microorganisms were kept in 20% glycerol medium at -20°C and on slants at 4°C.

2.5 Optimization of Fermentation Process for maximum metabolite production for anti-Mucorales activity

Prior to standardizing the subsequent parameter, the approach used for fermentation parameter standardization assesses the impact of each individual parameter and incorporates it at a standard level. Incubation temperature, initial substrate moisture content, initial pH adjustment with 1N HCl or 1N NaOH, and inoculum volume were the process parameters that were optimized. Additionally, the impact of extra supplements such as carbon sources, and nitrogen sources was investigated. Every experiment was carried out in triplicate, and the result given is mean of the three values ± standard deviation (13).

2.5.1 The effect of fermentation medium on the production of anti- Mucorales metabolite

Selected fungi were first grown in 100 milliliters of various growth media, such as Sabouraud dextrose broth (SDB), Czapex dox broth (CDB), Muller-Hinton broth (MHB) and potato dextrose broth (PDB), while all other parameters remained unchanged. The fermentation broth was filtered and examined for anti-*Mucorales* activity following a 7-10 days of incubation period at 30±2°C on PDA petiplates. Maximum anti-fungal activity was the criterion used to choose the optimized medium (14; 15). By adjusting a number of physiological and chemical parameters, including incubation time, growth temperature, medium pH, carbon and nitrogen sources, the percentage of selected carbon and nitrogen sources, and the size of the fungal inoculum, other cultural growth parameters were also optimized in the chosen growth medium. Similar to this, various physiological and chemical parameters were optimized to maximize other cultural growth parameters in the selected growth medium.

2.5.2 The effect of pH on the production of anti- Mucorales metabolite

In order to examine the impact of pH, the pH was varied between 4, 6, 7, and 8 under static conditions and incubated at room temperature. After seven days, the culture fluid reached a pH of neutral and was tested using the agar well diffusion method to evaluate for antifungal activity.

2.5.3 The effect of temperature on the production of anti- Mucorales metabolite

The selected endophytic fungi were cultured in 100 milliliters of PDB at temperatures between 25 and 40 degrees Celsius with a 5 degree Celsius interval under stationary conditions while keeping all other parameters constant. Following a seven-day incubation period, the bioactive metabolite's for antifungal activity was evaluated.

2.5.4 The effect of different carbon sources on the production of anti- Mucorales metabolite

200 g of thin potato slices were boiled for 30 minutes in one liter of distilled water to create a potato infusion, which was then filtered through cheesecloth.100 ml of potato infusion and 2 g of various carbon sources, such as galactose, sucrose, fructose, and glucose, are contained in each 250 ml flask. After incubation period of seven days, the antifungal activity for maximum bioactive metabolites was evaluated.

2.5.5 The effect of different nitrogen sources on the production of anti- Mucorales metabolite

In potato dextrose medium supplemented with varying concentrations of nitrogen sources, such as ammonium sulphate, peptone [(NH4)2S0], sodium nitrate {NaN03}, and urea, bioactive metabolite was examined. The selected fungi were incubated for seven days at 30±2°C after being inoculated in media supplemented with different nitrogen sources.

2.5.6 The effect of inoculum size on the production of anti- Mucorales metabolite

By conducting fermentation at varying inoculum levels, the impact of inoculum level was examined. In various flasks, the substrate was inoculated with a disc of fungi of 10mm-16mm size. For 7-10 days, the substrate was incubated at 30 ± 2°C. Following fermentation, the broth was removed and its antifungal activity examined.

3. RESULTS AND DISCUSSION

In the present study, based on macroscopic analysis (growth pattern, colony color, aerial mycelium, pigmentation, and surface texture) and microscopic analysis (hyphae, conidia, conidiophores, and spore structure), isolated endophytic fungi were identified as, *Mucor* sp, *Fusarium* sp, *Alternaria* sp, *Rhizopus* sp, *Aspergillus* sp., *Colletotrichum* sp, *Cladosporium* sp, and *Penicillium* sp. The most promising fungal isolated, which inhibited he growth of both test microbes was found to be *A. terreus*. The most promising isolated endophyic fungi was subjected to CSIR MTCC, IMTECH Chandigarh for characterization and molecular identification based on ITS sequencing data (18s sequencing) (Figure 1 and 2). The phylogenetic tree shows ancestral relations of *A. terreus* (Figure -1).

