In vitro Antimicrobial activity and Qualitative Enzymatic assays of *Cladosporium pseudocladosporidiales*, AVNK4, an endophytic fungus isolated from seeds of *Nigella sativa*

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Abstract
Endophytes are vital reservoirs of bioactive compounds with pharmaceutical and industrial significance. This study reports isolating and characterising a novel fungal endophyte, AVNK4, from surface-sterilised Nigella sativa seeds, identified as Cladosporium pseudocladosporidiales using morphological observations and ITS-rDNA sequencing. AVNK4 is the first reported isolate of Cladosporium pseudocladosporidiales from Nigella sativa seeds, showing both diverse lytic enzyme production and broad-spectrum antimicrobial activity against human and plant pathogens. Ethyl acetate extracts exhibited strong inhibition zones and low minimal inhibitory concentrations, indicating potent bioactivity. Enzymatic assays revealed substantial production of cellulase, amylase and protease, which enhance the endophyte's bioactive potential. These hydrolytic enzymes enhance the endophyte's bioactive potential by degrading cell walls and assisting in metabolite delivery, synergising with antimicrobial compounds.

Furthermore, some enzymes are directly involved in secondary metabolite biosynthesis such as non-ribosomal peptide synthetases and polyketide synthases, which are responsible for the production of antibiotics and antifungals. The dual functionality of AVNK4, as a producer of both antimicrobial metabolites and industrially relevant enzymes, underscores its promise for biotechnological applications in pharmaceuticals, agriculture and enzyme-based industries as novel drugs and biocatalysts. This study lays the foundation for further metabolite purification and mechanistic investigations to support drug development and industrial enzyme deployment.

Keywords: Bioactive secondary Metabolites, Optimisation, Anticancer, Agar-well diffusion method.

Introduction

The global rise in multidrug-resistant pathogens and the limitations of synthetic drugs have intensified the search for alternative, natural bioactive agents with therapeutic and industrial relevance[11,28]. Over the past few decades, there has been a tremendous increase in population globally encountering numerous obstacles in the healthcare stream including various disorders, cardiovascular diseases, cancer, diabetes and multiple medication resistance[2,43]. Even in the age of vaccinations and treatments, infectious illnesses account for a significant death rate. Medicinal plants and their associated endophytes have emerged as promising sources of such compounds due to their ecological adaptability and metabolic diversity[3,23].

Cancer is the world's second-most prevalent cause of death due to its high prevalence among all ages of mankind. Every year, tumour-bearing cells kill millions of people and the number is constantly increasing with every passing year. The treatment of such a dreadful disease includes either partial or complete excision or treatment with powerful chemotherapeutics, which results in a worsening of the quality of life. To avoid these circumstances, cancer can be treated with alternative natural procedures involving the use of safe, economical, biocompatible, natural compounds derived from endophytic microbes with very minimal side effects. These natural compounds have anticancer properties and can treat a wide range of cancers.

Plants produce a variety of secondary metabolites which can be classified chemically as phenolics, terpenes, or alkaloids[3]. *Nigella sativa*, commonly known as black cumin, is a medicinal plant extensively used in traditional medicine. While its phytochemical constituents are well studied, the microbial endophytes inhabiting its seeds remain underexplored. Endophytes are microorganisms that colonise the internal tissues of plants without causing harm and they are known to produce a diverse array of secondary metabolites including antimicrobial agents, anticancer compounds and plant-growth regulators[32,40].

Endophytes release a variety of pharmacokinetic compounds which were biologically potent and active as metabolites from plant cells and help them to survive in stressful conditions and to defend against plant pathogens[5]. Endophytic fungi are used by the biochemical and pharmaceutical industries as they possess significant potential medical and agricultural applications.

The word endophytes were used in the nineteenth century[6]. Endophytic fungi have been left to be a relatively unexplored source of metabolites, which might be helpful in the

pharmaceutical and agricultural industries. Among endophytes, fungi are particularly notable for their ability to secrete extracellular enzymes such as cellulases, proteases and amylases which enable them to create symbiotic connections with host plants[10]. Such extracellular enzymes produced by the endophytic organisms were more durable and can function at a wide range of cultural conditions like pH, temperature, pressure and minimal energy sources when compared to the enzymes made using standard synthetic catalysts[11].

