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Research Article
Physiology of Endophytic *Aspergillus nomius* EF8-RSM Isolated from *Aloe vera* Western Ghats of Karnataka India

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Abstract

Background and Objective: Fungal taxonomy is lagging behind from decade due to their unrevealed physiology as compared to the bacteria and viruses. Therefore, the present investigation aimed to the physiology of endophytic fungus isolated from *Aloe vera* Western Ghats of Karnataka, India. **Materials and Methods:** The fresh and healthy samples of *Aloe vera* were collected and submerged for the enumeration and purification of fungal endophytes. Most purified and potent endophytic fungus were investigated for their physiology and identified on the basis of ITS sequencing. **Results:** A total of 32 fungal endophytes were isolated and *Aspergillus nomius* EF8-RSM (MN871698) was reported as core fungus. The 14th day was reported as generation time by R2 value at the rate of 0.0841 with direct biomass at the rate of 2.88±0.02 as wet and 0.26±0.01 as dry weight while, indirect biomass was 13.69±0.41 mg gdfs^{-1} with $R^2 = 0.9107$. The fungus utilized cellulose at the rate of 16.27±0.06 cm and the production of different enzymes with $R^2 = 0.2543$ and AgNPs was within 5.86±0.41 h. **Conclusion:** The present research investigation is the first report available from the *Aloe vera* for an endophytic *Aspergillus nomius* with physiological characterization under optimized conditions.

Key words: *Aspergillus nomius*, *Aloe vera,* growth curve, biomass, enzymes, AgNPs

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fungal endophytes are the greatest symbionts in the nature[1-4]. They used to stay with plant tissues without harming their life cycle which results in the formation of mutuality symbiosis[5]. These fungal endophytes were isolated from tissues of different body parts of plants and used for the production of drugs[6]. One such example is the Taxol, which is an anti-cancer drug and isolated from the bark of *Taxus brevifolia*[7-10]. To date, not only Taxol got endophytic drug identity, but also other bioactive compounds potentially used in the preparation of different drugs as a central ingredient or molecule[11]. Endophytic fungi are widely known for the production of the commercial antibiotic and those are active as anti-viral, anti-oxidants[12], anti insecticidal, anti-diabetic agents[13], anticancer agents[14-17] and antiurolithiasis agent[18], etc. The plant selection for the isolation of endophytic fungi is a very tough job as far as plant taxonomy is considered and the isolation of endophytic fungi from such explants is a very keen investigation[19]. This investigation always takes place for months with standard protocols[20]. These plants are mainly known for effective Ayurvedic plants in curing diseases[21]. They are used as astringent, deobstruent, stomachic, diuretic and febrifugal[22]. The aerial parts of all plants have been reported to have alkaloids[23], flavonoids[24], phenols[24], coumarins, tannins[25], terpenoids and lignins[26]. Therefore, the present study was focused on the physiology of potent fungal endophyte from *Aloe vera*.

MATERIALS AND METHODS

Study area and plant material: *Aloe vera* leaves and roots were collected in sterile polythene bags from the different sites of Western Ghats of Karnataka, India, during August-September, 2018 and stored at 4°C. The plant was 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.

Isolation and identification of fungal endophytes: Isolation of fungal endophytes was performed by using the leaves and root samples of *Aloe vera*. After collection of samples, all samples were washed under tap water[1] and surface sterilized with ethanol[2] (75%) for 2 min, sodium hypochlorite (10%) for 3 min and sodium azide[3] (2%) for 5 min. Further, surface sterilized samples were cut and placed on potato dextrose agar media plates[4]. Then all plates were incubated in dark at 25±2°C for 15 days. After incubation, grown fungal endophytes were characterized on the basis of microscopic examinations and the potent one identified on the basis of molecular sequencing[5]. The potent characterized endophytic fungus sequences were corrected by using Bio-Edit software and compared in the NCBI with available data. Further phylogeny was drawn with NCBI website[6-10].

