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Impact of submerged culture conditions on growth and bioactive metabolite produced by endophyte *Hypocrea* spp. NSF-08 isolated from *Dillenia indica* Linn. in North-East India

Effet des conditions de culture en milieu liquide sur la croissance et la production de métabolite bioactif d'*Hypochrea* sp. NSF-08 un champignon endophyte isolé de *Dillenia indica* Linn. au Nord-Est de l'Inde

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Summary

An endophytic fungus, associated with tropical tree species *Dillenia indica* Linn., has a broad-spectrum antimicrobial property against human and plant pathogenic microorganisms. The fungus was identified as *Hypocrea* spp. NSF-08, a teleomorphic genus of *Trichoderma*, based on morphological, sporulation and molecular characteristics. The effect of various culture conditions, supplementary carbon, nitrogen sources and amino acid amendment on growth and antimicrobial agent production by the fungus was determined. The basal medium, enriched with glycerol (2%) as carbon source and ammonium nitrate as nitrogen source, promoted the biosynthesis of secondary metabolite. Optimum temperature required for maximum production of antimicrobial agent was 25 °C and pH 5.5, respectively, in an incubation period of eight days, while specific rate of product formation was at maximum on the 4th day. Amendment of amino acid alanine with glucose and glutamic acid in combination with glycerol, sucrose and fructose enhanced the production of bioactive metabolite by the fungus. The antimicrobial agent produced by *Hypocrea* spp. was extracted with ethyl acetate as solvent and purified by TLC. The purified active compound with UV λ-max 242 nm got the lowest minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and *Fusarium oxysporum*, whereas no activity recorded against *Pseudomonas aeruginosa*.

Résumé

Un champignon endophyte, isolé d'un arbre tropical, *Dillenia indica* Linn., possède un large spectre d'activité antimicrobienne contre des microorganismes pathogènes pour l'homme et pour les plantes. Ce champignon est identifié sur des critères morphologiques et moléculaires comme *Hypocrea* sp. NSF-08, un genre téléomorphe de *Trichoderma*. Les effets de diverses conditions de culture, sources de carbone, d'azote et d'acides aminés, sont évalués sur sa croissance et sa production de métabolites antimicrobiens. Le milieu de base, enrichi par du glycérol (2 %) comme source de carbone et de nitrate d'ammonium comme source d'azote, développe la synthèse de métabolites secondaires. La température et le pH optimum requis pour la biosynthèse d'agents antimicrobiens est de 25□C et de 5,5 respectivement, avec la production d'un taux spécifique maximum au quatrième jour. L'apport d'alanine et d'acide glutamique en combinaison avec le glycérol, le glucose, le sucrose et le fructose augmente la production de métabolite bioactif par ce champignon. L'agent antimicrobien produit est extrait par l'acétate d'éthyle et purifié par chromatographie en couches minces (CCM). Le composé purifié avec un UV *λ*-max à 242 nm donne la plus faible concentration minimale inhibitrice (CMI) contre *Staphylococcus aureus* et *Fusarium oxisporum*, alors qu'il n'a aucune activité contre *Pseudomonas aeruginosa.*

Keywords: Endophyte; *Hypocrea* spp. NSF-08; Antimicrobial agent; Minimum inhibitory concentration
Mots clés: *Hypocrea* sp. NSF-08; Endophyte; Composé antimicrobien; Concentration minimale inhibitrice

Article Outline

Introduction

Endophytic microorganisms that asymptomatically live in the intercellular spaces of higher plants [38] have recently been intensified due to their potentialities in the production of bioactive metabolite [31] and [37], immunosuppressant [38], anticancer compound [36] and [43], cytotoxin [42] and biocontrol agent [39]. The fungal endophytes have been studied predominantly in temperate region [32] rather than tropical region [30]. Virtually,

very few reports are available on the association of endophytic fungi from tropical medicinal tree species [27]. In spite of numerous reports on synthesis of antimicrobial metabolites, including cyanide and cyano-like compound by endophytes, we are yet to exploit their potentialities in both pharmaceutical and agriculture sectors [24].

Supplement of carbon and nitrogen source [2], amendment of amino acid, sodium chloride [28], mineral salt [6] and various culture conditions like temperature, pH, incubation period [41] play a major role on growth and production of antimicrobial agent [25] by microorganisms.