These enzymes assist in plant colonisation, pathogen suppression and nutrient acquisition and they are also applicable in various biotechnological processes such as biodegradation, enzyme-based industry and agricultural waste recycling[12,13,24,35].

In this study, we report the isolation and functional characterisation of a novel fungal endophyte, AVNK4, from surface-sterilised seeds of *N. sativa*. The strain was identified as *Cladosporium pseudocladosporidiales* through ITS rDNA sequencing and morphological analysis. Its antimicrobial activity against human and plant pathogens and production of extracellular lytic enzymes were investigated. Fungal species was isolated from *N. sativa* seeds exhibiting dual bioactive potential, suggesting its value in pharmaceutical and industrial biotechnology.

Material and Methods

Sample Collection: Healthy, well-dried *Nigella sativa* seeds were collected from a certified herbal supplier in Riyadh, Saudi Arabia (collected in March 2023). The seeds were sorted to remove damaged or infected ones by sieving out the contaminants or any damaged seeds. The selected seeds were washed thoroughly under running tap water for 10 minutes and rinsed completely with double-distilled water, sun-dried and kept in an airtight container to maintain aseptic conditions for further research purposes.

Isolation of Endophyte Fungi from *Nigella sativa* Seeds: 30 g of well dried seeds of *Nigella sativa* were cleaned thoroughly before being surface sterilised with 20% hydrogen peroxide (20 minutes), 95% ethanol (5 minutes), 4% sodium hypochlorite (3 minutes), 0.1% mercuric chloride solution (1 minute) and 20% formaldehyde (2 minutes), followed by 3 sterile water rinses. To verify sterilisation efficacy, an aliquot of the final rinse (100 µL) was plated on Sabouraud dextrose agar (SDA) as a control. Sterile seeds were macerated in a sterile mortar and pestle with 5 ml of double-distilled water, serially diluted (up to 10^{-5}dilutions) and 100 µl of each dilution was inoculated on SDA plates with chloramphenicol (100 µg/ml) to inhibit bacterial growth via the pour plate method.

Triplicates of these plates were incubated at room temperature until the emergence of the endophytic fungi on the surface of the medium and the well-developed endophytic fungal colonies were subcultured individually based on morphological distinctions to produce pure cultures[10]. AVNK4 is one of the isolated endophytic fungi preserved on SDA slants at 4˚C for further analysis.

Molecular Identification of AVNK4: Genomic DNA was extracted using the CTAB method[21]. The internal transcribed spacer (ITS) region was amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR reactions were carried out in 25 µl volumes with 1× buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Taq polymerase and 10 µM of each primer. Thermal cycling included denaturation (94 °C, 5 min), 35 cycles of 94 °C (30 s), 55 °C (30 s), 72 °C (1 min) and final extension at 72 °C (7 min). Sequencing was outsourced to Macrogen (Seoul, South Korea).

The molecular identification of AVNK4 was done through 18S rRNA sequencing (Sanger dideoxy sequencing) using Location/Qualifiers source 1..1302 /organism= "Cladosporidium pseudocladosporidiales"/ mol_type=" genomic DNA" /isolate="AVNK4" /db_xref="taxon:5334" /geo_loc_name="Saudi Arabia" /collection_date="24-Mar-2023" rRNA <1..>1302 /product="small subunit ribosomal RNA" by Macrogen, a commercial business in Seoul, South Korea, followed by phylogenetic analysis using the neighbor-joining method[16]. The resulting ITS sequence was submitted to NCBI GenBank (Accession No PP957910) and analysed using BLAST and neighbour-joining phylogenetic analysis[18].

