RESEARCH PAPER

Optimization of Culture Conditions for Antimicrobial Metabolite Production by Endophytic Fungi Associated with Calotropis procera

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ABSTRACT

Endophytic fungi associated with medicinal plants are recognized as promising sources of bioactive secondary metabolites with antimicrobial potential. The present study aimed to isolate endophytic fungi from Calotropis procera and optimize culture conditions for enhanced production of antimicrobial bioactive metabolites. Endophytic fungal isolates were cultured in Potato Dextrose Broth (PDB), and the effects of different physicochemical parameters including pH, temperature, carbon source, nitrogen source, and nutrient concentrations were evaluated. Antimicrobial activity was assessed against Candida, Fusarium, and Alternaria using inhibition zone analysis, while fungal biomass production was determined gravimetrically. Among the tested isolates, EPF-04 and EPF-06 showed significant antimicrobial activity. Maximum metabolite production and biomass formation were achieved at pH 7 and temperature 30°C. Glucose (1%) served as the most effective carbon source, whereas sodium nitrate (0.8%) significantly enhanced antimicrobial metabolite production. Response Surface Methodology (RSM) further confirmed the interaction between pH and temperature, demonstrating maximum biomass production at near-neutral pH and moderate temperature conditions. The findings suggest that optimized culture conditions significantly improve antimicrobial metabolite biosynthesis in endophytic fungi associated with Calotropis procera, indicating their potential application in pharmaceutical and antimicrobial research.

Keywords: Endophytic fungi, Calotropis procera, antimicrobial metabolites, Media optimization, RSM, Biomass Production.

How to cite this article: Khan R, Lal M, Tia N, Jain NK, Chauhan S, Bhadouriya A, Shrivastav L. Optimization of Culture Conditions for Antimicrobial Metabolite Production by Endophytic Fungi Associated with Calotropis procera. Int J Drug Deliv Technol. 2026;16(61s):1561-1571. DOI: 10.25258/ijddt.16.61s.176

Source of support: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest: The authors declare no competing financial interests or personal relationships that could have influenced the work reported in this paper.

INTRODUCTION

2.1 ENDOPHYTIC FUNGI AND THEIR BIOLOGICAL SIGNIFICANCE

Endophytes are microorganisms that inhabit healthy internal plant tissues without causing visible disease symptoms. These microorganisms colonize plant tissues either intercellularly or intracellularly and may remain associated with the host throughout part or all of their life cycle [1]. Endophytes are widely distributed among diverse plant groups, including angiosperms, gymnosperms, ferns, and mosses [2]. Nearly every higher plant species is believed to harbor one or more endophytic microorganisms, making them an extensive and relatively unexplored source of natural bioactive compounds [3].Among endophytes, endophytic fungi have gained increasing attention because of their ability to synthesize structurally diverse secondary metabolites with significant biological activities. These fungi establish a mutualistic relationship with host plants, where they receive nutrients and shelter while simultaneously contributing to host defense through the production of protective metabolites. Several studies have demonstrated that endophytic fungi can produce compounds similar to those synthesized by their host plants, thereby enhancing their pharmaceutical importance [4]. Due to their remarkable metabolic diversity, endophytic fungi are increasingly recognized as promising sources of antimicrobial, antioxidant, anticancer, anti-inflammatory, and antiviral compounds.

2.2 MEDICINAL IMPORTANCE OF CALOTROPIS PROCERA AND ITS ENDOPHYTIC ASSOCIATION

Calotropis procera (Ait.) W.T. Aiton is an erect, branched medicinal shrub belonging to the family Apocynaceae and is characterized by the presence of milky latex throughout all plant tissues. The plant is widely distributed in arid and semi-arid regions and has considerable ethnomedicinal significance in the Indian subcontinent. Traditionally, C. procera has been utilized for the treatment of numerous ailments including skin disorders, inflammation, digestive disorders, respiratory diseases, and microbial infections [5].

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The pharmacological significance of C. procera is largely attributed to the presence of diverse phytochemicals, including alkaloids, flavonoids, glycosides, terpenoids, phenolic compounds, and cardenolides, which exhibit potent antimicrobial, antioxidant, anti-inflammatory, and anticancer properties [6]. In addition to its medicinal importance, C. procera represents an important ecological niche for diverse endophytic microorganisms capable of producing valuable secondary metabolites [7]. The unique biochemical environment of medicinal plants may influence the metabolic potential of associated endophytes, making them promising candidates for the discovery of novel therapeutic compounds.

