RESEARCH ARTICLE

Protection And Bio-Prospection Of *Bombax Ceiba* By Revealing Their Endophytic Fungi Diversity With Phytochemical And Enzyme Analysis

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ABSTRACT

The objective of the present study was to protect *Bombax ceiba* by revealing their endophytic fungi diversity with phytochemical and enzyme analysis. In the present study, a total of 64 fungal endophytes were enumerated and were belonging to *Aspergillus niger*, *Alternaria alternata (A. alternata), Phomopsis* Spp*., Nigrospora* Spp*,* and *Fusarium* Spp. with the colonization frequency of 75, 30, 22.5, 10, and 17.5% respectively. The endophytic *Aspergillus niger* showed the highest average linear growth rate (6.2 mm/day) and isolation frequency (80%) on tomato dextrose agar while lowest on water agar. The fungus showed white to dark brown to black or purple-brown to yellowish-green color variations with mycelial growth in between 65–90 mm on the media and 8-31 numbers of spores/ microscopic field. Further, fermentation and extraction of the same fungus revealed 2.86 g/100g of total wet biomass and 0.25 g/100g of the dried biomass of corn bran with 5.30 g/100mL of the aqueous concentrated crude extract while the simultaneous plant showed 2.96 g/100mL of aqueous crude extract. Phytochemical screening of both extracts showed alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols. The total phenolic content of the plant crude extract was 27.0090 ± 0.04129 mg, and flavonoids content was 20.3568 ± 0.05064 mg, while the fungus showed 29.0060 ± 0.03223 mg of phenolic and 12.2549 ± 0.02345 mg of flavonoids. Thin layer chromatography showed alkaloids [0.29], flavonoids [0.41] terpenoids [0.15] and saponins [0.91]. The fungus produced chitinase, amylase, protease, and cellulases enzymes. It is concluded that the *Bombax ceiba* has the potential to purify the potent fungal endophytes and their bioactive compounds that may be helpful to evaluate in vitro models against different diseases.

Keywords: *Bombax ceiba*, conservation, fungal endophytes, phytochemical and enzyme analysis

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INTRODUCTION

Bombax ceiba (*B. ceiba*) Linn. is a bulky, deciduous, elegant tree which is found in Africa, Australia, temperate and tropical Asia. It belongs to the family Bombacaceae. It has been applied in inflammations, diabetes, diarrhea, helnintic, leprosy, muscular injury, wounds, asthma, birth control, and sexual diseases.[1] Besides these, this same plant is used in agroforestry, providing food, fodder, fuel, and fiber.[2] In the past, *B. ceiba* was said to be the Yamadruma, Yama's tree (the lord of hell).[3] Also, tribes from Udaipur prohibit the use of *B. ceiba* explants because of unknown myths such as poison in the leaves, flowers, and stem; therefore, it led to natural protection of the same plant but due to this features tribes considered *B. ceiba* as a god tree and they use to burn them during Holi festival as a traditional Dahan of Holika.[3] Day by day, due to ethnobotany and commercial uses of *B. ceiba* led to an endangered zone, therefore, need to plan an effective protection strategy.

However, in the path of evolution, the fungi developed various types of associations with the plants. One of these associations is 'endophytes'.[4] This association was supported by the fossil records showing that plants are allied with endophytic and mycorrhiza fungi for roughly 400 million years.[5] During the isolation of fungal endophytes in the lab, they show different growth requirements; like nutrients, pH, osmotic conditions and temperature. These requirements may change from one fungus to another fungus.[6] Mycologists revealed natural media as the best choice of isolating media under any environmental condition, and these are composed of natural substrates, such as herbaceous or woody stems, seeds, leaves, corn meal, wheat germ, fruit pulps, and oatmeal.[7]

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Synthetic media contain ingredients of a known composition such as carbohydrates, nitrogen, and vitamin sources.[8] Widely fungal endophytes are isolated from explants of plants in the lab; therefore, sometimes they may not get sufficient nutrients from natural or synthetic media in the lab.[9] Critical and comprehensive knowledge of nutritional patterns and factors influencing the growth of endophytic fungi is a precondition for several studies foremost to the sympathetic of host-pathogen association[10]. Nowadays lab used media are costly and inefficient so industrialists have started to use several common cheap natural media.[11] The objectives of the present work were.[1] to purify fungal endophytes from *B. ceiba*[2] to produce, extract, purify bioactive compounds from an endophytic *Aspergillus niger*[3] To characterize endophytic *Aspergillus* species on a different media[4] To analyze phytochemicals and enzymes from the same fungus.