Morphological and Microscopic Characterisation of AVNK4: The isolated fungi, AVNK4, were grown on SDA for 7 days at 28˚C. Colony characteristics such as colony morphology (surface and texture), mycelia, colony colour, margins, pigmentation and conidia position and structure were documented. Slide cultures were prepared using the method of Rosana et al[30] as described in the taxonomy guide and microscopically identified by examining internal properties of mycelia and sexual/asexual spores under a light microscope. This approach involves inoculating a 2x2 cm agar block on a sterile glass slide with *Cladosporium pseudocladosporidiales*, covering it with a sterile cover slip and placing it in a sterile Petri plate. *C. pseudocladosporidiales* is incubated till fully grown. The coverslip was then hyphal and conidial structures were stained with lactophenol cotton blue and placed on a clean, sterile slide for examination under the microscope at 40x and 100x magnification[15].

Preliminary screening of antibacterial activity with the dual-culture method: The dual-culture technique for screening of antagonistic activity was developed by Bell et al[5] and modified by Devi et al[8]. It was utilised to evaluate the antagonistic effect of AVNK4 against selected plant pathogenic fungus species, namely *Aspergillus brasiliences*, *Aspergillus niger* and *Fusarium oxysporum*[5,8,14]. In this procedure, 5 mm discs of AVNK4 and pathogenic fungi were spot inoculated from actively growing colonies of both

pathogenic and endophytic fungi on opposite sides of SDA plates and incubated at 28 ± 2 °C for 10–14 days. The plate with only pathogenic fungi serves as a negative control. Triplicates of the dual-culture plates were incubated for approximately 12-14 days at room temperature.

The antagonistic activity was assessed by comparing the growth inhibition of pathogenic fungi to that of negative control plates[31] and rating the degree of antagonism on a scale of 1 to 5.

Class 1: Highly antagonistic endophytic fungi which cover the entire medium surface by outcompeting the pathogen completely.

Class 2: Moderately Antagonistic endophytic fungi colonise at least two-thirds of the medium surface.

Class 3: Mild antagonistic Endophytic fungi covers almost half of the medium surface and colonize with the pathogen without dominating it.

Class 4: Poor antagonistic fungi colonise at most one-thirds of the medium surface and could not dominate the invasion by endophyte.

Class 5: Non- antagonistic endophytic fungi could not grow on the medium and get outcompeted by the actively growing pathogen. The efficiency of the antimicrobial activity of AVNK4 was further validated using the agar well diffusion method.

Secondary screening of antimicrobial activity using the agar well diffusion method: The secondary screening of antimicrobial activity of AVNK4 was evaluated against Gram-positive bacteria *Staphylococcus aureus* (NCIM2079), *Clostridium sporogenes* (NCIM5113), Gram-negative bacteria *Salmonella typhimurium* (NCIM2501), *Pseudomonas aeruginosa* (NCIM2200) and the pathogenic fungus *Candida albicans* (NCIM3471). The pathogens are pre-cultured in Mueller Hinton broth and standardised to 0.5 McFarland turbidity (~10^8 CFU/ml)[16]. The AVNK4 inoculum was generated by inoculating the 3-day-old culture into Sabouraud's broth and incubating it for 21 days. After incubation, the fully grown AVNK4 culture was filtered using Whatmann no. 1 filter paper and the filtrate was collected and tested for antibacterial properties.

1 ml of freshly developed pathogenic bacterial culture was mixed well into Mueller-Hinton agar medium, poured onto a Petri plate and allowed to solidify by leaving it undisturbed for some time. After proper solidification, a sterile cork borer was used to create 6 mm wells. 100 µl of ethyl acetate extract, methanol extract and 100 µl of standard streptomycin solution (100 µg/ml) served as positive control, while the empty well served as a negative control. Plates were incubated at 37 °C for 24 h and zones of inhibition (mm) were recorded in triplicate[13]. The bioactivity was assessed using the zone of inhibition (ZOI) (including well diameter) that formed after 18-24 hours of incubation[30].