2.3 NEED FOR MEDIA OPTIMIZATION IN ENDOPHYTIC FUNGAL METABOLITE PRODUCTION

Despite the remarkable antimicrobial potential of endophytic fungi, one of the major challenges associated with their industrial utilization is the comparatively low production yield of bioactive metabolites under normal laboratory conditions. In several studies, metabolite production has been reported at microgram or low milligram levels, which is insufficient for large-scale commercial applications. Therefore, optimization of culture conditions becomes essential to improve fungal growth and secondary metabolite biosynthesis [8].

Culture parameters such as pH, temperature, incubation period, carbon source, and nitrogen source play a significant role in regulating fungal physiology, nutrient uptake, enzymatic activity, and metabolite production. Optimization of culture media is considered a crucial strategy for improving both fungal biomass and metabolite yield. Several studies have reported that nutrient composition significantly influences the diversity and abundance of cultivable endophytes. Supplementation of media with plant extracts or host-derived nutrients has been shown to enhance endophyte cultivation by providing essential growth factors absent in synthetic media, thereby facilitating the growth of difficult-to-culture fungal strains [9].

3.MATERIALS AND METHODS

3.1 COLLECTION OF PLANT MATERIAL

Healthy root samples of Calotropis procera were collected from behind Jiwaji University, Gwalior, Madhya Pradesh, India (26.207523° N, 78.191789° E) in March 2024. Root samples were collected aseptically in sterile polyethylene bags, transported to the laboratory in an icebox, and stored at 4°C until processing.

3.2 ISOLATION OF ENDOPHYTIC FUNGI

Endophytic fungi were isolated from root tissues using a modified method of Schulz et al[10].

3.2.1 SURFACE STERILIZATION

Root samples were washed thoroughly under running tap water, followed by detergent and sterile distilled water. Surface sterilization was performed using 70% ethanol for 1 min and 5% sodium hypochlorite for 15 min, followed by rinsing with sterile distilled water. [11] Sterilized root segments (5 × 5 mm) were placed on Potato Dextrose Agar (PDA) supplemented with streptomycin (500 mg/L) and incubated at 27°C.

3.2.2 STERILITY CHECK

The final rinse water was inoculated onto PDA plates and incubated at 32°C for 48 h to confirm surface sterilization. Absence of microbial growth indicated successful sterilization.

3.2.3 ISOLATION, PURIFICATION, AND PRESERVATION

Surface-sterilized root segments were placed on PDA medium containing chloramphenicol (150 µg/mL) and incubated at 25 ± 2°C. Emerging fungal colonies were purified by repeated subculturing on fresh PDA plates.[12] Pure isolates were identified based on colony morphology and preserved on PDA slants at 5–10°C.A Total of six endophytic fungal isolates were obtained, among which EPF-04 and EPF-06 exhibited maximum bioactive metabolite production and were selected for further study.

3.3 MEDIA OPTIMIZATION STUDY

Media optimization was performed following the method of Lal et al. (2024) with slight modifications to enhance antimicrobial bioactive metabolite production by endophytic fungal isolates. Potato Dextrose Broth (PDB) was prepared according to the manufacturer's instructions, and 100 mL of medium was dispensed into sterile 250 mL Erlenmeyer flasks. [13]The medium was sterilized at 121°C for 15 min and allowed to cool to room temperature.

3.3.1 INOCULATION OF FUNGAL CULTURE

Under aseptic conditions, selected endophytic fungal isolates (EPF-04 and EPF-06) were inoculated into sterile PDB flasks and incubated under shaking conditions for optimization studies.

3.3.2 OPTIMIZATION OF PH

The pH of the PDB medium was adjusted to 6.0, 7.0, and 8.0 using 0.1 N HCl or 0.1 N NaOH prior to sterilization. Inoculated flasks were incubated at 25°C for 7 days at 150 rpm.

3.3.3 OPTIMIZATION OF TEMPERATURE

Fungal cultures were incubated at different temperatures (20°C, 30°C, and 40°C) under optimized pH conditions for 7 days at 150 rpm.