MATERIALS AND METHODS

Study Area, Survey and Sample Collection

The ethnobotany and commercial use information of *B. ceiba* was collected from the literature.[1] To confirm and to get more information about *B. ceiba* myths and traditions, we carried out interviews, a survey from the local tribal people, women's, elder individuals, and worshipers during March to June 2018 in the Udaipur city, Rajasthan. Further, the information was also collected from schools, colleges, and Ayurvedic teachers. The fresh and healthy samples of *B. ceiba* were collected from different areas of Udaipur city, Rajasthan. The explants were identified and authenticated by Dr. Kotresha K., Department of Botany, Karnatak Science College, Dharwad, Karnataka, India and a voucher specimen (N0-01/2018) was deposited. All samples were immediately brought to the laboratory and processed for isolation of endophytic fungi.

Isolation, Identification, and Characterization Of Fungal Endophytes

The explants were surface sterilized as per standard protocols,[12] then inoculated and incubated on Czapek Dox agar media plates[13] at 25°C ± 2°C for 15 days under dark conditions. After incubation, purification and growth study was carried out according to standard methods.[13] Morphological methods[14] performed identification of fungal endophytes and the selected fungus was identified by 18S rRNA analysis[15]. The endophytic *Aspergillus niger* was selected for further characterization study on different media. The tomato dextrose agar, potato dextrose agar, cabbage dextrose agar, malt dextrose agar, water agar, and sabouraud dextrose agar were prepared and endophytic *Aspergillus niger* culture was inoculated, incubated at room temperature (25°C ± 2°C) for 15 days[16]. Further, the growth of the same was observed on all grown media with naked eyes such as the color of the colony and substrate, the margin of the colony, the topography of the mycelium. The sporulation was measured by cutting a single block of 5 mm diameter from grown media and inoculated into 5 ml sterile distilled water in a test tube for spore suspension. Then a smear of spore suspension was prepared on a slide and observed under a microscope for their sporulation and conidial number[16].

Fermentation, Extraction, and Purification of Bioactive Compounds

The production of bioactive compounds was carried out by solid-state fermentation in which corn bran was used as a substrate[9]. Three pieces of the grown pure culture of endophytic *Aspergillus niger* were cut from the culture plate and inoculated in a 1000 ml Erlenmeyer flask containing 200 g of corn bran and 50 ml of distilled water and incubated in the dark at 28°C ± 2°C for 15 days at static condition. At the end of the incubation period, the fermented media were processed for the extraction of bioactive compounds. Further extraction was done by Soxhlet apparatus by using an aqueous solvent. In brief, 100 ml of distilled water was added in fermented media and kept on the rotary shaker for 24 hrs. After 24 hrs the mycelium and culture media were separated from each other by vacuum filtration. In the first hand, the filtrate was extracted three times with equal volume of aqueous solvent for the complete extraction of metabolites from fungal biomass for 18–20 hours at 40°C in Soxhlet apparatus. Then the concentration of the extract was performed on Rota evaporator and dried under oven at 40°C, weighed and stored at 15°C. On the other hand, obtained mycelium was air-dried, weighed and recorded as mg/100mL.[18]

Phytochemical Analysis and Thin Layer Chromatography of an Aqueous Crude Extract of *Bombax ceiba* and the Fungus

Qualitative phytochemical analysis of both extracts was performed for alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, and phenols, coumarins, quinones, and glycosides by following standard protocols. Quantitative analysis of flavonoids and phenol content was performed as per standard protocols[19].