Dillenia is an evergreen tree, belongs to the family Dilleniaceae and occurs in the moist evergreen forests of sub-Himalayan tract, including Assam, India [1]. The ripe fruits are rich in nutrients and their extracts have got antioxidant property [1]. *Hypocrea* is teleomorphic genus of its asexual anamorph *Trichoderma* [22] and both individuals have got special economic interest in biological control [29], in the production of cellulolytic and other hydrolytic enzymes [21] and antibiotics [16].

There are sporadic reports on endophytic fungus growing on *Taxus* sp. and some other gymnosperms [5] and [33] in North-East India. Since entire North-East India belongs to the Indo-Burma mega-biodiversity hot-zone [26], possibilities can never be rolled out for exploration of novel bioactive metabolite from the microbial wealth of this region. It has already been reaffirmed by isolation of a novel antibiotic 2-methylheptyl isonicotinate from a soil *Streptomyces* spp. 201 [7]. Considering the emerging problem of multidrug resistance and increasing demand of alternate novel drugs to control dominant infectious diseases, it was our endeavour to characterize the antimicrobial property of an endophytic fungus associated with *Dillenia indica* from Nambor wild life sanctuary (WLS), India. In this study, effort has been made to characterize a bioactive molecule synthesized by endophyte *Hypocrea* spp. NSF-08 and analyze the impact and subsequent optimization of submerged culture conditions to facilitate improved production of the antimicrobial agent.

Materials and methods

Isolation and identification of the endophyte

The endophytic fungus NSF-08 was isolated from the bark of *D. indica* Linn., an inhabitant of Nambor WLS of Assam, India. Nambor WLS is an evergreen tropical forest, covering an area of 32 square km, which is known for four natural hot-water springs viz. Borpung, Gelipung, Charaipung and Garampani. The endophyte was isolated by the method as described earlier [43] and maintained on potato dextrose agar (PDA) slant (Hi-media Ltd., Mumbai, India). For isolation, the inner bark was cut into small pieces (~0.5 × 0.5 × 0.5 cm) and then treated with 70% (v/v) ethanol followed by 3% sodium hypochlorite treatment (Rankem). Afterward, the outer bark was removed with a sterilized sharp blade and placed on PDA surface, then incubated at 25 °C in stationary condition for two weeks. During incubation, hyphal tips of the fungus protruding from the inner bark on the plates were further purified and transferred to slants.

The identification of the fungus was carried out on the basis of culture characteristics and morphological characters like mycelial properties, spore bearing organ with spore arrangement observed under microscope (ZEISS, Axioskop 40) [3] and 18S rDNA homology carried out by National Centre for Biotechnology Information (NCBI) blast service and through the courtesy of Microbial Type Culture Collection (MTCC) and Gene Bank, Chandigarh, India.

Test organisms

Six bacterial test pathogens, out of which three were gram positive – *Bacillus subtilis* (NCIM2063), *Staphylococcus aureus* (MTCC737), *Mycobacterium phlei* (ATCC19420) – and three gram negative bacteria – *Escherichia coli* (MTCC739), *Klebsiella pneumoniae* (MTCC109) and *Pseudomonas aeruginosa* (MTCC741) – were used for bioassay. The fungal pathogen *Candida albicans* (MTCC 3017) including the bacterial pathogens were obtained from MTCC and Gene Bank, Chandigarh, India.

The plant pathogens *Rhizoctonia solani*, *Fusarium oxysporum* spp. Raphani Schlect and *Fusarium semitectum* Berkeley and Ravenel were obtained from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India.

In vitro antibiosis

Dual plate culture technique was done to screen the antagonistic effect of the endophyte NSF-08, whereas agar well-diffusion method [34] was followed to assay the active compound isolated from the fungus. Muellar hinton agar (MHA) and potato dextrose agar (PDA) medium was used for bioassay against bacterial and fungal test pathogen, respectively.

Extraction and purification of bioactive compound

For extraction of the bioactive compound, the fungal isolate NSF-08 was cultured on above standardized media and mycelial part was separated by centrifugation at 7796 × g (SS-34 rotor, RC5-B Plus Centrifuge, Sorvall) for 10 minutes. The supernatant was extracted by mechanical shaking thrice with equal volume of ethyl acetate (1:1). The solvent part was treated with Na_2SO_4 (Rankem), evaporated in rotary evaporator (Buchi R-114) and then under high vacuum to give 8.4 g crude compound. Purification was carried out by thin layer chromatography (TLC) device on silica gel (Merck Ltd.) using petroleum ether-ethyl acetate (Rankem) gradient (30:1 → 1:30) as running solvent system. TLC purified fractions were recovered and bioassayed to find out the pure active compound.