ITS gene sequence data is as follows:

> *Aspergillus terreus* strain (TT01) small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GCGGCCAGCAACGCGGGCGGGCCCGCCGAAGCAACAAGGTACTATAGTCACGGGTGGG
AGGTTGGGCCATAAAGACCCGCACTCGGTACTTGATCTTGGTGAACCTGCGGAAGGATC
ATTACCGAGTGCGGGTCTTTATGGCCCAACCTCCCACCCGTGACTATTGTACCTTGTTGC
TTCGGCGGGCCCGCCAGCGTTGCTGGCCGCCGGGGGGCGACTCGCCCCCGGGCCCGTGC
CCGCCGGAGACCCCAACATGAACCCTGTTCTGAAAGCTTGCAGTCTGAGTGTGATTCTTT
GCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCA
GCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACG
CACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCA
AGCCCGGCTTGTGTGTTGGGCCCTCGTCCCCCGGCTCCCGGGGGACGGGCCCGAAAGGC
AGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTCGTCTTCCGCTCCGTAGGC
CCGGCCGGCGCCCGCCGACGCATTTATTTGCAACTTGTTTTTTTCCAGGTTGACCTCGG

Following ethyl acetate extraction, extracts from *Aspergillus terreus* fungal strain was examined for anti-Mucorales activity against a range of mucoalean fungi. Extracts of ethyl acetate were reconstituted in 10% of DMSO and was tested against *Rhizopus* and *Absidia*. The presence of an inhibition zone, which was assessed using Hi-media Antibiotic Zone scale, validated the bioactive metabolites mixture's anti-Mucorales activity. The zone of inhibition of anti-Mucorales metabolite observed was 22±1.50 mm and 21 ± 1.35mm respectively against *Rhizopus* and *Absidia.* Antifungal fluconazole was used as positive control and DMSO (10%) as negative control.

3.1.1 The effect of fermentation medium on the production of anti- Mucorales metabolite

A the statistical data generated by growing *Aspergillus* sp. in different growth media showed that PDB (20.5± 1.2 mm of growth of zone of inhibition against test microbes) had the highest fungal growth and metabolite production, followed by SDB (18.25±1.1mm of growth of zone of inhibition) and CDB (11.65±4.4mm of growth of zone of inhibition) (Figure 3). Antifungal fluconazole was used as positive control and DMSO (10%) as negative control.

According to Puji Astuti et al. (2017), the study showed that altering carbon and nitrogen supplies and adjusting the pH and incubation temperature might enhance the production of pyrophen. In an alternative medium, the production rose about ten times. After 12 days of incubation, the endophytic fungus strain *A. fumigatus* reached its maximal growth in potato dextrose medium, and additional components were found in ethyl acetate extracts suggesting its possible research for further bioactive chemicals with pharmacological properties (16).

3.1.2 The effect of temperature on the production of anti- Mucorales metabolite

The most significant physiological factor influencing the rate of growth and yield of secondary metabolites is temperature. It has been demonstrated that temperature has a direct impact on an organism's overall

growth and development. It also has an impact on the physiology, which in turn influences the production of different secondary metabolites. Since it can impact cell viability, inhibition, and death, the incubation temperature is crucial to the development of the fermentation process. The present research demonstrated the impact of varying temperatures on the production of anti-Mucorales metabolites by the *Aspergillus* strain. The maximum production of secondary metabolites observed at 35 ℃ (22.01±0.5 mm) followed 40 ℃ (20.9±1.1 mm) and 30℃ (20.0±0.577 mm) indicating the optimal temperature range (Figure 4). The minimum zone of inhibitions were observed at 25 ℃ (19.33±0.5 mm). Raising the incubation temperature from 25 ℃ to 30 ℃ enhanced the *Aspergillus* growth and secondary metabolite synthesis. Antifungal fluconazole was used as positive control and DMSO (10%) as negative control. According to Puji Astuti et al. (2017), *A. fumigatus* strain exhibited the maximum growth and generation of antibacterial metabolites at 29 °C. The fungus *Rhizoctonia solani* produced the most antibacterial metabolites around 20 ℃ to 25 ℃, according to Ritchie et al. (2009). The study demonstrated that the ideal temperature for *Aspergillus niger* to produce secondary metabolites was 30 °C (17).

