

## Isolation and Molecular Characterization of Indian Isolates of Nematode Trapping Fungi

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**Abstract:** Two nematode trapping fungi were isolated from aerated soil samples collected from Anand district (Gujarat) India and identified on the basis of their culture –morphological characteristics and 18S rRNA gene sequencing as *Arthrobotrys oligospora* and *Duddingtonia flagrans*. These fungal isolates were assessed for their trapping efficiency further. Trapping structures such as ring like structures were observed under microscope. Growth conditions with respect to media, temperature and pH were optimized. This study showed that the *Duddingtonia flagrans* and *Athrobotrys oligospora* are nematophagous fungi.

**Key words:** *Arthrobotrys conoides*, *Duddingtonia flagrans*, biocontrol, nematophagous fungi.

### INTRODUCTION

Nematophagous fungi are a diverse group of carnivorous fungal species with the ability to infect and parasitize nematodes for the benefit of nutrients. [33,34]. These fungi are capable of developing specific trapping devices such as adhesive networks, adhesive knobs, and constricting rings to capture nematodes and then extract nutrients from their nematode prey [46,62]. Most nematode-trapping fungi can live as both saprophytes and parasites [45, 46, 55]. They play important roles in maintaining nematode population density in diverse natural environments. Their broad adaptability and flexible lifestyles also make them ideal agents for controlling parasitic nematodes of plants and animals [45, 46, 83, 84].

The nematophagous fungus *Duddingtonia flagrans*, the only species in family Orbiliaceae. Is one of the best-studied nematode-trapping fungi?

The ability to trap nematodes makes it an attractive candidate agent for controlling parasitic nematodes of plants and animals. *Duddingtonia flagrans* are capable of surviving passage through the gastro-intestinal tract of ruminant livestock. This has opened up the possibility of exploiting this fungus as a biological control agent of nematode parasites of livestock because it provides the opportunity to

incorporate the fungus into a variety of practical deployment options.

Chemical nematicides are widely used to control the nematode population due to their instantaneous result although, the excess uses of nematicides have resulted in the development of resistance in nematode population and moreover, these chemical are toxic to plant, soil and environment [3]. These incidents have directed the attention of agricultural scientists to develop bioproducts to control the parasitic nematodes. In fact use of biological products to protect plant form infectious diseases has got momentum in the field of agriculture as it is an ecofriendly and economically feasible approach.

The activities of soil microorganisms shape the soil characteristics as well as plant health and productivity [9]. Soil harbours a diverse range of fungi and many of them are rivals of nematodes [69]. Nematode-trapping fungi form a peculiar trapping structures by which it trap the nematodes and kill them. These fungi are possible candidate to be used as a biocontrol agent. Nematophagous fungi nowadays become a research of interest for the scientists all over the globe to control parasitic nematodes. Many researchers have isolated and studied their antagonist potential. Species of *Arthrobotrys* have been found to show the predatory

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activity against both plant and animal parasitic nematodes [10, 81]. On the other hand, *Duddingtonia flagrans* has been extensively studied against animal parasitic nematodes [7, 42, 60] and it been also reported to produce chlamydospores on large scale which can undergo gut passage in small ruminants [24, 75].

As discussed above, increasing population of world has end up in the excess use of chemicals in agriculture due of high demand of food. Now a day's pesticides are extensively used in agriculture to protect crop plants from pathogen. However, majority of these pesticides does not seem to be specifically targeting the specific organism but conjointly have an effect on non target organisms [13, 21, 30, and 64]. So it may happen that when one apply nematophagous fungi together with such pesticides, pesticides may interfere with their performance. Thus, for it to be successfully integrated into field, nematophagous fungi have to be assess their compatibility with the variety of fungicides, insecticides and fungicides which are currently widely employed in agriculture to protect agriculture crop form infectious diseases. Fungi which may resist such pesticides are often successfully implemented in the field for biocontrol purpose. In the present work, effect of different fungicides, herbicides and insecticides on growth of two nematode-trapping fungi i.e. *A. conoides* and *D. flagrans* was also checked under laboratory conditions. The pesticides that we have selected in the present work are well known and extensively used in agriculture crop protection.

Many researches from India have reported various biological agents to control root-knot nematodes [63, 71]. However, in India less attention has been paid on biocontrol of plant parasitic nematodes using nematophagous fungi. In India till date *Arthrobotrys oligospora*, *Dactylaria brochopaga* and *Monacrosporium eudermatum* have been reported for the control *Meloidogyne* spp. [65, 66, 67, 68, 69, 77]. In the present work, attention has been paid on the control of *Meloidogyne* spp. of root-knot nematodes which cause major crop loss. In the present study, two nematode trapping fungi form agriculture soil of Anand district of Gujarat was isolated and characterized based on morphology and 18S rDNA gene sequences. Additionally, their predatory activity against *Meloidogyne* spp. was investigated.