Minimum inhibitorial concentration (MIC) and minimum fungicidal concentration (MFC)

MIC and MFC were determined according to Boruwa et al. [8] by adding an inoculum size of 3×10^2 colony forming units (CFU)/µl of test organisms to 5 ml of nutrient broth (NB) and potato dextrose broth (PDB), respectively, on different tubes. Serial dilutions of the active compound were added at the same time. MIC of the bacterial test pathogens were determined after 48 h of incubation by removing 10 µL of the contents from each tube and spreading them onto NA plates. After 24 h of incubation at 35 °C, growth of any colony was observed. MFC were determined in molten M_2 agar base (Hi-media Ltd.) by following the same procedure and only the incubation period was extended up to 48 h at 28 °C.

Optimization of media

In order to standardize the impact of culture conditions and media supplements on growth and bioactive metabolite production, the stem-leaf extract of *D. indica* was considered as basal medium. Growth was determined as dry mycelial weight in mg/ml by drying the mycelial mat at 70 ± 1 °C until a constant weight was obtained.

Estimation of bioactive metabolite

The *λ*-max of the purified active compound in ethyl acetate was determined by using a UV spectrophotometer (SPECORD 210). A standard curve was calibrated from metabolite concentration versus optical density and biosynthesis of the active metabolite was estimated by comparing the optical density at recorded *λ*-max. The following parameters were considered for optimization characterizations.

Carbon and nitrogen supplement

Glucose, starch, lactose, mannitol, sucrose, glycerol, galactose, fructose, maltose, ribose and xylose as carbon source and beef extract, yeast extract, peptone, $(NH_4)_2SO_4$, NH_4Cl, NH_4PO_4 and NH_4NO_3 were used as nitrogen source, which were supplemented separately into the basal medium in 1% concentration. The

endophytic isolate NSF-08 was inoculated to the carbon and nitrogen amended basal media separately and incubated at 25° ± 1 °C for 10 days at 150 rpm in gyratory shaker (Kuhner ISF-1-W). The respective biomass vis-à-vis metabolite production was recorded.

Incubation period, temperature and pH

The fungus was inoculated into the basal medium supplemented with glycerol and $(NH_4)_2SO_4$, then incubated in shaking condition (150 rpm) up to 15 days at 25 °C. For temperature effect on growth and active metabolite production, the fungus was cultured in various temperatures ranging from 15 °C to 50 °C. In addition, to determine the effect of initial media pH, the endophyte was grown in different pH range 3–11 at 25 °C for 10 days. The growth and the bioactive metabolite production in comparison to control were determined individually by comparing mycelial dry weight and UV *λ*-max of the culture filtrate.

Salinity

The effect of salinity on growth and antimicrobial agent produced by NSF-08 was carried out by culturing the fungus in various NaCl concentrations, ranging from 1 to 10%, in to the basal media amended with 1% glycerol. The biomass accumulation and bioactive metabolite production for each NaCl concentration were estimated as stated above.

Amino acid amendment

A number of amino acids were amended at a concentration 0.1 mg/ml in combination with four carbon sources – glucose, glycerol, sucrose and fructose. The effect on growth and bioactive metabolite production was estimated after incubation at optimum conditions.

Specific rate of product formation (*qp*)

The specific rate of active metabolite production (*qp*) was calculated according to following equation:

$$qp = \frac{1}{X}\left(\frac{dP}{dt}\right),$$

where *X* is the biomass concentration (μg/ml), *P* is antimicrobial agent concentration and *t* is time, respectively. The derivative d*P*/d*t* was calculated according to the method proposed by Le Duy and Zajic [23] and software for graphical analysis Origin PRO-7.5.

Statistical analysis

Statistical analyses were carried out by calculation of the means and standard deviations of the results. Data were subjected to analysis of variance to test the null hypothesis. Duncans multiple range test (DMRT) was done to compare that the sample means were significantly different from each other at a significant level of $P > 0.001$ [17].