3.3.4 OPTIMIZATION OF CARBON SOURCE

Different carbon sources, including glucose, sucrose, and dextrose, were incorporated separately into modified PDB medium. Cultures were incubated under optimized pH and temperature conditions for 7 days at 150 rpm.

3.3.5 OPTIMIZATION OF NITROGEN SOURCE

Different nitrogen sources, including peptone, yeast extract, and ammonium nitrate, were evaluated for metabolite production. Inoculated cultures were incubated under optimized conditions for 7 days at 150 rpm.

3.3.6 BIOMASS ESTIMATION

After incubation, fungal biomass was separated using pre-weighed Whatman No. 3 filter paper and dried at 40–50°C until constant weight was achieved. Biomass production was determined gravimetrically.

3.3.7 EXTRACTION OF INTRACELLULAR METABOLITES

Dried fungal biomass was powdered and extracted with ethyl acetate for 24 h under shaking conditions. The extract was filtered, and the solvent fraction was collected for further analysis.

3.3.8 EXTRACTION OF EXTRACELLULAR METABOLITES

Culture filtrates were mixed with an equal volume of ethyl acetate in a separating funnel and shaken vigorously for 5–10 min. The organic phase was collected, and extraction was repeated three times for maximum metabolite recovery.

4.RESULTS AND DISCUSSION

4.1 IN VITRO CULTURE OF ENDOPHYTES (SCREENING OF PURE CULTURE)

Endophytic fungi were successfully isolated from the root tissues of Calotropis procera and purified on PDA medium. Different fungal colonies exhibited diverse morphological characteristics,

indicating endophytic diversity. Methanolic crude extracts of fungal isolates showed variable antifungal activity against Alternaria, Fusarium, and Candida species. [14]Among the isolates, EPF-04 and EPF-06 exhibited the highest zones of inhibition against the tested pathogens and were selected for further studies due to their strong antifungal potential. The inhibitory activity increased with extract concentration, with maximum activity observed at 100 µL concentration. No inhibition was observed in the negative controls. These findings suggest that endophytic fungi from C. procera roots are potential sources of bioactive antifungal metabolites.

4.2 MEDIA OPTIMIZATION

4.2.1 EFFECT OF PH ON FUNGAL BIOMASS AND ANTIMICROBIAL METABOLITE PRODUCTION

The pH of the culture medium significantly influenced fungal biomass and antimicrobial metabolite production in endophytic fungal isolates. Among the tested pH levels, pH 7 was found to be optimum for both EPF-04 and EPF-06, showing maximum antimicrobial activity and biomass production. EPF-04 exhibited inhibition zones of 24 mm, 17.4 mm, and 20.7 mm against Candida, Fusarium, and Alternaria, respectively, with biomass of 1.655 g/mL. Similarly, EPF-06 showed highest activity at pH 7 with inhibition zones of 24.2 mm, 17.8 mm, and 20.9 mm and biomass of 1.88 g/mL.Low pH conditions (pH 3–4) resulted in poor fungal growth and reduced antimicrobial activity, while alkaline pH (pH 9) also showed decreased metabolite production and biomass. The results indicate that neutral pH favors nutrient uptake, enzyme activity, and secondary metabolite biosynthesis. Therefore, pH 7 was considered the optimum condition for antimicrobial metabolite production in endophytic fungal isolates.

4.2.2 EFFECT OF TEMPERATURE ON FUNGAL BIOMASS AND ANTIMICROBIAL METABOLITE PRODUCTION

Temperature significantly influenced fungal growth and antimicrobial metabolite production in endophytic fungal isolates. Among the tested temperatures, 30°C was found to be optimal for biomass accumulation and antimicrobial activity. EPF-04 showed maximum biomass production (2.485 g/mL) at 30°C, with strong inhibition zones against Candida (23.2 mm) and Alternaria (18.9 mm).[17] Although maximum inhibition against Fusarium (24 mm) was observed at 40°C, fungal biomass decreased considerably, indicating temperature-induced stress. Lower temperatures (10°C and 20°C) resulted in reduced fungal growth and antimicrobial activity due to slower metabolic and enzymatic processes. At 50°C, both biomass and metabolite production declined significantly, suggesting adverse effects of excessive heat on fungal viability. Therefore, 30°C was identified as the optimum temperature for efficient fungal growth and antimicrobial metabolite production.