## MATERIALS AND METHODS

### Isolation of nematophagous fungi and its morphological characterization

A total 20 soil samples were collected from the vicinity of tomato roots from various agricultural fields

infected with nematodes in Anand district of Gujarat during November 2014 to January 2015.

### Isolation of nematode-tapping fungi

Nematode-trapping fungi were isolated on water agar (Hi-Media, India), pH 7.0 as per the method described by Nagee *et al* (2001). Briefly, 1g of each soil samples were sprinkled on 2% water agar plates amended with tetracycline (50µg/mL) to avoid bacterial contamination. Plates were incubated at 25±1°C in dark. After 10 days, as different fungi started growing, 1mL of nematode suspension (~2000-3000 live nematodes) were added to each plate and further incubated. Afterwards plates were regularly monitored under a light microscope to visualize trapping of nematodes. Fungi which showed trapping of nematodes were subsequently purified by sub-culturing on Czapek Dox Agar (CDA) and Corn Meal Agar (CMA). Purified cultures were maintained and preserved on 1.7% CMA by inoculating and incubating at 28±1°C for 6 days. Pattern of trapping structures and morphology of conidiospores were used to identify the fungi on the basis of morphology according to [16, 19]. Microscopy and micrometry of fungi were made in light microscope under magnification 10X and 40X attached with camera (Leica, MD2500). For further morphological based identification, conidial size was measured after growing fungi on nine different media described by [65]. Length of 10 conidia form each medium was measured and average length was calculated. Nematode-trapping fungi were partially identified based on their morphology.

### OPTIMIZATION OF GROWTH CONDITIONS

As a fact, environmental factors viz., temperature, nutrient source, pH of soil as well as other parameters affect the performance of biocontrol agent under the field. Optimum growth conditions i.e. suitable medium, pH and temperature was evaluated for the isolates.

### Effect of media

Effect of nine different media described by [65] and other was assessed to know which medium promotes luxurious growth of our isolates. For all the media, agar plates were prepared and inoculated centrally with 8mm diameter disc with mycelial growth from previously grown fungi. Plates were incubated at 28±1°C for 5 days. After 5 days, radial diameter of growth was measured and mean from three plates were calculated and compared.

### Effect of pH

To investigate which pH supports healthy growth of the isolated nematode-trapping fungi, isolates were subjected to grow on corn meal agar having different pH i.e. 4, 5, 6, 7, 8 and 9. The desired pH of medium was adjusted by adding 0.1N NaOH or 0.1N HCL. Plates were incubated at 28±1°C for 5 days and effect of pH was assessed by measuring the radial diameter of growth.

### Effect of temperature

Both the isolates were further subjected to different temperature conditions for determination of optimum temperature for growth. Corn Meal Agar (CMA) (pH7) was prepared, inoculated centrally and incubated at variable temperatures viz., 4±1, 15±1, 25±1, 28±1, 37±1 and 42±1°C and incubated in the dark for 5 days. Effect of temperature was evaluated as described above. All the above experiments i.e. effect of media, pH and temperature were performed in triplicate.

### MOLECULAR CHARACTERIZATION OF FUNGI

Fungi were identified based on 18S rDNA gene sequencing. For DNA isolation, 70 mL of 2.4% potato dextrose medium (PDB) (Hi-media, India) was prepared in 250 mL Erlenmeyer flask. Medium was inoculated with spore suspension and allowed to grow on rotary shaker 125rpm at 28±1°C for 7 days. Mycelia were harvested by filtering the medium using sterilized Whatman filter paper No.1. After filtering, approximately 100-150mg of mycelia were crushed to fine powder in mortar-pestle using liquid nitrogen followed by grinding with glass beads (Sigma) in lysis buffer containing 0.1M Tris (pH 7.1), 0.3M EDTA (pH 8.0) and 1% SDS. To this 70µL of 0.1% β mercaptoethanol was added per 1 mL of lysis buffer. Homogenized mycelia were incubated at 70°C for 1hr. After this the mixture was centrifuged at 10,000rpm for 10minutes. Supernatant was transferred to new 2mL microfuge tube and protein was precipitated using equal volume of Tris saturated phenol (pH 8) and centrifuged at 10,000rpm for 10 minutes. This was followed by mixing of aqueous phase with an equal volume of phenol-chloroform and again centrifuged at 10,000rpm for 10 minutes. Finally DNA was precipitated from the aqueous phase using double volume of chilled ethanol (75%) and incubated at -20 °C for 1hr. Precipitated DNA was palleted by centrifuging at 12,000rpm for 10minutes. DNA was washed with an absolute alcohol and again collected by centrifuging as mentioned above. Plates were

allowed to air dry and resuspended into 25µL nuclease free water. Quality of DNA was assessed using gel electrophoresis and quantified using NanoDrop 1000 spectrophotometer (Thermo scientific, USA). The partial sequence of 18S rDNA encoding gene was amplified by using the forward 5' AGGGTTCGATTCCGGAGA 3' and reverse 5' TTGGCAAATGCTTTCGC 3'. Primers were synthesized from Sigma, India. 25 µL PCR reaction mixture was prepared as follow.