Qualitative estimation of primary metabolites of AVNK4 utilising enzymatic assays: Endophytic fungi can produce antimicrobial agents and commercially important enzymes such as amylases, lipases and cellulases, which can either act directly on pathogens by breaking their cell walls, interrupting the protein synthesis and cell wall synthesis or by producing intermediate compounds with antimicrobial properties[17]. Screening of extracellular lytic enzymes produced from the endophytic fungi was done using the qualitative approach using agar plate technique and the quantitative method using liquid culture technique. Extracellular enzymes were qualitatively assessed in specific growth medium using agar plate-based methods by evaluating the zone of clearance (ZOC) produced by the endophytic fungi and the plates which were left without inoculation of fungi, served as control plates[19,38].

Amylolytic Activity: To qualitatively analyse amylolytic activity, Sabouraud's agar medium supplemented with 1% starch at pH 6.0 was used. After incubation, the plates were completely flooded with iodine on the top surface of the media. A distinct zone around active colonies demonstrated amylolytic activity of AVNK4[28,45].

Proteolytic Activity: To qualitatively assess protease activity, the endophytic fungal strain AVNK4 was cultured on Sabouraud's medium supplemented with 0.4% gelatin. After incubating for approximately 7-9 days, the plates were completely flooded with saturated ammonium sulphate, revealing a zone of clearance surrounding the active colonies[19,21].

Cellulolytic Activity: To qualitatively determine cellulolytic activity, Sabouraud's medium is supplemented with 0.5% carboxymethylcellulose (CMC). After an incubation period of 5 days, the plates were flooded with 0.2% aqueous Congo red solution and then destained with 1M NaCl and left undisturbed for a period of 15 minutes. The presence of a translucent zone appears in yellow colour around the fungal colony whereas the leftover region of the media in red colour demonstrates the cellulose activity[19,23].

Lipase Activity: To qualitatively determine lipase activity, the endophytic fungi were cultivated on Sabouraud's agar medium, which is mixed with 1% tween 20. After the end of the complete incubation period, a sedimentation ring formed at the site of the active fungal colony as calcium salts precipitated, indicating lipase activity[23,24,41].

Laccase activity: It was determined qualitatively using Sabouraud's medium containing 0.05g of 1-napthol. After a period of incubation, the colour of the media surrounding the colony changed from colourless to blue, suggesting that the laccase enzyme was produced[7].

Chitinase activity: Sabouraud's medium supplemented with 0.2-1% of colloidal chitin and 0.5 ml of conc. HCl is used to determine the chitinase activity of endophytic fungi AVNK4 qualitatively. After the incubation period, the Petri plates were deeply flooded with 0.1% Congo red solution or

Gram's Iodine solution. The presence of a tough zone surrounding the developing colony suggests the activity of chitinase, which breaks down the colloidal chitin polysaccharide to N-acetylglucosamine (GlcNAc) units[1,7].

L- Asparaginase activity: Qualitative enzymatic screening of L-Asparaginase activity is determined by cultivating the endophyte on Sabouraud's media supplemented with L-Asparagine and phenol red (pH Indicator). The colour change from yellow to pink around the colony demonstrates the increase in pH and the production of L-Aspartic acid by the action of L-Asparaginase enzyme[18,22].

L-Glutaminase activity: Qualitative enzymatic screening of L-Glutaminase activity is determined by cultivating the endophyte on Sabouraud's media supplemented with L-Glutamine and phenol red (pH Indicator). The colour change from yellow to pink around the colony demonstrates the increase in pH and the production of L-Glutamic acid by the action of L-Glutaminase enzyme[18,22]. The percentage of the degradation of *C. pseudosporidiales* using the lytic enzymes production was evaluated using the following equation:

Percentage of Degradation (%) = x (in mm) / x (in mm) + y (in mm) Degradation area.

where x = colony diameter, y = halo diameter (ZOC). Enzyme potential was scored as: +++ (very high, <0.33), ++ (high, 0.33–0.69), + (weak, 0.69–0.99), – (no activity = 1).

Results and Discussion

Sampling: Samples were collected and stored aseptically for future research.

Isolation of Fungal Endophytes from *Nigella sativa* Seeds: Eight fungal endophytes (AVNK1-AVNK8) were isolated from surface-sterilised seeds of *N. sativa* and subcultured to form pure cultures. Among these, AVNK4 showed distinct morphological features and potent antimicrobial activity and was selected for further analysis.