Characterization of the endophytic fungus

Determination of growth curve pattern: Tiny pieces of solid medium (0.5×0.5 cm) bearing mycelia of the endophytic fungus were cut from the preserved solid culture stock and used to inoculate in 100 mL of potato dextrose broth in 250 mL Erlenmeyer flasks. The fungus was incubated at 25±2°C on an orbital shaker (120 rpm). Throughout the growth of the fungus in potato dextrose broth, 5 mL of suspension were withdrawn from the orbital shaker on 0-30th days of the incubation period. This suspension was programmed for transmission of 90% at 540 nm of wavelength on photo-colorimeter. The optical density was recorded and graphs were plotted to obtain growth curve[11].

Determination of biomass

Direct method: The wet and dry weight of the endophytic fungus were determined by harvesting method[12-14]. In this study, tiny pieces of endophytic fungus were incubated in potato dextrose broth at 25±2°C on an orbital shaker with 120 rpm for 15 days. After incubation, mycelia of the fungus were harvested by separating from the culture liquid by filtration through a Whatman No. 1 filter paper. The mycelial pellet was frequently washed with distilled water and determined their wet growth. Further same mycelia were dried at 30°C overnight. The wet and dry weight of the fungus were designed by using the following formula:

Wet/dry weight = Weight of filter paper+Mycelia-Weight of filter paper

Indirect method: Glucosamine content was determined by following the standard methods[15,16]. Dried 0.5 g of fermented sample was taken in a 500 mL conical flask and 2 mL of concentrated sulphuric acid was added to it. This mixture was kept at 25°C for 24 h. It was then diluted with distilled water to make 1N solution and auto-claved at 15 lb pressure for 1 h and filtered through Whatman no. 1 filter paper to remove the sediments. The solution was neutralized with 5N NaOH and made up to 100 mL with distilled water. About 1 mL of the sample was measured for absorbance at 530 nm against reagent blank. The N-Acetyl D-Glucosamine was used as a standard. The value was expressed as mg/glucosamine g^{-1} of

the dried fermented substrate. The glucosamine content was measured for 30 days by taking 3 days gap during the incubation period.

Utilization of carbon sources: The utilization of carbon sources by the endophytic fungus was studied by using different carbon sources such as; glucose, sucrose, cellulose, starch and xylose. The pure culture of endophytic fungus was transferred in potato dextrose broth which was with 2% of each carbon source separately and incubated at 25±2°C for 15 days. After the incubation, grown endophytic fungal plates were used for further morphological characterization[17-20].

Production of extracellular enzymes: Primary screening of the fungus was performed for cellulase, chitinase, amylase, lipase, protease and xylanase on potato dextrose agar at 25±2°C for 15 days by following the standard methods[23,24].

Production of silver nanoparticles: About 20 mL of fungal filtrate was added to 80 mL of 1 mM $AgNO_3$ solution and incubated at 25±2°C for about 2-4 days. After incubation, the silver nanoparticles were detected by the color change of the solution and estimated spectrophotometrically from 200-700 nm[25-27].

Statistical analysis: All experiments were performed in triplicates (n = 3) and the data are presented as the Mean±Standard Deviation.

RESULTS

Isolation and identification of fungal endophytes: A total of 32 fungal endophytes were isolated (Fig. 1a-b) from 100 pieces of 200 explants such as; leaves and roots of *Aloe vera*. Purification of endophytic fungi was done on the basis of macroscopic and microscopic characterization of fungus mycelium and spores (Fig. 1c-f). Most of them were belonging to the classes Deuteromycetes, Ascomycetes, Zygomycetes and Basidiomycetes. The endophytic *Aspergillus nomius* found a core group with the colonization frequency of 63.20% as presented in Fig. 2. Further DNA of the fungus was isolated by chemical method and further sequenced by PCR. The endophytic *Aspergillus niger* found the second-largest core group with colonization frequency of 41.60% followed by *Aspergillus ceremeus* (38.33%), *Sterile mycelia* (33.20%), *Plectosphaerella cucumerina* (27.20%), *Nigrospora* species (20%) and *Trichoderma citrinoviride* (15.30%). The colonization frequency of leaf explants was varied between 0-46.6% and roots explants were varied from 0-20%. The leaves showed the highest colonization frequency by *Aspergillus nomius* (63.20%) and lowest colonization frequency by *Phomopsis* species (0%) while, the root showed the highest colonization frequency by *Phomopsis* species (20%) and lowest colonization frequency by *Nigrospora* species (0%) (Fig. 2). Amplified sequence was used for identification by ITS region and phylogeny was obtained as presented in Fig. 3.