3.1.3 The effect of pH on the production of anti- Mucorales metabolite

Microbial growth and the production of secondary metabolites were significantly impacted by the growth medium's pH. The pH affects microbial cell permeability, which in turn affects the rate at which nutrients are absorbed from the medium. A pH range of 4–10 was used to cultivate a specific fungus, *Aspergillus terreus*, in order to evaluate growth and metabolite synthesis. As shown in Fig. 5, the maximum anti-Mucorales activity was observed at pH 6 (22.5±0.52 mm and 20.81±0.31 mm). At pH 8 antifungal activity was recorded 19.53±0.5 mm against *Rhizopus.* Least anti-Mucorales activity was observed at pH 10 due to basic nature of the medium (16.9±86 mm). At high acidic pH-4 no fungal growth was recorded as shown in Figure 3. Antifungal fluconazole was used as positive control and DMSO (10%) as negative control. The p-value 0.0011 and 0.0046 indicates significant results.

Secondary metabolite production is influenced by pH in *Streptomyces* species. According to their research, *Streptomyces carpaticus* produced the most metabolites at a neutral pH. According to Ripa et al. (2009), Streptomyces sp. is unable to produce secondary metabolites at pH 3.0. (Kokare et al. (2007), Thongwai and Kunopakarn (2007) found that the pH range of 5.5 to 8.5 was in which a significant number of microorganisms produced the most antibiotic metabolites. At pH 6.0, *Aspergillus terreus* produced the most secondary metabolites, according to various studies. According to reports, 6.0 pH is optimal for *Fusarium solani* growth and metabolite production. Bundale *et.* al., (2015) examined the process by which pH affected the development and synthesis of a bioactive metabolite by the endophyte *Hypocrea* sp. and found that isolation from *Dillenia indica Linn.* in North-East India achieved a maximum at pH 6.0 (18).

3.1.4 The effect of different carbon source on the production of anti- Mucorales metabolite

Among the tested carbon sources, glucose was the best suitable carbon source for the maximum fungal growth and metabolite production against both *Rhizopus* and *Absidia* observed (22.2±0.3 mm and 22.1±0.2 mm) (Figure-6). On the other hand, sucrose, fructose and galactose produced 20.3±0.57 mm and 18.6±0.5, 11.6 mm against *Rhizopus* and, 18.63±0.54, and 12.00±0.93 mm, 12.00±0.9 mm and 11.33±1.04 mm against

Absidia respectively. Fermentation carried out using a range of glucose concentrations, from 0.5% to 3.5% (w/v), to examine the effects of glucose concentration on the fermentation medium. Based on the data, it can be said that the optimal glucose concentration of 2% (w/v) resulted in the highest synthesis of metabolite. Enzyme synthesis decreased when the concentration of glucose increased further; this could be because larger amounts had an inhibitory effect. Antifungal fluconazole was used as positive control and DMSO (10%) as negative control.

Mannitol supported high rates of *Streptomyces* sp. metabolite synthesis, followed by sucrose and glycerol (18). In the medium supplemented with maltose (1%) as the only carbon source, *A. fumigatus* exhibited high amounts of antimicrobial metabolites. Regarding carbon source species, *Streptomyces* species may exhibit particular variations in cell development and the generation of secondary metabolites (17).

3.1.5 The effect of different nitrogen source on the production of anti- Mucorales metabolite

Nitrogen sources can have a significant impact on fungal growth and the production of antimicrobial metabolites. The current study demonstrated how nitrogen sources affect growth and the synthesis of antimicrobial metabolites. Ammonium sulphate was the most effective nitrogen source for achieving the maximum amount of anti-Mucorales activity.