4.2.3 EFFECT OF CARBON SOURCE ON FUNGAL BIOMASS AND ANTIMICROBIAL METABOLITE PRODUCTION

Carbon sources play a vital role in fungal metabolism as they serve as the primary source of energy and carbon skeletons required for cellular growth and biosynthesis of secondary metabolites. In the present study, glucose, sucrose, and dextrose were evaluated for their effect on biomass and extract production.Carbon supplementation significantly affected the production of antimicrobial metabolites and fungal biomass.[18] Among the evaluated carbon sources, glucose proved to be the most efficient substrate for fungal growth and metabolite synthesis. The highest inhibition zones were obtained with glucose, measuring 22 mm against Candida, 21 mm against Fusarium, and 16 mm against Alternaria, accompanied by biomass production of 1.655 g/mL.Sucrose supported moderate fungal activity, whereas dextrose produced relatively lower inhibition zones and biomass values. The enhanced performance observed with glucose may be linked to its direct utilization in primary metabolic pathways, thereby supplying sufficient energy and precursor molecules required for secondary metabolite biosynthesis.Optimization of glucose concentration further demonstrated that 1% glucose concentration was ideal for metabolite production. At this concentration, EPF-04 exhibited maximum inhibition zones of 22.3 mm, 17.8 mm, and 20.7 mm against Candida, Fusarium, and Alternaria, respectively. EPF-06 also displayed highest biomass production (1.78 g/mL) and improved antimicrobial activity at the same concentration.Increasing glucose concentration beyond 1% caused a reduction in biomass and inhibition activity, possibly due to substrate inhibition and osmotic imbalance. These findings indicate that glucose at 1% concentration is the most suitable carbon source for antimicrobial metabolite production by endophytic fungal isolates.

4.2.4 EFFECT OF NITROGEN SOURCE ON FUNGAL BIOMASS AND ANTIMICROBIAL METABOLITE PRODUCTION

Nitrogen is an essential nutrient required for protein synthesis, nucleic acid formation, enzyme production, and other metabolic activities in fungi. In this study, yeast and mold extract, peptone, and sodium nitrate ($NaNO_3$) were evaluated as nitrogen sources.Nitrogen source optimization showed a marked effect on fungal biomass and antimicrobial metabolite production. Among the tested nitrogen supplements, sodium nitrate ($NaNO_3$) promoted the highest antimicrobial activity. In EPF-04, $NaNO_3$ supplementation resulted in inhibition zones of 25.5 mm against Candida, 17.9 mm against Fusarium, and 20.8 mm against Alternaria, with biomass production of 1.357 g/mL.Peptone favored fungal biomass accumulation because of its rich organic nutrient composition, including amino acids and peptides that support mycelial growth. However, its antimicrobial activity remained lower than that observed with $NaNO_3$. Yeast extract demonstrated intermediate effects on both growth and metabolite production.Further optimization of $NaNO_3$ concentration showed that 0.8% $NaNO_3$ yielded maximum antimicrobial activity in both EPF-04 and EPF-06 isolates. EPF-04 produced inhibition zones of 26.5 mm, 18.8 mm, and 21 mm against Candida, Fusarium, and Alternaria, respectively, at this concentration. EPF-06 also demonstrated enhanced inhibition activity at 0.8% $NaNO_3$. Biomass production increased progressively with rising $NaNO_3$ concentration, indicating improved fungal growth under optimized nitrogen availability.The Enhanced antimicrobial activity under inorganic nitrogen supplementation may be associated with stimulation of nitrogen-sensitive metabolic pathways responsible for secondary metabolite formation. Overall, sodium nitrate at 0.8% concentration was found to be the most effective nitrogen source for antimicrobial bioactive metabolite production.

CONCLUSION

The present study demonstrated that endophytic fungi isolated from Calotropis procera possess significant potential for antimicrobial secondary metabolite production. Among the isolated strains, EPF-04 and EPF-06 exhibited maximum antimicrobial activity. Optimization of physicochemical parameters revealed that pH 7, temperature 30°C, glucose (1%), and sodium nitrate (0.8%) were the most favorable conditions for enhanced fungal biomass and metabolite production. Response Surface Methodology (RSM) further confirmed the significant interaction of culture parameters in improving metabolite yield. These findings highlight the pharmaceutical potential of Calotropis procera-associated endophytic fungi as promising sources of antimicrobial bioactive compounds for future therapeutic applications.