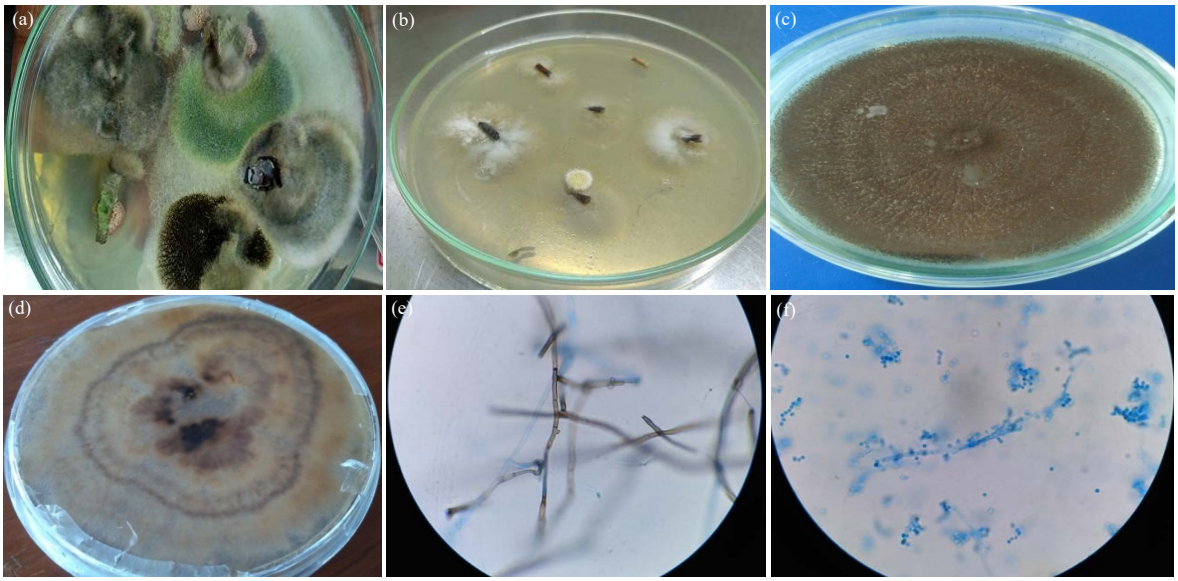

Fig. 1(a-f): Isolation and characterization of fungal endophytes from leaves and roots of *Aloe vera*, (a) Purification of endophytic *Aspergillus nomius*, (b-d) Microscopic characterization of fungus mycelia and (e-f) Spores
Samples were sterilized with the help of sterilants and inoculated on PDA media plates and incubated in dark at 25±2 for 15 days

Growth curve: The fungus was subjected to growth curve study in potato dextrose broth up to 30 days of incubation period at 25±2°C. During 30 days of incubation period, optical density of the fungus was measured spectrophotometrically at 540 nm. On 0th day of incubation, OD was 0.00 and it was considered as an inoculation day, but growth had initiated increasing from day 2nd at the rate of 0.02 therefore, this growth was considered as measurable growth. The period from inoculation to the measurable growth considered as the lag phase. In this phase, the fungus was trying to adjust with new growth conditions. On the 7th day, OD was 0.07 and suddenly on 8th day OD was increased up to 0.18. This growth increased up to day 11th with OD of 0.23, therefore, the period from day 7-11th known as log phase due to the increased growth and production of primary metabolites in the broth for their growth. The growth of the fungus was steady from the day of 12-16th with OD of 0.25, therefore, this phase was considered as stationary phase. On 17th day, slightly the fungus growth was declined and it was continued till 30th day, therefore, this phase was considered as death phase and at last reported generation time of the fungus as 13 days. During this phase, different reasons are established to compensate the death of the fungus. One of the reasons was the exhaustion of nutrients and production of autolysins (Fig. 4).