In current research of *Aspergillus terreus* strain, Ammonium sulphate amended medium delivered maximum production for antifungal activity (21.6±0.2 mm and 21.8±0.76 mm against both mucoralren fungi respectively). Urea (19.7±0.6 mm, 19.16±01.2 mm) and sodium nitrate (17. ±0.25 mm, 19.86±0.23) produced moderate amount of anti mucorales metabolite production. Peptone exhibited least anti Mucorales activity (18±0.76 mm against *Rhizopus*) (Figure 7). Antifungal fluconazole was used as positive control and DMSO (10%) as negative control.

To evaluate the effects of ammonium sulphate concentration on the fermentation medium, fermentation was performed using a range of ammonium sulphate concentrations, from 0.5 to 3.5% (w/v). The findings demonstrated that a 2% (w/v) ammonium sulphate concentration produced the highest amount. Because ammonium sulphate has a repressive effect at greater concentrations, further increases in its concentration led to a decrease in the production of enzymes.

Atta et. al., (2011), found that adding sodium nitrate to the Str*eptomyces albidoflavu*s 143 culture medium produced highest antibacterial activity (19). According to Puji Astuti et. al. (2017), biomass was high with sodium nitrate, but bioactive metabolite synthesis from A. *fumigatus* strain was high in medium using yeast extract as a nitrogen source. One of the main elements of complex macromolecules found in living things is nitrogen. It is an essential component of the amino acids needed for the production of a number of bioactive substances (20; 21).

3.1.6 The effect of Inoculum size on the production of anti- Mucorales metabolite

Another significant element affecting the metabolite production in fermentation is inoculum volume. A sufficient inoculum can reduce contamination from other organisms by accelerating mycelium development

and product generation. The metabolite was definitely impacted by the inoculum quantity. Because low spore densities result in insufficient biomass and end product synthesis and allow for the growth of undesired contaminants, and because high spore densities may cause a rapid and excessive biomass production, which in turn leads to rapid nutrient depletion and ultimately a reduction in the quality of the final product, fermentation requires optimization of the inoculum volume. A smaller inoculum level might not be enough to start growth, whereas a higher level might result in competitive inhibition.

The fungal strain cultures in 250 ml flask showed maximum metabolite production (as per Figure-8) at 12 mm (21.6±0.5 mm, 21.9±0.7 mm against *Rhizopus* and *Absidia*) at after 7 day of incubation. Antifungal fluconazole was used as positive control and DMSO (10%) as negative control. A declining shift was observed as per inoculum days increased due to accumulation of toxic substance (22).

CONCLUSION

Ethyl acetate extracts of fungal strains associated with the medicinally valued Neem plant, which is indigenous to Karnal, Haryana, India, were the main focus of the current study. According to the results of microscopic and morphological analysis, numerous endophytic fungi from various genera were isolated. *Mucor* sp, *Fusarium* sp, *Alternaria* sp, *Rhizopus* sp, *Aspergillus* sp., *Colletotrichum* sp, *Cladosporium* sp, and *Penicillium* sp. were identified as fungal genus. The results of agar well diffusion method showed that endophytic fungal crude ethyl acetate extracts were efficient against opportunistic test microorganisms used in the study. Analysis of the impact of physiological and chemical factors on *Apergillus* sp. growth and metabolite production was a further objective of the study. The maximum production of the antibacterial metabolites by *A. terreus* strain was achieved in Potato dextrose broth medium supplemented with glucose (2%) as carbon and ammonium sulphate (2%) as nitrogen source. Further process parameters, like incubation temperature at 35 ℃, pH-6, found to be optimum for the maximal production of anti Mucorales metabolites. The endophytic microbial population found in medicinal plants may be a useful resource for bio prospecting novel bioactive chemicals, according to this study.

Acknowledgments

Authors are grateful to the Director, University Institute of Engineering and Technology (UIET) and Vice-chancellor of Kurukshetra University Kurukshetra for providing lab facilities. Thanks are also due to two anonymous referees for their comments and suggestions for the improvement of the manuscript.