ACKNOWLEDGMENTS

The authors acknowledge ITM University, Gwalior, for providing instrumental facilities and technical assistance during the experimental studies.

DISCLOSURE STATEMENT

The authors declare no competing financial interests or personal relationships that could have influenced the work reported in this paper.

FUNDING & ETHICAL APPROVAL

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Ethical approval was not required for this study.

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FIGURES AND TABLES

Figure Large-scale production of antimicrobial bioactive metabolites by selected endophytic fungal isolates (EPF-04 and EPF-06) under optimized culture conditions.

Tables

Table 1. Effect of initial pH on antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-04.

Effect of Endophytic isolate (EPF-04) initial pH on antimicrobial bioactive metabolites production.				
pH	***Candida***	***Fusarium***	***Alternaria***	**Biomass**
3	1	1.5	2	0.2
4	5	4.5	5.5	0.4
5	15	10	14	0.68
6	20	15	16.3	1.2
7	24	17.4	20.7	1.655
8	16.5	15	17.1	1.265
9	6	5	7	0.58

Table 2. Effect of initial pH on antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-06.

Effect of Endophytic isolate (EPF-06) initial pH on antimicrobial bioactive metabolites production.				
pH	***Candida***	***Fusarium***	***Alternaria***	**Biomass**
3	1	1.6	2.5	0.2
4	5.2	4.5	5.5	0.4
5	15	10	14	0.68
6	20	16	16.8	1.28
7	24.2	17.8	20.9	1.88
8	16.5	15	17.1	1.265
9	7	5.5	7.1	0.58

Table 3. Effect of incubation temperature on antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-04.

Temperature (°C)	*Candida*	*Fusarium*	*Alternaria*	Biomass (g/mL)
10	**9**	**6.5**	**8.8**	**0.5**
20	**16**	**12**	**14.8**	**1.497**
30	**23.2**	**16.8**	**18.9**	**2.485**
40	**13.6**	**24**	**17.5**	**0.827**
50	**8**	**6**	**6.2**	**0.4**

Table 4. Effect of different carbon sources on antimicrobial bioactive metabolite production by selected endophytic fungal isolates.

Effect of Carbon source on antimicrobial bioactive metabolites production from Endophtic isolate				
Carbon Source (1%)	***Candida***	***Fusarium***	***Alternaria***	**Biomass (g/mL)**
Glucose	22	21	16	1.655
Sucrose	13	12.5	8.5	1.112
Dextrose	10	9	1	1.145

Table 5. Effect of glucose concentration on antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-04.

Effect of Carbon source on antimicrobial bioactive metabolites production from Endophtic isolate EFM-04				
Glucose (%)	***Candida***	***Fusarium***	***Alternaria***	**Biomass**
0.2	8	7	9	0.68
0.5	18	14.8	16.3	1.2
1	22.3	17.8	20.7	1.655
1.5	20.5	15	17.1	1.265
2	12.5	8.8	8	0.58

Table 6. Effect of glucose concentration on antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-06.

Effect of Carbon source on antimicrobial bioactive metabolites production from Endophtic isolate EFM-06				
Glucose (%)	***Candida***	***Fusarium***	***Alternaria***	**Biomass**
0.2	8.2	7.5	9.1	0.88
0.5	18.5	14.9	16.6	1.2
1	22.8	17.8	20.9	1.78
1.5	20.7	15.2	17.6	1.35
2	12.7	8.9	8.3	0.59

Table 7. Effect of different nitrogen sources on antimicrobial bioactive metabolite production by selected endophytic fungal isolates.

Nitrogen Source (0.3%)	**Candida**	**Fusarium**	**Alternaria**	**Biomass (g/mL)**
Yeast Extract	18	18.8	12.5	1.119
Peptone	19.2	20	14.8	1.295
$NaNO_3$	25.5	17.9	20.8	1.357

Table 8. Effect of sodium nitrate ($NaNO_3$) concentration on antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-04.