Determination of biomass: Wet and dry biomass of the fungus were determined by harvesting their mycelia after 15 days of the incubation period. The glucosamine content was effective up to 30 days of the incubation period of the fungus at 25°C for 24 h. The highest glucosamine content in the fungus was observed on 15th day at the rate of 13.69±0.41 mg gdfs^{-1} while after 15 days of incubation period. The peaks decreased their intensity such as on 20th day showed 12.87±0.06 mg gdfs^{-1}, 25th day showed 12.06±0.05 mg gdfs^{-1} and 30th day showed 9.54±0.51 mg gdfs^{-1} of glucosamine content. The R2 value was recorded as $R^2 = 0.9107$ as presented in Fig. 5.

Effect of carbon source: The effect of carbon sources was studied on the fungus and mostly fungus utilized as cellulose on 15th day at the rate of 16.27±0.06 cm while, other carbon sources showed an effect on the growth of the fungus. The xylose affected the growth of fungus at the rate of 15.44±0.64, glucose at the rate of 13.50±1.02, starch at the rate of 12.09±0.09 and sucrose at the rate of 9.37±0.46. The results are shown as xylose (Fig. 6a), glucose (Fig. 6b), cellulose (Fig. 6c), starch (Fig. 6d) and sucrose (Fig. 6e).

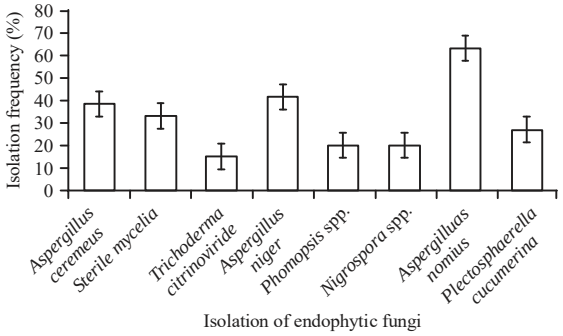

Fig. 2: Isolation and identification of endophytic fungi isolates from *Aloe vera* explants