Molecular Identification of AVNK4: The ITS rDNA gene sequencing confirmed the identity of AVNK4 as *Cladosporium pseudocladosporidiales* by using amplification with PCR and with Sanger dideoxy sequencing. The blast sequence of the AVNK4 strain in ex-taxon was subjected to a pairwise sequence similarity search which revealed that the 18s rRNA gene sequence from AVNK4 had 100% similarity with reference strains. Phylogenetic analysis using the neighbour-joining method grouped AVNK4 with the *Cladosporium* clade was illustrated by the dendrogram in figure 1, corroborating its taxonomic placement[18]. It was deposited in NCBI as *Cladosporidium pseudocladosporidiales*(AVNK4) with Gene Bank accession number PP957910.

Morphological and Microscopic Attributes of *Cladosporium pseudocladosporidiales*: Morphological examination of AVNk4 reveals confined folded superficial colonies that are velvety to dusty, smole grey to olivaceous and felty-floccose, with a dark green-black reverse. Maximum growth occurs at room temperature and sporulation occurs after 9 days of inoculation without colouring of the agar. Microscopically, the Lactophenol cotton blue mount of *C. pseudocladosporidiales* revealed Hyphae that are pale, olivaceous and have smooth or somewhat rough walls. Conidiophores are slightly or distinctly cylindrical, upright, solitary and frequently flexuose, with conidia on dispersed, cylindrical to conical denticles. Following detachment, an inconspicuous frill frequently remains on both the denticle and the conidium base.

Conidiogenous cells are generally terminal but sometimes intercalary, somewhat attenuated, cylindrical or oblong, 10-30 µm long, septate, pale olivaceous, smooth, with a base width of 2-3 µm. Terminal conidia are tiny, obovoid, ovoid, or ellipsoid, measuring 3-6 × 1-3 µm, whereas intercalary conidia are ovoid, ellipsoid, or sub-cylindrical, olivaceous to pale brown, consistent with *Cladosporidium pseudocladosporidiales*[3,30]. Macroscopic and microscopic characteristic features were given in figure 2.

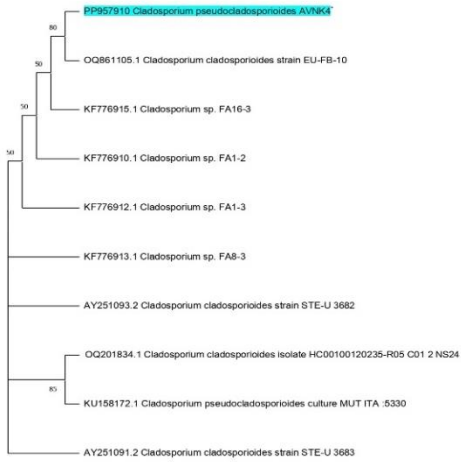


Figure 1: Dendrogram of (AVNK4) *Cladosporium pseudocladosporidiales.*

The preliminary screening of antibacterial activity with the dual culture method: Figure 3 depicts the moderate antagonistic activity of *Cladosporium pseudocladosporidiales*, AVNK4, against phytopathogens evaluated using the dual culture technique by comparing the pathogenic fungal growth radius in a control plate (pathogenic fungi alone) to endophytic fungi in a dual culture plate. After 21 days, *C. pseudocladosporidiales* demonstrated significant growth inhibition on *Aspergillus brasiliensis*, indicating its antibacterial efficacy. The radius of pathogenic fungi alone in the control plate = 84 mm. The radius of pathogenic fungi along with *C. pseudocladosporidiales* in a Dual culture plate = 8.82 mm

Radius of growth inhibition (RGI): Radius of pathogenic fungal colony without *C. pseudocladosporidiales* (in mm) – Radius of pathogenic fungal colony with *C. pseudocladosporidiales* (in mm)

RGI of *C. pseudocladosporidiales* = 84 – 8.82 = 75.18 mm

% of Growth inhibition (GI) of *C. pseudocladosporidiales* = (RGI / Radius of Fungal Colony without *C. pseudocladosporidiales*) × 100

% of GI of *C. pseudocladosporidiales* = 75.18/84 × 100 = 0.895 × 100 = 89.5%

The inhibition % of *C. pseudocladosporidiales* against harmful fungal pathogens was calculated as 89.5% with an RGI of 75.18 mm as depicted in figure 3.