Effect of $NaNO_3$ on antimicrobial bioactive metabolites production from Endophtic isolate EPF-04.				
$NaNo_2$ (%)	***Candida***	***Fusarium***	***Alternaria***	**Biomass (g/L)**
0.2	10	8	4	0.9
0.4	13.5	12.5	7.5	1.3
0.6	23	22	13.5	1.4
0.8	26.5	18.8	21	1.5
1.0	19	18	16	1.8

Table 9. Effect of sodium nitrate ($NaNO_3$) concentration on antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-06.

Effect of $NaNO_3$ on antimicrobial bioactive metabolites production from Endophtic isolate EPF-06.				
$NaNo_2$ (%)	***Candida***	***Fusarium***	***Alternaria***	**Biomass (g/L)**
0.2	9.5	8	5.2	0.8
0.4	12.8	12.1	6.9	1.2
0.6	22.8	21.6	13.1	1.38
0.8	25.9	17.3	20.8	1.45
1.0	19	18.1	16.3	1.1

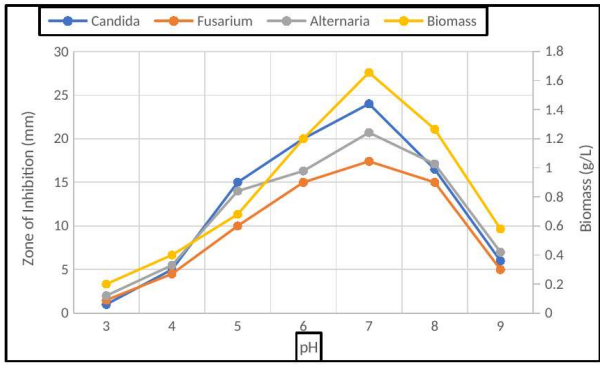

Figure Influence of initial pH on fungal biomass and antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-04.

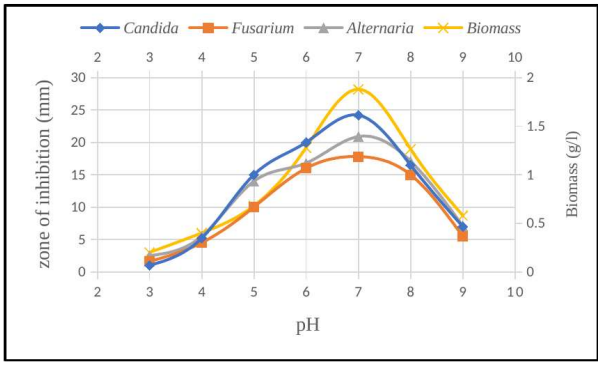

Figure Influence of initial pH on fungal biomass and antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-06.

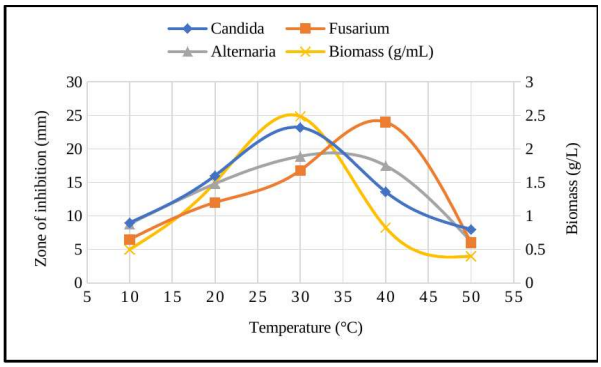

Figure Influence of incubation temperature on fungal biomass and antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-04.

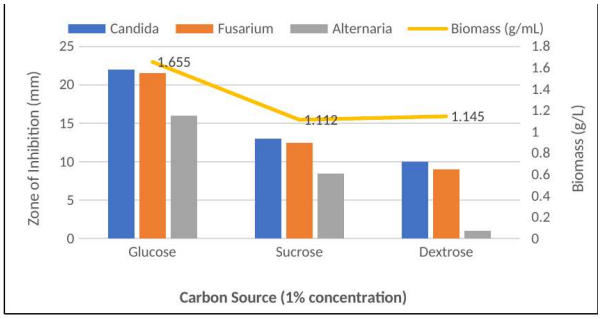

Figure Influence of different carbon sources on fungal biomass and antimicrobial bioactive metabolite production by selected endophytic fungal isolates.