References

1. Caruso, D. J., Palombo, E. A., Moulton, S. E. and Zaferanloo, B. (2022). Exploring the promise of endophytic fungi: A review of novel antimicrobial compounds. Microorganisms 10: 1990.
2. Badawy, B., Gwida, M., Sadat, A., EL-Toukhy, M., Sayed-Ahmed, M., Alam, N., Ahmad, S., Ali, M. S. and Elafify, M. (2022). Prevalence and antimicrobial resistance of virulent *Listeria monocytogenes* and *Cronobacter sakazakii* in dairy cattle, the environment and dried milk with the in vitro application of natural alternative control. Antibiotics 11: 1087.

3. Kaur, N., Kumar, R., Alhan, S., Sharma, H., Singh, N., Yogi, R., Chhokar, V., Beniwal, V., Ghosh M., Chandraker, S., Rustagi, S. and Kumar, A. (2024). Lycium shawii–mediated green synthesis of silver nanoparticles, characterization and assessments of their phytochemical, antioxidant, antimicrobial properties. Inorg Chem Com. 159: 111735.
4. Cui, G., Pan, C., Xu, P., Li, Y., Wang, L. and Gong, B. (2020). Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* CG9 from human saliva. Biotechnol.Biotec. Equipment. 34: 1224-1233.
5. Harrison, J. G. and Griffin, E. A. (2020). The diversity and distribution of endophytes across biomes, plant phylogeny and host tissues: How far have we come and where do we go from here? Environ. Microbiol. 22: 2107-2123.
6. Ahmed, A. M., Ibrahim, A. M., Yahia, R., Shady, N. H., Mahmoud, B. K., Abdelmohsen, U. R. and Fouad, M. A. (2023). Evaluation of the anti-infective potential of the seed endophytic fungi of *Corchorus olitorius* through metabolomics and molecular docking approach. BMC Microbiol. 23: 355.
7. Jain, P., & Pundir, R. K. (2010). *International Journal of Pharmaceutical Sciences Review and Research*, **5**(3), 72–76.
8. Gu, W., & Qiao, C. (2012). Furandiones from an Endophytic *Aspergillus terreus* residing in *Malus halliana*. *Chemical and Pharmaceutical Bulletin*, **60**, 1474–1477.
9. Mbilu, M., Wanyoike, W., Kangogo, M., Bii,C. C., Agnes, M. and Kihia, C. M (2018). Isolation and characterization of endophytic fungi from medicinal plant, *Warburgia ugandensis*. J. Biol. Agri. Healthcare 8: 57- 66.
10. Mikolajczyk, O. M., Drozdzynski, P., Polewczyk, A., Smulek, W. and Antczak, T (2021). Biosurfactant from endophytic *Bacillus pumilus* 2A: Physico-chemical characterization, production and optimization and potential for plant growth promotion. Microb Cell Fact. 20: 01-11.
11. Boghsani, T. Y., Ganjeali, A., Cernava, T., Müller, H., Asili, J. and Berg, G. (2020). Endophytic fungi of native *Salvia abrotanoides* plants reveal high taxonomic diversity and unique profiles of secondary metabolites. Front. Microbiol. 10: 3013.
12. Samapti, M. M. S., Afroz, F., Rony, S. R., Sharmin, S., Moni, F., Akhter, S., Ahmed, S. F. U. and Sohrab, M. H. (2022). Isolation and identification of endophytic fungi from *Syzygium cumini Linn*. And investigation of their pharmacological activities. Sci. World J. 2022: 9529665. doi: 10.1155/2022/ 9529665.
13. Balakrishnan, K., & Pandey, A. (1996). Production of biologically active secondary metabolites in solid state fermentation. Journal of Scientific and Industrial Research, **55**,365–373.
14. Verma, S. K., Lal, M., & Debnath, M. (2017). Optimization of process parameters for production of antimicrobial metabolites by an endophytic fungus *Aspergillus* sp. cpr5 isolated from *Calotropis procera* root. *Asian Journal of Pharmaceutical and Clinical Research, 10*(4), 225.
15. Kalyani, P., Botsa, S. M., Laxmi, K. V. D., & Saragonda, A. (2023). Optimization of cultural conditions for biomass and antibacterial metabolite production by *Aspergillus fumigatus* strain MF1. *Hybrid Advances*, *2*, 100016.