**Table 2.1**  
PCR reaction mixture used to amplify the 18S rDNA gene sequence

Reagent	Quantity (µL)
Genomic DNA (50-70ng/µL)	1.0
10X <i>TaqA</i> assay buffer	2.5
Forward primer (10 µM)	1.0
Reverse primer (10 µM)	1.0
dNTPs mix (2.5 mM each)	2.5
<i>Taq</i> DNA polymerase (2.0U/ µL)	0.5
MiliQ water	16.5
Total	25.0

*Taq* DNA polymerase, 10X *TaqA* assay buffer and dNTPs were purchased from Genei, Bangalore, India. PCR conditions was optimized and amplification of the target sequence was carried out in thermocycler (Corbett, Korea) with cycling profile describe below.

**Table 2.2**  
PCR cycling conditions applied to amplify the 18S rDNA gene sequence

Temperature °C	Time (Minutes)	Cycles
94	5	1
94	1	} 35
58	1	
72	1	
72	7	

PCR products were electrophoreses at 100V in submarine system in 1% agarose gel amended with ethidium bromide dye along with 100 bp DNA marker and visualized on UV transiluminator. Amplified products were sent to Eurofins Genomics India Pvt. Ltd., Bangalore for sequencing from both the sides using prime pair which was used to amplify the target sequence.

## RESULTS AND DISCUSSION

### Isolation and morphological characterization of nematophagous fungi

Isolation of nematophagous fungi from soil is a little bit complicated due to its less abundance and over

**Table 2.2**  
**18S rDNA gene sequences downloaded from NCBI nucleotides collection for phylogenetic analysis**

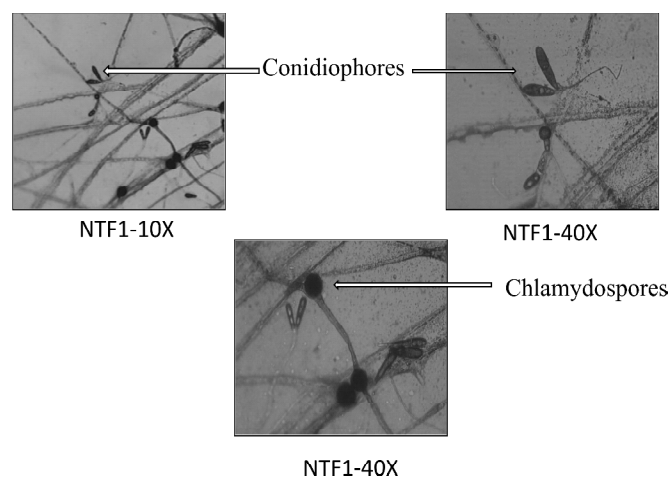
Name of fungi	Mode of killing nematodes	Accession No.	Reference
<i>Arthrobotrys oligospora</i> NBAII AO1	Adhesive network	JX403728	Direct submission
<i>Orbilia auricolor</i>	Adhesive network	DQ471001	(Spatafora <i>et al.</i> , 2006)
<i>A. musiformis</i>	Adhesive network	AJ001985	Direct submission
<i>A. robusta</i>	Adhesive network	AJ001988	
<i>Monacrosporium doedyoides</i>	Adhesive network	AJ001994	Direct submission
<i>A. superba</i>	Adhesive network	EU977561	Direct submission
<i>Dactylella oxyspora</i>	Adhesive knob	AY902793	(Li <i>et al.</i> , 2006)
<i>Gamsyella gephyropaga</i>	Adhesive columns	EF445990	(Smith and Jaffee, 2009)
<i>Hirsutella minnesotensis</i>	Endoparasitic	DQ078757	Direct submission
<i>Pochonia chlamydosporia</i>	Egg parasitic	EU266591	Direct submission
<i>Verticillium chlamydosporium</i>	Egg parasitic	AJ291806	(Morton <i>et al.</i> , 2003)
<i>Paecilomyces lilacinus</i>	Egg parasitic	FR775537	Direct submission
<i>Nematoctonus concurrens</i>	Endoparasitic	EF546658	Direct submission