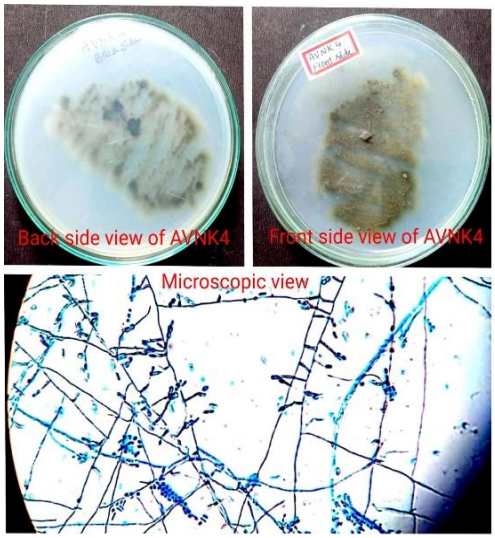


Figure 2: Macroscopic and microscopic view of AVNK4 *Cladosporium pseudocladosporidiales*.

According to Bell et al[5], such high inhibition suggests class 1 antagonism, highly competitive and aggressive colonisation by the endophyte. Similar findings have been reported for *C. pseudocladosporidiales* isolated from soil which showed 86.47% growth inhibition, which is lower than the growth inhibition of 89.5% of AVNK4, *C. pseudocladosporidiales* isolated from *N. sativa* seeds.[24,39]

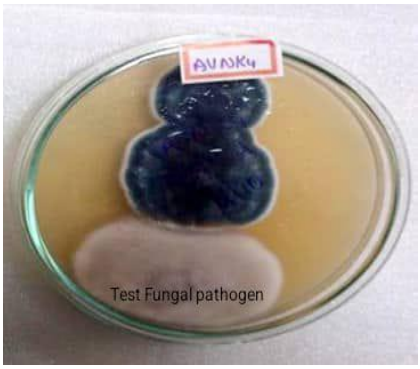


Figure 3: Antimicrobial activity of AVNK4 *Cladosporium pseudocladosporidiales* using the Dual-culture method.

Antimicrobial activity of AVNK4 as determined by the Agar Well-Diffusion Method: The antibacterial activity of ethyl acetate extract of *C. pseudocladosporidiales* against several human infections was measured by the zone of inhibition (clearing zone in mm) using the Agar Well-Diffusion method. The highest zone of inhibition (ZOI) was observed against Gram-positive bacteria (*Staphylococcus aureus (48mm), Clostridium sporogenes (42mm)*) than Gram-negative bacteria (*Salmonella typhimurium (26mm), Pseudomonas aeruginosa (20mm)*) followed by *Candida albicans* (18mm), depicted in figure 4.

According to the American Society of Microbiology guidelines, the zone of inhibition (> 19mm) shows strong antimicrobial activity, moderate antimicrobial activity with ZOI (>15-18mm) and low antimicrobial activity with ZOI (< 14mm), showing resistance of harmful microorganisms. Table 1 shows that *C. pseudocladosporidiales* extracts have higher antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus, Clostridium sporogenes*) than Gram-negative bacteria (*Salmonella typhimurium, Pseudomonas aeruginosa*) due to simpler peptidoglycan structure, consistent with earlier reports[11,36]. As shown in table 1, Endophytic fungi, *C. pseudocladosporidiales*, exhibit significant antimicrobial activity against pathogenic bacteria, with the highest clearance zone of 48 mm, which is significantly larger than the clearance zone of 11 mm against *Staphylococcus aureus*[32,42] supporting our findings.