Thin layer chromatography (TLC) was performed by using precoated TLC plates with silica gel 60 F-254 for the investigation of bioactive compounds from the aqueous crude extract of *B. ceiba* and endophytic *Aspergillus niger* by using different solvent systems. Alkaloids [Methanol: conc.NH_4OH (17:3)], flavonoids [Chloroform: methanol (18:2)], terpenoids [Benzene: Ethyl acetate (1: 1)], saponins [Chloroform: glacial acetic acid: methanol: water (6:2:1:1)] were used. TLC plates were spotted with aqueous crude extract with standard solutions of alkaloids, flavonoids, terpenoids, and saponins, and then developed in their respective eluent solvent systems. The chromatogram was developed in the closed TLC chamber in the selected solvent system for 5 minutes. After 5 minutes, plates air dried and observed under sun light and UV light (254 and 366 nm) for the observation of compound bands. Retention factor (Rf) value was calculated by using the following formula,

$$Rf = A/B$$

A = distance between sample spot and central point of the observed spot.

B = distance between the sample spot and the mobile phase front.

Primary Screening of Enzymes by Endophytic *Aspergillus niger*

Primary screening of chitinase[9], amylase,[20] cellulases,[12] and protease[9] was performed by an endophytic *Aspergillus niger* on selective media and enzyme production were confirmed by the observation of zone of hydrolysis.

Data Analysis

Determination of Average Linear Growth Rate

Average Linear growth rate (mm) of the isolate was calculated on TDA, PDA, CDA, MDA, WA, and SDA media plates after 15 days of incubation period by using the following formula[21].

$$ALGR\ (mm/day) = (C15-C0)/15$$

Where,

C_{15}= Colony diameter after 15 days of the incubation period

C_0= Colony diameter at initial inoculation time

Determination Isolation Frequency

Isolation frequency was calculated for the isolates with the help of the following formula[22].

$$IF\ (\%) = (\text{Number of pieces showing growth/Total number of inoculated pieces}) \times 100$$

Determination of colonization frequency

For colony uniqueness, the mycelia were transferred into PDA agar media. Colonization Frequency (CF) was calculated as described[23].

$$CF\ (\%) = (\text{number of segments colonized by endophytic fungi/total number of segments analyzed}) \times 100$$

RESULTS AND DISCUSSION

The Holi, the color festival comes in March and the *B. ceiba* is also blooming at the same time with red flowers and horny stem, therefore, the stem of the same plant is used to burn in Holi as a Holika-Dahan by Bhil, Garasia and Damor tribes in Udaipur. *B. ceiba* stem is considered as virtuous Prahlad and planted almost a month before the festival day. Even the image of Holika and Prahlad is also prepared and attached over the prepared Holi[1]. In Bhils, before cutting a *B. ceiba* stem, a coconut is fixed on the branch, liqueur trickled and vermillion is applied and the tree is cut to have a head and two arms and usually the stem is removed from the ablaze pile. This conventional two armed Holi is still prepared and planted. But in some Bhil villages of Banswara district, they use bamboo with a red cloth tied on it, representing as Prahlad and there Bombax indicates the wicked aunt Holika, therefore it is allowable to be burnt and bamboo is detached presentation continued existence of Prahlad[3]. Due to the tradition, the lethal ax always cascades on the *B. ceiba*. This unreasonable custom of wounding the plant for the purpose of Holika-Dahan is detrimental to the environment, troubling the eco-system and proving overwhelming for human health[2]. A survey in Udaipur and nearby forest areas has revealed many illegally *B. ceiba* tree were cut (Figure 1).