16. Grabka, R., d'Entremont, T. W., Adams, S. J., Walker, A. K., Tanney, J. B., Abbasi, P. A. and Ali, S. (2022). Fungal endophytes and their role in agricultural plant protection against pests and pathogens. Plants 11: 384.
17. D.K. Gogoi, H.P. Deka Boruah, R. Saikia, T.C. Bora, Optimization of process parameters for improved production of bioactive metabolite by a novel endophytic fungus *Fusarium* sp. DF2 isolated from *Taxus wallichiana* of North East India, World J. Microbiol. Biotechnol. 24 (1) (2008) 79–87.
18. S. Bundale, D. Begde, N. Nashikkar, Optimization of culture conditions for production of bioactive metabolites by *Streptomyces* spp. isolated from Soil, Adv. Microbiol. 5 (2015) 441–451.
19. H.M. Atta, M.H. El-Sehrawi, N.M. Awny, N.I. El-Mesady, Microbiological studies on cultural, physiological characteristics and antimicrobial activities of Streptomyces cyaneus-AZ-13Zc, Researcher 3 (2) (2011) 80–90.
20. Mikolajczyk, O. M., Drozdzynski, P., Polewczyk, A., Smulek, W. and Antczak, T (2021). Biosurfactant from endophytic *Bacillus pumilus* 2A: Physico-chemical characterization, production and optimization and potential for plant growth promotion. Microb Cell Fact. 20: 01-11.
21. Vo, T. N. M., Nguyen, D. N., & Nguyen, V. T. (2020). *Optimization of Culture Conditions on the Proliferation of Aspergillus terreus N-GL1 Strain Isolated from Curcuma longa L. by Design-Expert 6.0.6 and BC Pharsoft Software.*
22. Varalakshmi, V., & Karothi, J. R. (2013). Optimization of L-asparaginase production by *Aspergillus terreus* MTCC 1782 using Bajra seed flour under solid state fermentation. *International Journal of Research in Engineering and Technology*, *2*(9), 2321–7308.

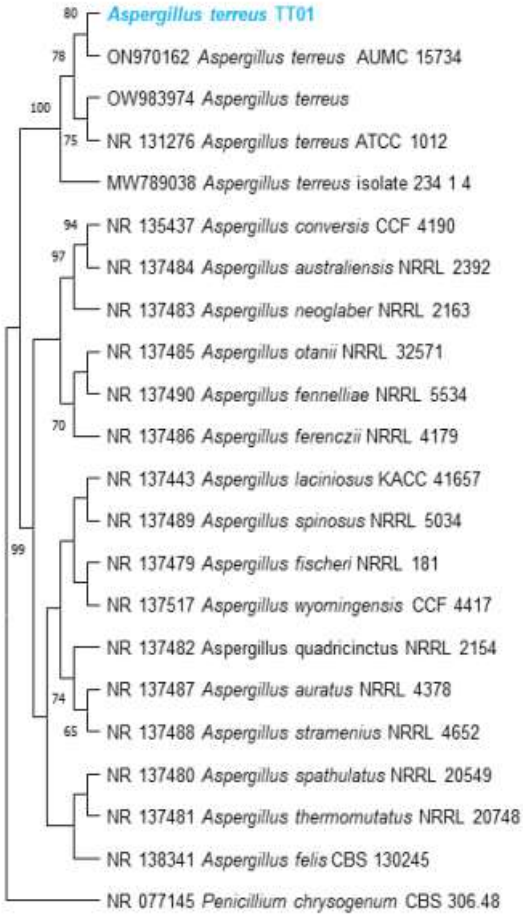

Figure 1: Phylogenetic placement of *Aspergillus terreus* TT01 by a Maximum parsimony (MP) analysis of a dataset of the ITS region.