Qualitative estimate of Primary metabolites utilising enzymatic assays: Various qualitative enzymatic screenings of the endophytic fungal strain, AVNK4, *Cladsporium pseudocladosporidiales*, are performed using various appropriate media and the Halo zone or zone of clearance (ZOC) is precisely measured to calculate the percentage of degradation of the extracellular enzyme produced by AVNK4.

Fungal colony diameter (in mm) = x mm
Degradation area (Zone of Clearance, ZOC) = y mm

Percentage of Degradation (%) = x (Diameter of the endophytic fungal colony (in mm)) / x (Diameter of the

endophytic fungal colony (in mm)) + y (Degradation area by the lytic enzymes around the colony/ (ZOC) (in mm))

% of Enzymatic degradation = x/x+y.

The score of percentage degradation of the enzyme produced by *Cladsporium pseudocladosporodiales* to synthesise various enzymes was investigated and is reported in table 2. Strong production (++/+++) was observed for lipase, cellulase, amylase, L-asparaginase and L-glutaminase, while minimal production was recorded for Protease and laccase. Several investigations have shown that endophytic fungi produce extracellular enzymes such as amylase, pectinase, cellulases, laccase and protease as a defensive approach for resistance against the pathogens and to develop sustenance from host plants[25,27].

Bioremediation procedures based on microorganism enzyme systems, particularly endophytic fungi, have gained a lot of interest, as they can be employed for detoxification of industrial and agricultural waste, as well as other harmful compounds[26].

According to certain research, endophytic fungi's amylase activity aids in the destruction of starch in the host plant as it ages. Amylase was first used medicinally to treat digestive issues and it was the first enzyme produced for commercial use[34]. It is also employed in the food business to improve the taste and flavour of pastries, textiles, paper and detergent industries, as well as animal feed production.

The physiology of endophytic fungi depends on protease enzymes, which break down proteins in living tissues by breaking down extracellular large peptides. Through the breakdown of cell wall glycoproteins, they facilitate entry into host tissues. Protease enzymes play a role in blood coagulation, diabetes, wound healing and digestive health. Endophytic fungi have been shown to secrete cellulases, demonstrating that the fungus could penetrate live host cells as well as dissect dead components.

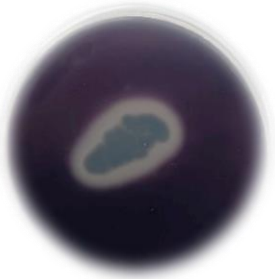
Figure 4: Antimicrobial activity of Ethyl acetate extract of AVNK4 *Cladosporium pseudocladosporidiales* against *Staphylococcus aureus* Agar well-Diffusion method

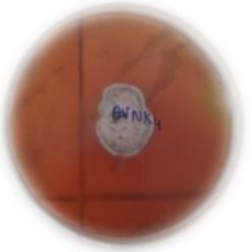
Table 1
Antimicrobial activity of *Cladosporium pseudocladosporidiales* against Human Pathogens using Agar well-diffusion method

S.N.	Human Pathogenic Organism	Zone of Inhibition in terms of mm	Antimicrobial Efficiency
01	*Staphylococcus aureus*	48	+++
02	*Clostridium sporogenes*	42	+++
03	*Pseudomonas aeruginosa*	26	++
04	*Salmonella typhimurium*	20	++
05	*Candida albicans*	18	+

+++ → Highly susceptible; ++ →Susceptible; + → Intermediate

Table 2
Qualitative enzymatic screening of AVNK4 *Cladosporium pseudocladosporidiales*.

S.N.	Enzymatic assay	Inference	Score of Percentage (%) of Enzymatic degradation	Potentiality of enzyme production of AVNK4	Observation
01	Amylase	The clear halo zone around the colony shows the presence of amylase.	0.55	High ++	
02	Protease	The clear halo zone around the colony shows the presence of protease.	0.72	Weak +	
03	Cellulase	The pale yellow coloured halo zone around the colony shows the presence of cellulase.	0.48	High ++	
04	Lipase	The zone of white precipitation around the colony shows the presence of lipase	0.30	Very high +++	
05	Chitinase	The orange shaded zone around the colony shows the presence of Chitinase.	0.46	High ++	