Results

Fungal identification

The mycelia of the fungus NSF-08, initially white in colour turned to light yellowish green then to bright olive green as the conidia matured. Reverse colour is off-white with wavy edges. Hyphae are septate and hyaline under microscope. Conidiophores are branched and produced singly or in tufts. Flask shaped phialides arranged in terminal whorls and conidia are echinulate, globose or oval and 5–10 μ in size. The 18S rRNA gene sequence homology of NSF-08 with the help of NCBI blast service showed 98% similarities with the most closely related

species *Hypocrea schweinitzii* AF455511.1. From these characteristics the endophytic isolate NSF-08 can be identified as *Hypocrea* spp. belongs to the fungal class Ascomycetes, order Hypocreales and further designated as *Hypocrea* spp. NSF-08.

Purification and activity

Five major fractions with different R_f values from preparative TLC plates were solvent eluted and dried in vacuo. They were dissolved in dimethyl sulfoxide (E Merck Ltd.) and bioassay was done to determine the active fraction for further purification. Fraction with R_f value 0.615 exhibited inhibition zones against almost all the test pathogens except *P. aeruginosa* (Table 1). The pure compound was soluble in methanol, ethyl acetate and chloroform, where as sparingly soluble in water. The TLC purified active compound showed UV λ-max in ethyl acetate at 242 nm (Fig. 1).

Table 1.

In vitro antibiosis (inhibition zone diameter in mm) of the five TLC purified fractions against the test organisms with respective R_f values.

Tableau 1 Activité antimicrobienne in vitro (diamètre d'inhibition en mm) de cinq fractions purifiées par CCM sur divers microorganismes en fonction des valeurs du R_f.

Test organisms	R_f values				
	0.28	**0.42**	**0.615**	**0.73**	**0.86**
Bacillus subtilis	–	–	18 (± 2.5)	–	–
Staphylococcus aureus	–	21 (± 1)	21 (± 1.5)	–	–
Mycobacterium phlei	–	–	17 (± 1)	–	–
Escherichia coli	–	–	18 (± 1)	–	–
Klebsiella pneumoniae	–	–	17 (± 1.5)	–	–
Pseudomonas aeruginosa	–	–	–	–	–
Candida albicans	18 (± 2)	–	25 (± 3)	–	–
Fusarium oxysporum	–	–	28 (± 2.5)	–	21 (± 1.5)
Fusarium semitectum	–	–	27 (± 1.5)	–	–
Rhizoctonia solani	–	–	23(± 2)	–	–

Concentration of the fractions = 100 µg/ml.

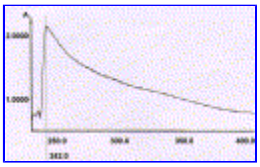

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Figure 1. UV λ-max of the bioactive compound isolated from endophyte *Hypocrea* spp. NSF-08.

Figure 1 UV λ-max du composé bioactif isolé de l'endophyte *Hypocrea* sp. NSF-08.

The bioactive compound has got lowest MIC of 35 µg/ml against *B. subtilis* and *F. oxysporum*, whereas highest was recorded against *R. solani* (60 µg/ml) (Table 2). Significant MIC and MFC was recorded against *B. subtilis* (40 µg/ml), *E. coli* (40 µg/ml), *C. albicans* (40 µg/ml), *M. phlei* (45 µg/ml) and *F. semitectum* (45 µg/ml), respectively (Table 2).

Table 2.

Minimal inhibitory concentrations (MIC) of the TLC purified secondary metabolite isolated from *Hypocrea* spp. NSF-08.

Tableau 2 Concentration minimale inhibitrice (CMI) du métabolite secondaire d'*Hypocrea* sp. NSF-08 purifié par CCM.

Test organisms	MIC/MFC (µg/ml)
Bacillus subtilis	40 (± 2)
Staphylococcus aureus	35 (± 2)
Mycobacterium phlei	45 (± 2)
Escherichia coli	40 (± 2)
Klebsiella pneumoniae	50 (± 2)
Pseudomonas aeruginosa	–
Candida albicans	40 (± 2)
Fusarium oxysporum	35 (± 2)
Fusarium semitectum	45 (± 2)
Rhizoctonia solani	60 (± 2)

Data were mean observation of three independent experiments.