According to local tribes and teachers, in the year 2017, around 2,300–3,000 trees or twigs or stem of *B. ceiba* were cut for Holika-Dahan. There was barely any anxiety concerning the forgo of such a big tree in the middle of Udaipur tribes. The seriousness of the circumstances can be further assessed as presently only 2,351 trees are surviving in the Udaipur district. A variety of ethnic conservation practices, in the shape of civilization, customs, myths, and folktales have made the continued existence of *B. ceiba* for so a lot of years. Now, there is requiring renewing these helpful societies for protection while discarding all other conventional practices that destroy the plant. The most significant part of the conservation strategy is to make people conscious of its various helpful therapeutic applications. It is to be supposed a combined responsibility of nongovernmental and governmental organizations, forest officials, local environmentalists, village heads and teachers at the local school level. For conserving the ritual, only a little stick of *B. ceiba* can be used emblematically. In this regard, it is not compulsory that an iron pole wrapped with dehydrated grassland and hay fabric in its place of the wood pole of Bombax can be used for on fire in Holi. Biotechnological applications can be used as a major tool to spread and preserve the species in a short time period. The plentiful species of fungal endophytes made an ecological place in the inner gap of plants. These ubiquitous fungi act together absolutely with their environment in a positive way and extremely superior for application in plant development and disease control.[20] The diversity of endophytic fungi was revealed from the different medicinal plants of rainfall area and their effect was studied on a host plant with their biochemical occurrence.[23] They revealed the diversity of leaves, flowers, stem, and roots for the isolation of fungal endophytes, except they have isolated only total 24 fungal endophytes with 54% as an isolation frequency.[24] In the present study, we have isolated a total of 64 fungal endophytes with an isolation frequency of 74% from leaves and lowest from roots at the rate of 27%. All isolates were belonging to *A. niger*, *A. alternata, Phomopsis* Spp., *Nigrospora* Spp, and *Fusarium* Spp. with the colonization frequency of 75, 30, 22.5,

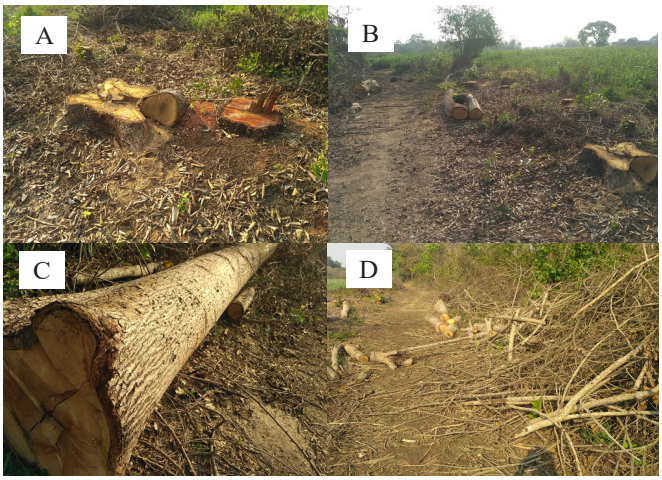

Figure 1: Cutting of the *B. ceiba* in Udaipur forests (A and B), Cut tree laying in the forests (C and D)

10, and 17.5%, respectively. The results are shown in Table 1 and Figure 2.

Endophytic *A. niger* showed core group in the isolation therefore, the same fungus was selected for further study. On the synthetic media endophytic *A. niger* showed initially, dark green conidia, which next conquered colony look. They were commonly plain and flat at the boundaries but were raised in the center and wrinkled in an almost cerebriform pattern. They produced droplets of liquid, which were either uncolored or brown. The colonies were encircled by a white border, and the colony diameter ranged between 40–65 mm. The undersides of the colonies were slightly pale. While on natural media, the same fungus showed somewhat different colony characteristics. The colonies were initially white and had a spongy velvety surface. After 8 days of the incubation period, the colonies became raised and turned floccose at the center. The colonies produced yellowish-green and olive conidia during sporulation. The conidia covered the complete exterior of the colonies apart from for the edges, where a white border was produced. The white border then moved out as the colonies became superior and shaped extra conidia. Sclerotia were produced and were originally white and turned a deep brown on the 13 days of the incubation period. No liquid bubbles were produced. The reverse sides of the colonies were furrowed and slightly pale brown or yellowish. The colony diameter on the natural medium was ranged between 65-90 mm (Figures 3 and 4).