06	Laccase	The formation of a bluish coloured zone around the colony shows the presence of Laccase	0.72	Weak +	
07	L-Asparaginase	The colour change of the media from pale yellow to orange colour especially around the colony, shows the presence of l-L-asparaginase.	0.29	Very high +++	
08	L-Glutaminase	The colour change of the media from yellow to pink colour especially around the colony, shows the presence of L-glutaminase.	0.24	Very high +++	

A score of % degradation of enzyme is 1→. Production of enzyme is not present and denoted by "-"(No)
If score is between 1 and 0.69→ Production of enzyme is low and denoted by "+"(weak)
If score is between 0.69 and 0.33→ Production of enzyme is high and denoted by "++"(high)
If the score is less than 0.33→, Production of the enzyme is very high and is denoted by "+++"(very high)

Endophytic fungi produce cellulase which has been demonstrated to inhibit pathogen development *in vitro* by hydrolysing the cell wall. The abundance of cellulosic materials on Earth represents a prospective and perfect energy source; therefore, considerable research has continued to demand renewable, environmentally benign fuel sources with the use of cellulose-degrading enzymes, making them one of the most commercialised energy sources in the world[35,38].

Lipases are used in numerous commercial applications such as cosmetic additives and detergents, wastewater treatment, industrial effluent treatment, leather defating and biodiesel production[4,7,29,33,36]. Lignolytic enzymes like xylanases and laccases produced from endophytic fungi can bio bleach agriculture waste-based pulps by cleaving the β-1,4 backbone of complex plant cell walls for papermaking[37,39]. Chitinase is found in microorganisms and is required for building up symbiotic associations between the endophyte and the plant, in the decomposition of dead plant materials[40], as well as protecting from the attack of pathogenic fungal microbes and because of which they gained considerable applications, particularly in the paper and food sectors and agricultural industry[37]. Endophytic microbes reside asymptomatically within plant tissues and contribute to host benefits by L-Asparaginase and L-Glutaminase, thereby helping in nitrogen cycling by releasing ammonia in nitrogen metabolism, combating biotic/abiotic stress tolerance and helping to maintain cellular homeostasis in plants[42]. They are also helpful in the growth promotion of plants by releasing growth hormones, antifungal metabolites, inducing to induced systemic resistance by altering nitrogen fluxes and thereby enhancing yield[35].

L- L-Asparaginase depletes L-asparagine which leukaemia cells cannot synthesise due to low asparagine synthetase expression, leading to cell death or apoptosis. L-Glutaminase targets glutamine addicted tumours by disrupting glutamine-dependent biosynthetic and energy pathways, showing promising actions in cancerous cells[9,22]. In addition to industrial and environmental applications, endophytic fungi gained scope in the field of medicine. According to several studies, endophytic fungi such as *Taxomyces andreanae*, *Seimatoantlerium nepalense, Alternaria alternata and Chaetomella raphigera* are involved in the manufacture of the anti-cancer medication paclitaxel[20]. It attaches to tubulin, preventing depolymerisation during cell division[44].

Many studies proved that potent anticancer compounds like camptothecin were produced from *Aspergillus niger* inducing apoptosis. Vinblastine produced by *Fusarium*

oxysporium is effective against lymphoblastic leukaemia and different cancer cell lines[41].

Conclusion

The seeds of *Nigella sativa*, a ubiquitous medicinal plant, contain several endophytic fungi capable of producing hydrolysed enzymes as primary metabolites and pharmacologically relevant antimicrobial chemicals as secondary metabolites. *Cladosporium pseudocladosporidiales'* antibacterial activity against human infections was demonstrated using the dual-culture and agar well-diffusion methods. The efficacy of antibacterial activity was demonstrated by growth inhibition or antagonistic activity (89.5%).

The strong secretion activity of numerous commercially relevant extracellular enzymes suggests that these fungi can be engaged as a promising source in a variety of applications following separation and characterisation. Endophytic fungi's ability to create extracellular enzymes, powerful and effective antimicrobial agents, demonstrates their potential for therapeutic uses in clinical microbiology and biotechnology.

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