Impact of culture conditions

Maximum growth (3.68 mg/ml) of spp. NSF-08, on the basis of dry weight estimation recorded at 25 °C, but active metabolite production (12.46 µg/ml) was highest at 27 °C (Fig. 2). Therefore, the temperature 27 °C was considered as optimum for the future studies. The fungus could not grow at the temperature below 20 °C and above 50 °C, moreover, growth as well as metabolite production almost ceased at 45 °C (0.084 mg/ml; 1.34 µg/ml). The favourable pH for growth (3.6 mg/ml) was optimized at pH = 5.5, whereas maximum synthesis of metabolite (12.46 µg/ml) recorded at pH = 6 (Fig. 3). No growth of the fungus could be detected at pH values below four and above ten.

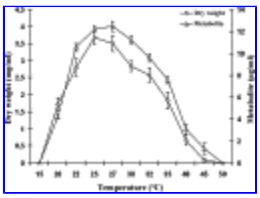

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Figure 2. Impact of temperature on growth and biosynthesis of secondary metabolite by endophyte *Hypocrea* spp. NSF-08.

Figure 2 Effet de la température sur la croissance et la biosynthèse de métabolite secondaire par l'endophyte *Hypocrea* sp. NSF-08.

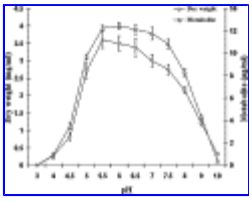

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Figure 3. Impact of pH on growth and biosynthesis of secondary metabolite by endophyte *Hypocrea* spp. NSF-08.

Figure 3 Effet du pH sur la croissance et la biosynthèse de métabolite secondaire par l'endophyte *Hypocrea* sp. NSF-08.

Although maximum growth (3.23 mg/ml) of the fungus, *Hypocrea* spp. NSF-08 was observed on the 6th day of incubation, highest amount of bioactive metabolite (12.52 μg/ml) was collected from eight days old culture filtrate (Fig. 4). The mycelial dry weight of the fungus and metabolite production remains almost constant after six and eight days of incubation, respectively. The specific rate of bioactive metabolite production was rapidly increased from one day after incubation and produced maximum (1.108 day^{-1}) at 4th day, then decreased gradually till the 8th day, no *qp* was found from 9th day of incubation (Fig. 5).

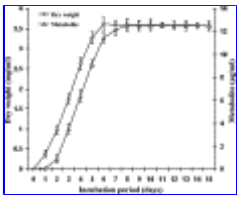

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Figure 4. Impact of incubation period on growth and biosynthesis of secondary metabolite by endophyte *Hypocrea* spp. NSF-08.

Figure 4 Effet de la période d'incubation sur la croissance et la biosynthèse de métabolite secondaire par l'endophyte *Hypocrea* sp. NSF-08.

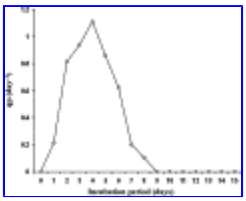

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Figure 5. Specific rate of bioactive metabolite production (*qp*) by endophyte *Hypocrea* spp. NSF-08.

Figure 5 Taux de production spécifique (*qp*) du métabolite bioactif.

Impact of carbon, nitrogen source and salinity

Carbon and nitrogen sources have got significant effect on growth as well as secondary metabolite production by *Hypocrea* spp. NSF-08 (Fig. 6). A higher amount of mycelial dry weight (3.4 mg/ml) was detected in glucose supplemented medium, while glycerol is the best nitrogen source for maximum production (13.4 μg/ml) of

antimicrobial agent among the carbon source used in this study. Although fructose, lactose, maltose, xylose, sucrose and starch could promote the growth of the fungus, metabolite production was less in comparison to control. As for growth of *Hypocrea* spp. NSF-08, yeast extract represents best (12.84 mg/ml) nitrogen supplement, but so far as antimicrobial agent production is concern, ammonium nitrate was met out in front (13.28 µg/ml). No significant enhancement on metabolite synthesis could bring out by rest of nitrogen supplements.

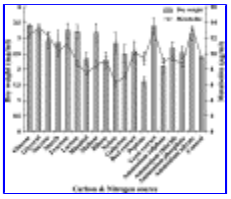

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Figure 6. Impact of carbon and nitrogen sources on growth and biosynthesis of secondary metabolite by endophyte *Hypocrea* spp. NSF-08.