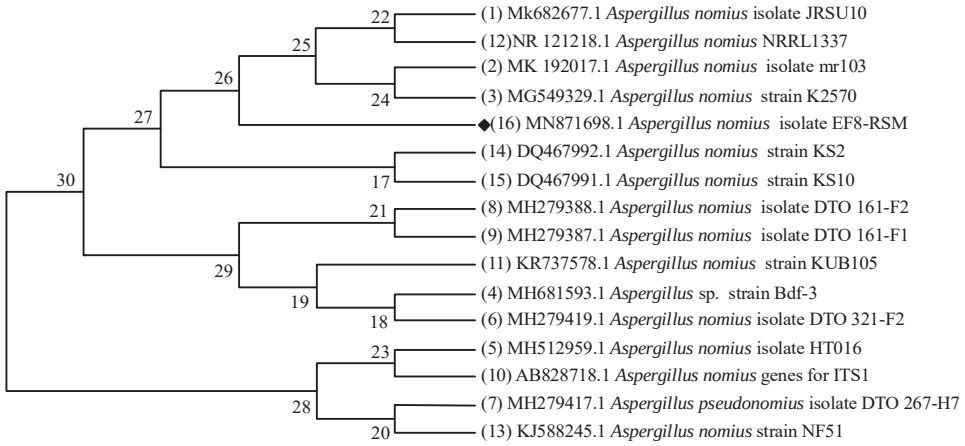

Fig. 3: Molecular identification of fungal endophytes and their phylogeny

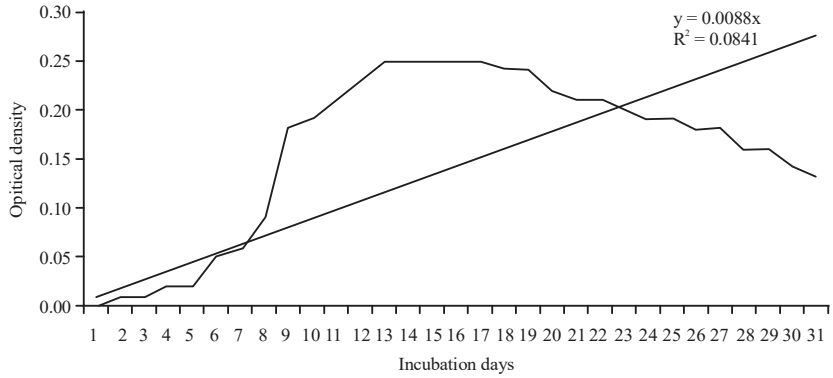

Fig. 4: Growth curve study of endophytic fungus at 540 nm under spectrophotometer shows $R^2 = 0.0841$

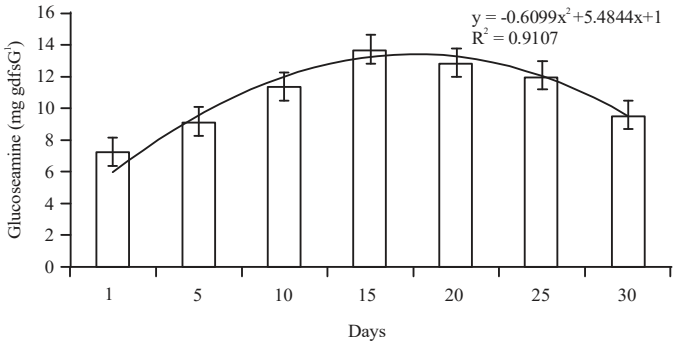

Fig. 5: Glucosamine estimation (mg $gdfs^{-1}$) of the fungus

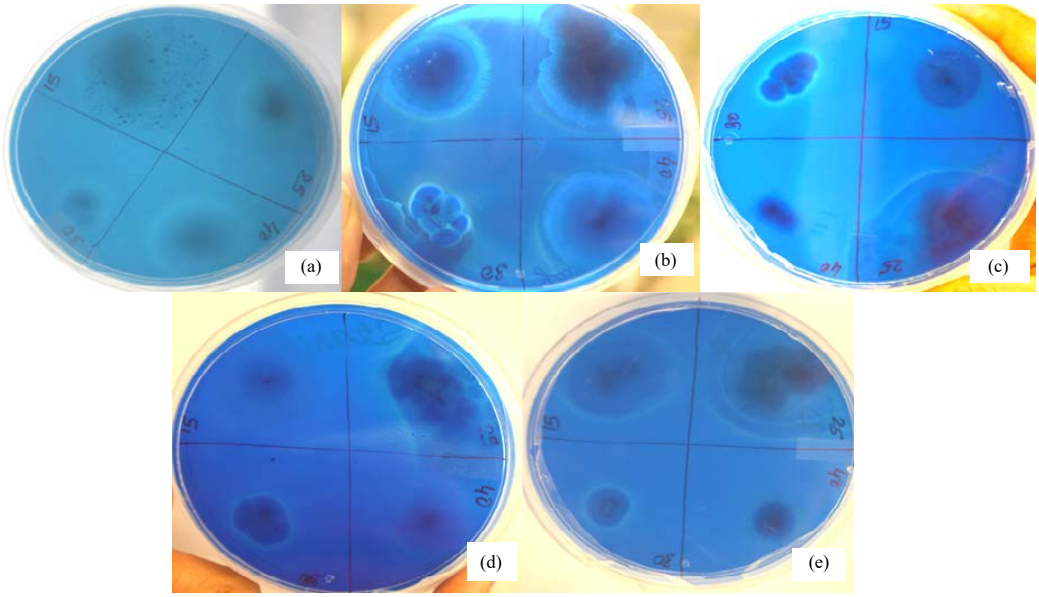

Fig. 6(a-e): Endophytic fungal disc were placed on basal media with different substrates (a) Xylose, (b) Glucose, (c) Cellulose, (d) Starch and (e) Sucrose and kept for incubation at 25 °C for 15 days