On the natural media, the conidiophores were uncolored, thick-walled, and thickly roughened and were vesicles bearing. They ranged 600 and 1000 µm in diameter. The vesicles were globose and were also variable in diameter, ranging between 1200 and 1600 µm. They were blackish colored with either uniseriate or biseriate or both (Table 2). An endophytic *Aspergillus niger* showed variations in sporulation on synthetic and natural media due to variations in nutrient compositions. They showed the highest sporulation on TDA in between 18–27 while lowest showed on SDA in between 4–9 per microscopic field. This result supports the findings that *Aspergillus niger* showed the lowest sporulation on SDA in the range 8-12 per microscopic field[24]. The average linear growth rate of *Aspergillus niger* on TDA was 6.2 mm/day while the lowest growth rate was 5.3 mm/day on WA; therefore, it indicates the

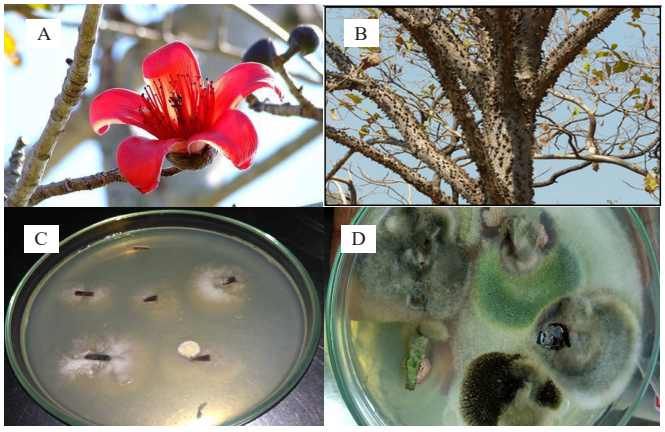

Figure 2: Explants of *B. ceiba* were collected from Udaipur study area (A & B) and surface sterilized with help of oxidants and chemicals at a definite concentration. Then all surface sterilized explants were implanted on czapek dox agar medium and incubated at 25°C ± 2°C for 15 days. After 15 days of incubation fungal endophytes were observed with actinomycetes (C and D).

Table 1: Fungal endophytes biodiversity within the explants of *B. ceiba*

Endophytic fungal species	*Explants*	*Total analyzed explants*	*Isolates*	*Colonization frequency (%)*
Aspergillus niger	Leaves	40	11	27.5
	Flowers	40	6	15
	Stem	40	9	22.5
	Roots	40	4	10
Alternaria alternata	Leaves	40	2	5
	Flowers	40	3	7.5
	Stem	40	4	10
	Roots	40	3	7.5
Phomopsis spp.	Leaves	40	4	10
	Flowers	40	1	2.5
	Stem	40	3	7.5
	Roots	40	2	5
Nigrospora spp.	Leaves	40	1	2.5
	Flowers	40	3	7.5
	Stem	40	1	2.5
	Roots	40	-	-
Fusarium spp.	Leaves	40	3	7.5
	Flowers	40	1	2.5
	Stem	40	1	2.5
	Roots	40	2	5

good and rapid growth of the fungus on TDA as compared to other media. The isolation frequency of *A. niger* was lowest at WA at the rate of 50% while the highest on TDA at the rate of 80%. Total wet biomass of endophytic *Aspergillus niger* was recorded as 2.86 g/100g of corn bran, and the dried biomass was 0.25 g/100g of corn bran. On the other hand, 80 mL of the filtrate were concentrated into 5.30 g/100mL of the aqueous solvent by using Rota evaporator at 40°C after 6–8 hrs rotation at 90 rpm and used for further processes. From plant, total 2.96 g/100mL of aqueous crude extract were obtained. Phytochemical screening of aqueous crude extract of *B. ceiba* and the fungus showed alkaloids, terpenoids, steroids, tannins, and saponins flavonoids, phenols and absence of coumarins, quinones, and glycosides. The total phenolic content of the aqueous crude extract of *B. ceiba* was estimated with Gallic acid as a reference standard, it showed high phenolic content (27.0090 ± 0.04129mg). The aqueous crude extract of *B. ceiba* showed high flavonoids content (20.3568 ± 0.05064 mg). The fungus aqueous crude extract showed 29.0060 ± 0.03223 mg of phenolic and 12.2549 ± 0.02345 mg of flavonoids content (Figures 2 and 3).