Figure 6 Effet des sources de carbone et d'azote sur la croissance et la biosynthèse de métabolite secondaire par l'endophyte *Hypocrea* sp. NSF-08.

The result recorded in Figure 7 revealed that sodium chloride at a concentration 2.5% produced maximum mycelial dry weight (3.58 mg/ml) as well as bioactive metabolite (13.12 µg/ml). Consequently, the NaCl concentration from 1 to 4% growth and secondary metabolite synthesis was considerable but detected to be decreased above 5% NaCl concentration.

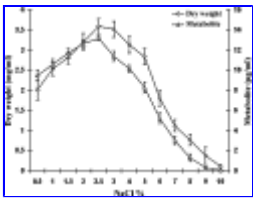

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Figure 7. Impact of NaCl concentration on growth and biosynthesis of secondary metabolite by endophyte *Hypocrea* spp. NSF-08.**Figure 7** Effet de la concentration en NaCl sur la croissance et la biosynthèse de métabolite secondaire par l'endophyte *Hypocrea* sp. NSF-08.

Amino acid amendment

Concerning the effect of amino acids amendment in combination with four carbon sources, the result showed that alanine in combination with glucose promoted growth (3.46 mg/ml) and antimicrobial agent production (13.45) by NSF-08 (Fig. 8). Glutamic acid in combination with sucrose enhanced growth (13.18 mg/ml) as well as metabolite (3.12 µg/ml) synthesis (Fig. 9). In addition, aspartic acid with glycerol (3.2 mg/ml) and fructose (3.42 mg/ml) are the best for growth, while glutamic acid with glycerol (13.56 µg/ml) and fructose (12.52 µg/ml) are best for metabolite production (Figure 10 and Figure 11).

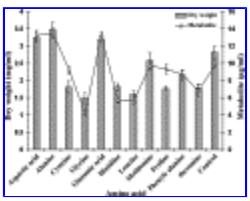

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Figure 8. Impact of amino acids amendment with glucose on growth and biosynthesis of

secondary metabolite by endophyte *Hypocrea* spp. NSF-08.

Figure 8 Effet de l'apport d'acides aminés et de glucose sur la croissance et la biosynthèse de métabolite secondaire par l'endophyte *Hypocrea* sp. NSF-08.

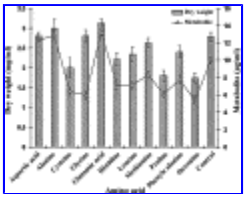

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Figure 9. Impact of amino acids amendment with sucrose on growth and biosynthesis of secondary metabolite by endophyte *Hypocrea* spp. NSF-08.

Figure 9 Effet de l'apport d'acides aminés et de sucrose sur la croissance et la biosynthèse de métabolite secondaire par l'endophyte *Hypocrea* sp. NSF-08.

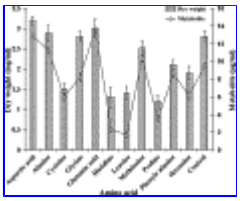

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Figure 10. Impact of amino acids amendment with glycerol on growth and biosynthesis of secondary metabolite by endophyte *Hypocrea* spp. NSF-08.

Figure 10 Effet de l'apport d'acides aminés et de glycérol sur la croissance et la biosynthèse de métabolite secondaire par l'endophyte *Hypocrea* sp. NSF-08.

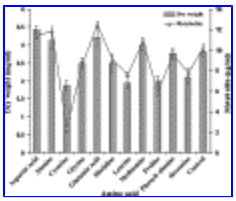

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Figure 11. Impact of amino acids amendment with fructose on growth and biosynthesis of secondary metabolite by endophyte *Hypocrea* spp. NSF-08.

Figure 11 Effet de l'apport d'acides aminés et de fructose sur la croissance et la biosynthèse de métabolite secondaire par l'endophyte *Hypocrea* sp. NSF-08.

Discussion

Out of all the bioactive secondary metabolites reported so far, almost 50% are isolated from microorganisms. Beginning from the historic achievement of Penicillin, 22% of total antibiotics are reported from filamentous fungi [12]. Subsequently, a stream of attention on antibiotic research is being directed to endophytes, some of which have been ascertained to possess antimicrobial potentialities.