After incubation, congo red revealed the zone of hydrolysis which indicates carbon utilization

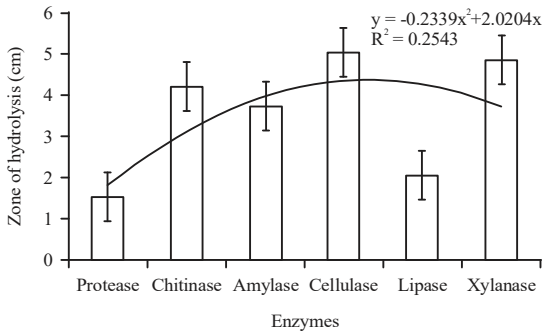

Fig. 7: Extracellular production of enzymes by the fungus

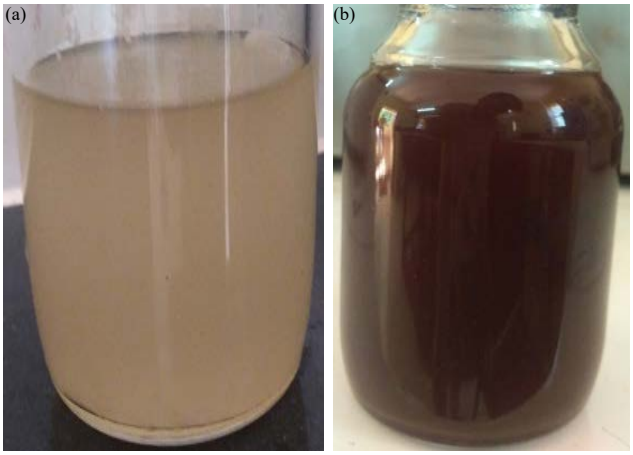

Fig. 8 (a-b): Color changes in the solution after 5.86±0.41 h
Formation of AgNPs under controlled conditions with the endophytic fungus extract

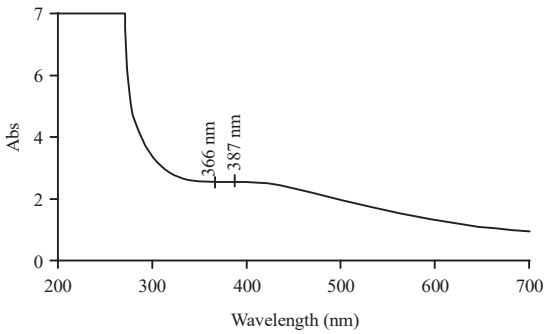

Fig. 9: Absorption bands of AgNPs observed in between 200-600 nm

Production of extracellular enzymes: The endophytic fungus showed the hydrolytic zone around the colony for the production of enzymes at 25±2°C after 15 days of incubation. After observing, zones were recorded and depicted in the Fig. 7.

Nanoparticle production: In the present study, the attempt was made to synthesize AgNPs with the help of endophytic fungus fermented extract and silver nitrate solution. The color change of the solution from light yellow to dark brown color was observed (Fig. 8 a-b). The fungus showed AgNPs production within 5.86±0.41 h. Further, the absorbance band at 366 and 387 nm was recorded due to Localized Surface Plasmon Resonance (LSPR) and confirmed the formation of silver nanoparticles (Fig. 9).