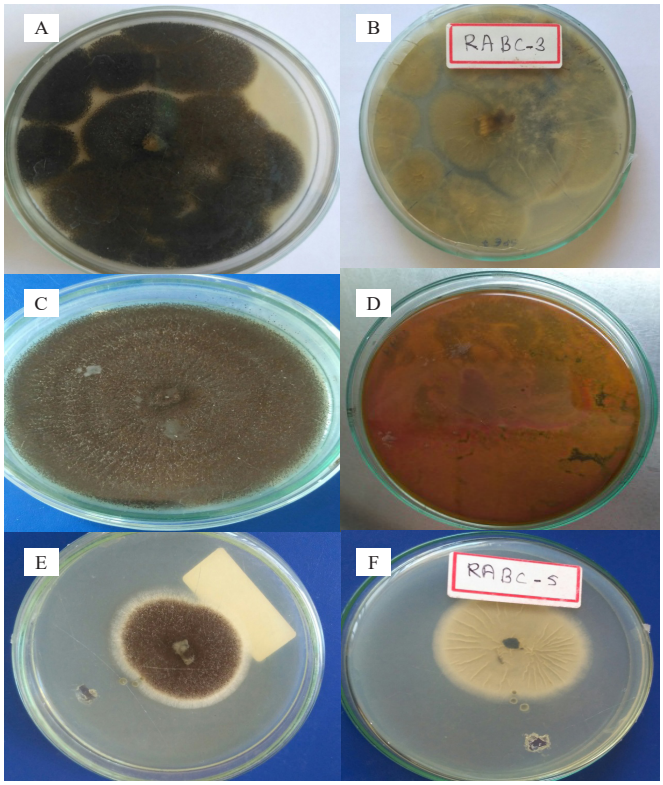

Figure 3: Characterization of endophytic *Aspergillus* species on different mediums. Front (A) and Back (B) side of the potato dextrose agar media plate with moderate growth of endophytic *Aspergillus* species, Front (C) and Back (D) side of the tomato dextrose agar media plate with an excellent growth of endophytic *Aspergillus* species, Front (E) and Back (F) side of the water agar with poor growth of endophytic *Aspergillus* species.

Both extracts were implanted in thin layer chromatography for the detection of different bioactive compounds. TLC of both crude extracts revealed the presence of four compounds having R*f* values of alkaloids [0.29] in Methanol: conc. NH_4OH- 17:3, flavonoids [0.41] in Chloroform: methanol-18:2, terpenoids [0.15] in Benzene: Ethyl acetate -1: 1, and saponins [0.91] in Chloroform: glacial acetic acid: methanol: water- 6:2:1:1 solvent systems. The results are shown in Figure 4.