The endophytic fungus *Hypocrea* spp. NSF-08, isolated from *D. indica*, produces antimicrobial agent with broad spectrum in vitro activity against some plant and human pathogenic organisms. Based on the results, the active

molecule synthesis by the fungus is more effective against pathogenic fungi and gram positive bacteria. Gram negative bacterial pathogens were less sensitive to the bioactive compound, for example, *P. aeruginosa* was found to be completely resistant. Similar type of results of bioactivity of endophytes has also been reported by Strobel et al. [39].

The biosynthesis of secondary metabolite is directly related to cultural conditions [12] that include biomass present in the production phase and duration of the incubation periods [14]. In this study, different carbon and nitrogen source supplement to the culture broth strongly influenced the growth and biosynthesis of active metabolite by *Hypocrea* spp. NSF-08. As many published reports [9] and [11] support, simple sugar such as glucose, fructose, sucrose, glycerol enhance growth as well as secondary metabolite production by microorganisms rather than complex carbon sources like starch, galactose, xylose, mannitol, etc. The simple carbohydrates through metabolic pathway affect on production of intermediates, leading to primary as well as secondary metabolites in addition to CO_2, water and energy [40]. After depletion of the favoured carbon source, the subsequent carbon source is used for metabolite biosynthesis. Rather it was also reported that readily utilized carbon sources yield less antibiotics than do slow utilizing one [13].

Concerning nitrogen sources, ammonium nitrate promoted the secondary metabolite biosynthesis, whereas yeast-extracts support the growth of *Hypocrea* spp. NSF-08. Nitrate as nitrogen source sometimes enhances the antibiotic production [15], but it represses antibiotic biosynthesis in *Aspergillus parasiticus* [19]. Maximum antibiotic production was obtained at 2.5% of NaCl concentration to culture media, while an increase in salt concentration reduced the secondary metabolite yield.

Regarding the temperature, incubation at 27 °C promoted the mycelial growth of the fungus and maximum concentration of bioactive metabolite recorded at 25 °C. Similarly, temperature lower than that required for optimum growth is reported to produce maximum production of enniatins [4] and aflatoxin [35] by *Aspergillus* spp. Huang et al. [18] also supported isolation of antifungal and antitumour agent from endophytic fungi at 25 °C.

It was found that *Hypocrea* spp. NSF-08 grew well in slightly acidic pH (6.0), whereas biosynthesis of antimicrobial agent was maximum in pH 5.5. According to Buchanan and Ayres [10] the initial pH of medium is not a determinant factor for antibiotic production, but Keller et al. [20] showed the reduction in secondary metabolite production as the pH was increased. The pH is related to permeability characteristics of the cell wall and membrane and hence, effect on either ion uptake or loss to the nutrient medium [28].

Maximum antimicrobial agent produced by the fungus was recorded on 8th day of incubation. The trophophase (growth phase) of the endophytic fungus lasted for six days, but the idiophase (production phase) reached its top at the 8th day of incubation and remain almost constant after nine days. Stinson et al. [37] reported maximum antimicrobial agent production on 10th days in case of endophyte *Gliocladium* spp.

Amended of some amino acids individually to glucose, glycerol, fructose and sucrose enhanced growth as well secondary metabolite production, while most of them found to possess negative effect. Out of 11 amino acids screened, aspartic acid, alanine, glutamic acid and methionine have got remarkable influence on enhancement of bioactive metabolite production by *Hypocrea* spp. NSF-08. Amino acid supplement may have some role by sharing their carbon ring or both carbon and nitrogen skeleton in to the primary or secondary metabolism processes of microorganisms [28].

The potentiality of the endophytic fungus *Hypocrea* spp. NSF-08 associated with *D. indica* Linn. is well-evinced by convincing MIC against virulent plant and human test pathogens. The optimum productivity of the antimicrobial agent was achieved with optimized process parameters such as glycerol (2% w/v) as carbon and NH_4NO_3 (2% w/v) as nitrogen sources, incubation period of eight days, incubation temperature at 25 °C, 2.5% (w/v) NaCl concentration, amendment of amino acids like aspartic acid, alanine, glutamic acid and methionine with carbon sources and initial pH 5.5. Further ongoing detail chemical characterization of the active compound

may be a new entity reported from this virgin untapped ecological area.

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