DISCUSSION

Scientists are trying to improve fungal taxonomy from decade by the new techniques including biochemical, physiological and molecular biological methods[1]. These methods may improve the taxonomical graph of fungi and may modify their applications in biotechnology and industry[2]. However, in these cases the significance methods are still unclear due to interrupted knowledge or results instability[3]. Therefore, the present research investigation was undertaken for the physiology of the endophytic fungi. The maximum isolation frequency showed by an endophytic *Aspergillus nomius* at the rate of 63.20% and furthermore the maximum parsimony analysis resulted in a cluster showed the close relationship between the organism that was isolated. This group of organisms was quite diverse due to their phylogenetic evolution. All the evidences confirmed that the EF8-RSM was *Aspergillus nomius*. Ranade *et al.*[4] isolated different fungal endophytes from *Azadirachta indica, Terminalia arjuna* and *Catharanthus roseus* belonging to *Aspergillus* species. Jena and Tayung[5] and Torres and dela Cruz[6] reported highest absorbance peak of *T. mentagrophyte*, *R. oryzae* and *S. schenckii* at 37°C after 10 days of incubation period. The study was undertaken by Mane *et al.*[7] and Khan *et al.*[8] and documented *Sporothrix schenckii* growth peak at 37°C on 13th day while, in the present study, the fungus showed highest peak at 25±2°C on 12th day and remained steady up to 16th day. The fungus recorded R2 value at the rate 0.0841 on the basis of graph while, Mane *et al.*[7] showed R2 value at 0.31 for the fungus (*S. schenckii*). The fungus cell wall is dynamic structures that are essential for cell viability, morphogenesis and pathogenesis and made up of different chemicals such as; chitin, peptidoglycan, starch, proteins and different carbohydrates[8-13]. This all constituents lead to the formation of thick walls and eventually the whole structure[14]. During the incubation of the fungus mycelia on the substrate, turgor pressure was created by the mycelia on to the substrate and easily will penetrate inside which leads to the high biomass of the fungus[15]. Carbohydrate utilization of

endophytic fungus was carried out on potato dextrose agar media containing five different carbon sources separately. Glucose was found to be a suitable source of carbon for the fungus[16]. The fungus utilized all carbon sources, but effective growth obtained on the media where glucose was used[17,18]. Further, Mane *et al.*[19] found that fungal isolates from *Ocimum sanctum* were found positive for protease, amylase and cellulose, while, Mahesh *et al.*[20] from mangrove angiosperm isolates found 66% cellulolytic and lipase activity by isolates of *Brucea javanica*[21-24]. The production of nanoparticles started after the addition of the fungus extract with the silver nitrate solution[25]. The visible color changes (light yellow to dark brown) the solution and spectral analysis confirmed the formation of silver nanoparticles. The absorption band of AgNPs was observed in between 300-400 nm. The color change and absorption spectra confirmed the production of AgNPs. The dark brown color of the solution established the reduction of silver nitrates into the silver nanoparticles. Additional, the configuration of silver nanoparticles was established by the UV-Vis spectrophotometer (300-700 nm). During the reaction, within 6 h the brown color formation took place indicated the formation of AgNPs. This kind of color change in AgNPs synthesis was also reported by Verma *et al.*[25] using an aqueous extract of fungi and plants. The typical peak for AgNPs observed generally ranges from 300-600 nm. UV-spectra obtained in the present study displayed an absorption peak at 387 nm which is specific to AgNPs. The results were somewhat similar to the previous study done by Lakshman *et al.*[26], who observed the absorbance peak at 390 nm. Absorption spectra of AgNPs formed in the reaction mixture have an absorption peak at 320-510 nm and the broadening of the peak indicated the polydispersed nature of particles[25-27].

CONCLUSION

The endophytic *Aspergillus nomius* showed different characteristics such as; slow growth, effective biomass via dry weight and glucosamine estimation, cellulose as a carbon source, plant growth-promoting traits, enzymes and AgNPs production under optimized conditions. All these characteristics indicate that the purified fungus is a potent candidate for the production of vital drug molecules and other agricultural-based products. Therefore, it was concluded that the present research investigation is the first innovative report available from the *Aloe vera* for an endophytic *Aspergillus nomius* with biological physiology from Western Ghats of Karnataka, India.

SIGNIFICANCE STATEMENT

This study discovers the physiology of the *Aspergillus nomius* that can be beneficial for fungal taxonomy. This study will help the researcher to uncover the critical areas of fungal taxonomy that many researchers were not able to explore. Thus, a new theory on fungal taxonomy by means of their physiology may be arrived at.

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