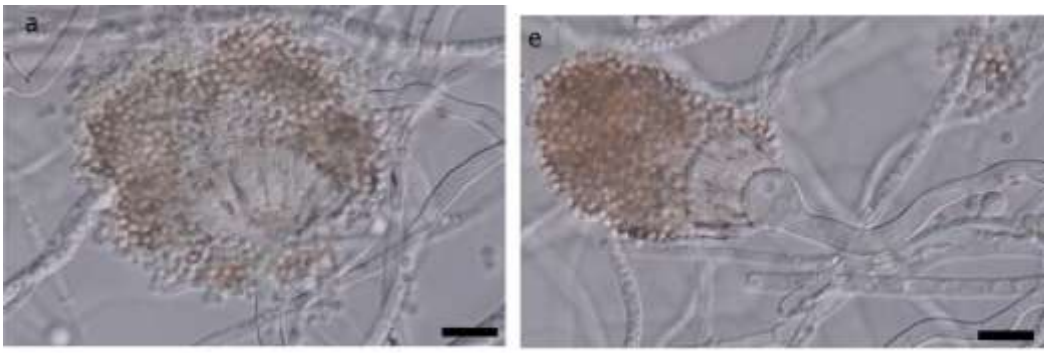

Figure 2: Microscopic morphological characteristics of *Aspergillus terreus* endophytic fungi isolated. (Microscopic images provided and Characterised from CSIR-Institute of Microbial Technology (CSIR-IMTECH), Chandigarh).

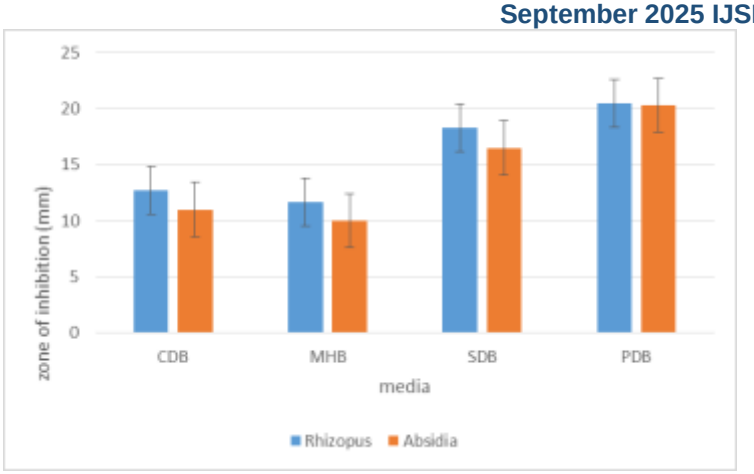

Figure 3: Growth media optimization for maximum metabolite production for anti-Mucorales activity (mm).

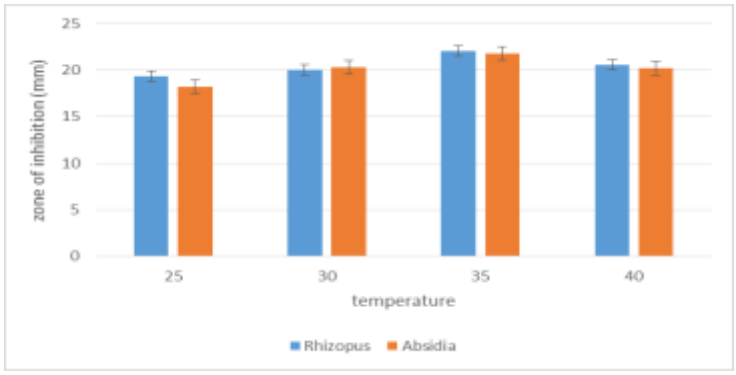

Figure 4: Effect of temperature for maximum metabolite production for anti-Mucorales activity (mm).