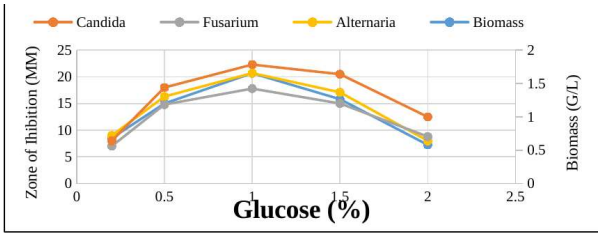

Figure Effect of varying glucose concentrations on fungal biomass accumulation and antimicrobial bioactive metabolite production by selected endophytic fungal isolates.

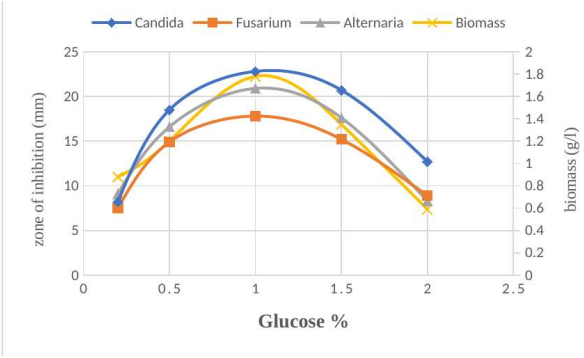

Figure Influence of glucose concentration on fungal biomass and antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-06.

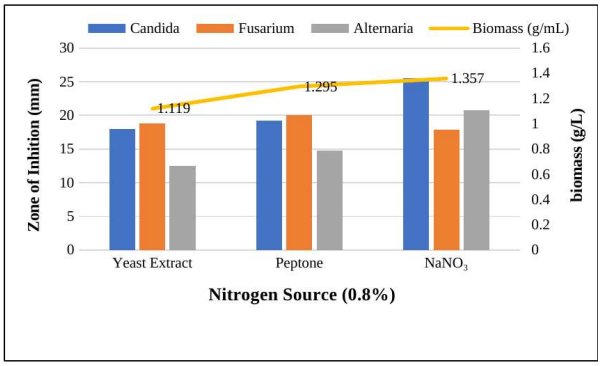

Figure Influence of different nitrogen sources on fungal biomass and antimicrobial bioactive metabolite production by selected endophytic fungal isolates.

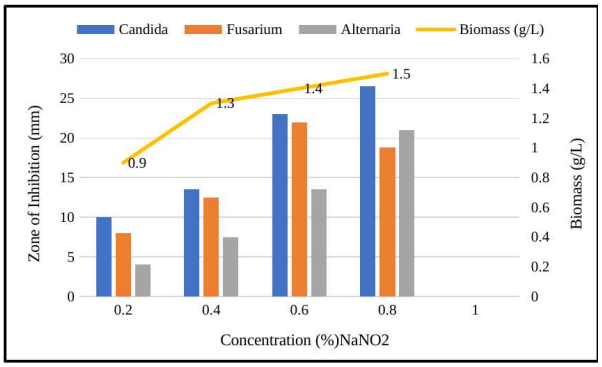

Figure Influence of sodium nitrate ($NaNO_3$) concentration on fungal biomass and antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-04.

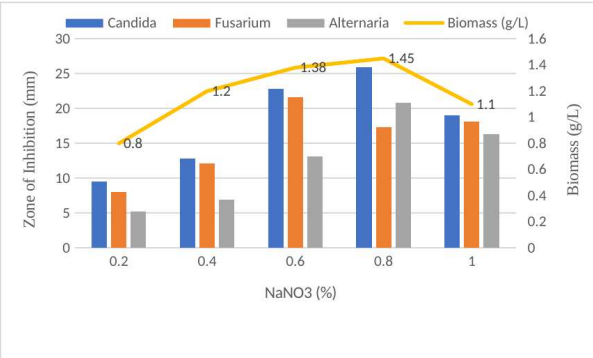

Figure Influence of sodium nitrate ($NaNO_3$) concentration on fungal biomass and antimicrobial bioactive metabolite production by endophytic fungal isolate EPF-06.