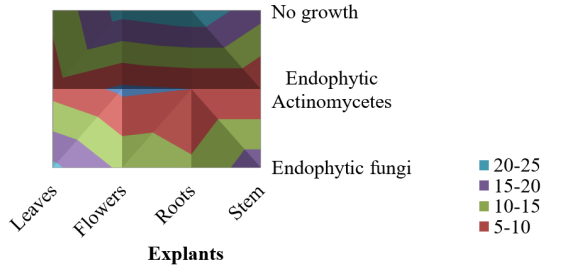

Figure 4: Isolation frequencies of explants of *B. ceiba*

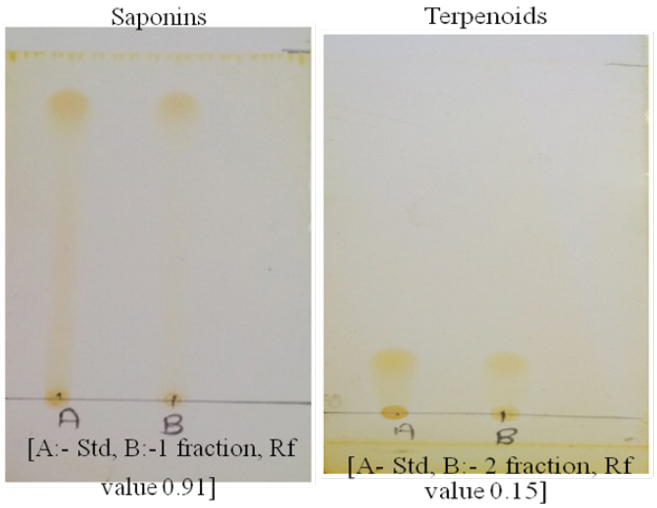

Figure 5: Thin layer chromatography

Table 2: Growth efficacy of endophytic *Aspergillus* species on the different media

Culture medium	*Mycelial growth (mm)*	*Colour of Colony*	*Sporulation*	*No of spores/microscopic field*
TDA	90	Dark green with black	+++	28-31
PDA	65	Blackish	+++	18-21
CDA	62	yellowish-green	++	8-10
MDA	58	Blackish	++	9-12
SDA	56	Lightly black	+++	14-18
WA	53	Black	+	4-8

[TDA= Tomato dextrose agar, PDA= Potato dextrose agar, CDA = Cabbage dextrose agar, MDA = Malt dextrose agar, SDA= Sabouraud dextrose agar, WA = Water agar.
+ = Poor, ++ = Moderate, +++ = Good]

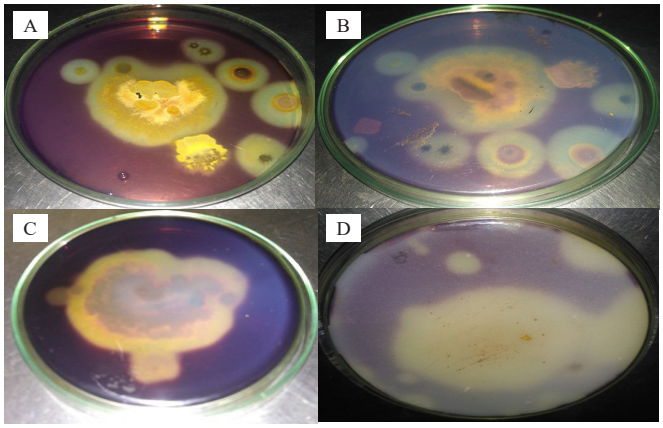

Figure 6: Enzymes Production by an endophytic *Aspergillus niger*. [A] Chitinase, [B] Amylase, [C] Protease, and [D] Cellulase

These bioactive compounds are considered as natural source of antioxidant, anti-microbial and anti-inflammatory agents which have been exposed to decrease the peril and series of many diseases such as cancer and diabetes. Particularly phenol compounds act as a hydrogen donor to radical and a potent radical terminator also it inhibits lipid oxidation[16]. Saponins are used as dietary supplements and nutraceuticals and play a very important role in producing an inhibitory effect on inflammation. Tannins are measured to be having antiviral, antibacterial, antiphrastic, anticancer, antiulcer and antioxidant agents. Even though Phenols and Flavonoids are well-thought-out to be a multitasking and broad group of natural components which possess a broad spectrum of biological activities including terminating free radicals, reducing the oxygen concentration, transforming primary goods of oxidation into non-oxidant molecules and acts as metal chelators.[19] The endophytic *Aspergillus niger* showed the ability for the production of chitinase, amylase, protease, and cellulases by primary screening. This result supports the findings that the *Penicillium* and *Aspergillus* spp showed the production of different enzymes[19]. The results are shown in Figure 6.

CONCLUSION

The present study concluded that the isolation of pharmaceutically potent fungal endophytes from *B. ceiba* could be a promising, safe, and cheap approach for protecting the same plant. This investigation revealed the diversity of fungal endophytes from *B. ceiba* with their multitasking abilities, including bioactive compounds production and enzyme analysis. The natural and artificial media proved as a novel culture medium and that it would be a rapid, effective nutrient house for the cultivation of an endophytic *Aspergillus niger,* which may help in the microbiology to have one more "golden age" with rebirth in curiosity in growth media.

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