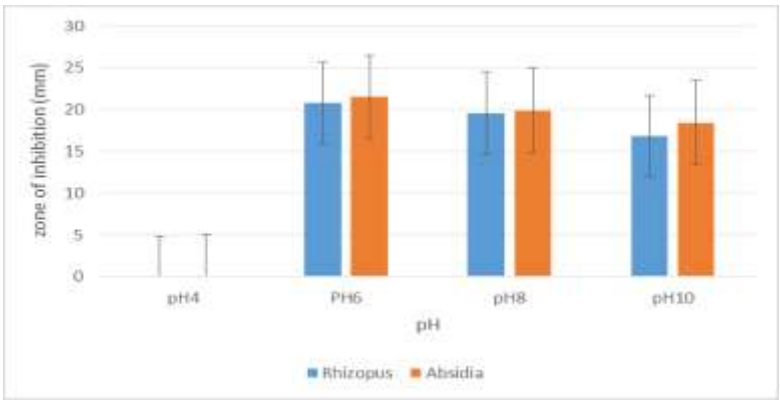

Figure 5: Effect of pH on maximum metabolite production for anti-Mucorales activity (mm). The p-value 0.0011 and 0.0046 indicates significant results.

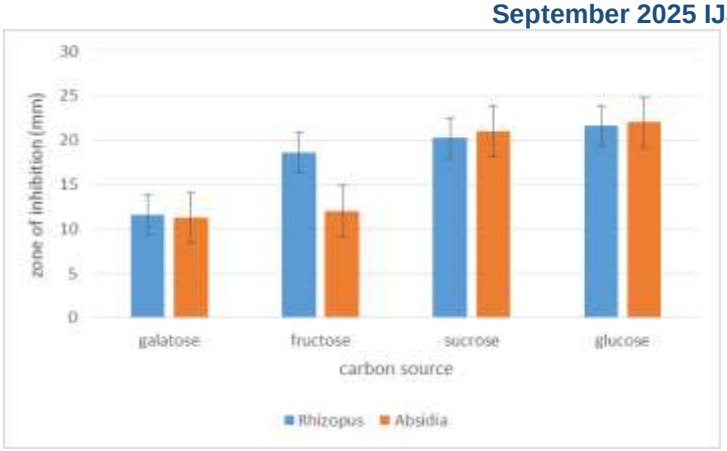

Figure 6: Effect of different carbon source on maximum metabolite production for anti-Mucorales activity (mm).

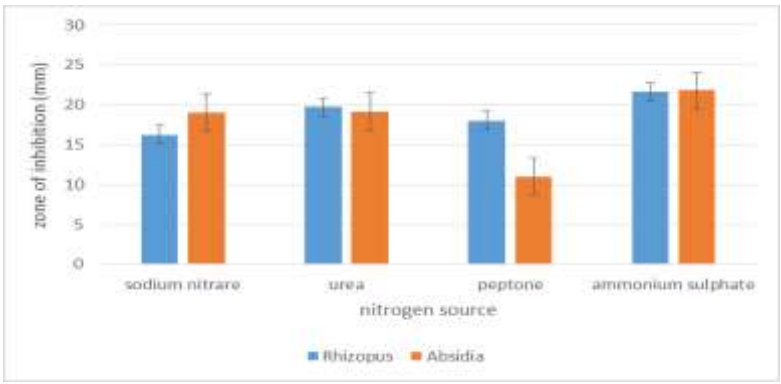

Figure 7: Effect of different nitrogen source on maximum metabolite production for anti-Mucorales activity (mm).

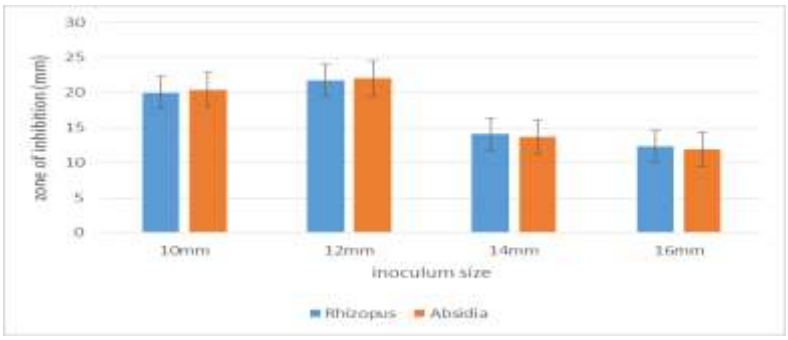

Figure 8: Effect of inoculum size on maximum metabolite production for anti-Mucorales activity (mm)