growth of other soil fungi. Water agar is nutritionally poor medium which permits sluggish growth of organisms so it can be used for isolation of scarce microorganisms. Two different nematode-trapping fungi were isolated from the soil samples collected from different fields of Anand district of Gujarat. After 20-25 days of incubation, two different fungi showed trapping of nematodes (Figure 3.1). Both the fungi were subsequently purified by sub-culturing on CMA/CZP agar plates. Isolates were designated as NTF1 and NTF2. Morphology of conidia and type of trapping structure forms the basis of identification of nematophagous fungi. Conidiophores are unbranched and have up to 18-20 conidia. These characteristics are found similar to *Arthrobotrys oligospora* described by [19] except for size of conidia which is reported 30-46  $\mu\text{m}$  and number of conidia on conidiophore upto 30. Size of conidia is also varying from *A. oligospora* and *Duddingtonia flagrans*.

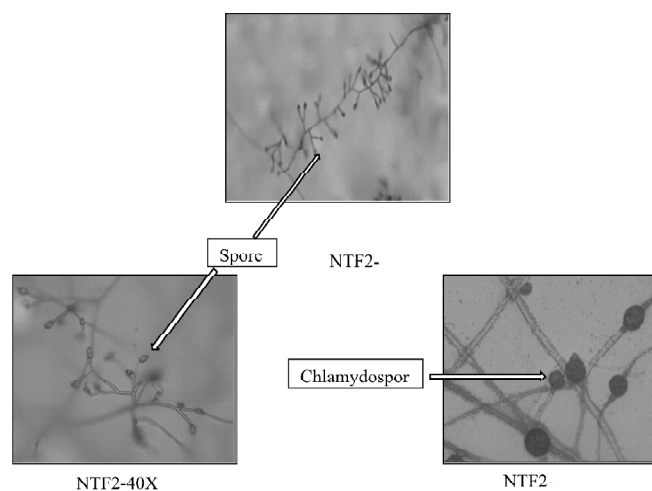
Another isolate, NTF 3 forms ring like trapping structure but not typical networks. Conidia are simple, single septate on erect conidiophore. The conidiophore produces consecutive conidia slightly below and to one side of elder conidia. These characteristics are analogous to *Duddingtonia flagrans* reported by [16] exception in size of conidia. Isolate NTF2 also produced thick walled chlamydospores and trapping structures (Fig.3.2). Based upon these morphological characteristics, isolate NTF1 & NTF2 may be *D. flagrans*.

It is well documented that chlamydospores of *D. flagrans* can survive gut passage of rumen and hence can be used as biocontrol agent against animal parasitic nematodes [5, 25, 75, 80].

#### NTF1



#### NTF2



NTF3

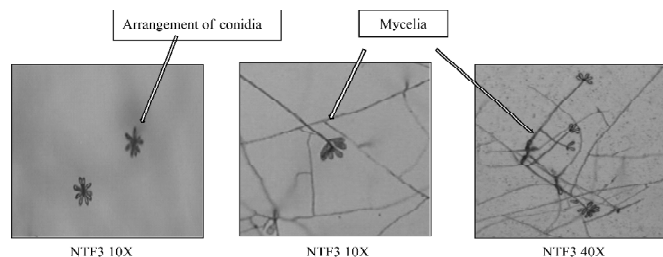


Figure 3.1: Morphological variation found in NTF1, 2, 3 on corn meal agar under 10X and 40X objective.

Previously *A. oviformis* and *A. musiformis* have been isolated from the Gujarat and their predatory activity against animal parasitic nematodes *Haemonchus contortus* is reported by [12, 42, 61].

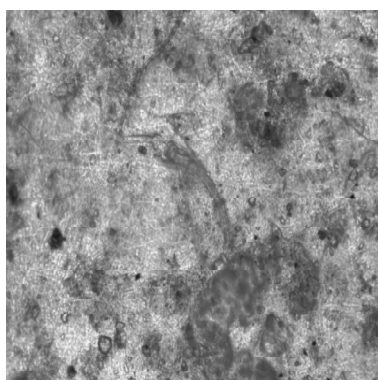


Figure 3.2: Morphological characterization of *D. flagrans* (NTF1 and NTF2) after growing on CZP agar at 28°C for 7 days. Ring like trapping structure, conidia and chlamydospores under 40X magnification

### OPTIMIZATION OF THE GROWTH CONDITIONS

For field application, large scale production of fungi is required. Further, different abiotic factors especially temperature and soil pH affect the efficiency of biocontrol agents under field conditions [28]. Here, different cultural conditions viz., pH, temperature and media for favorable growth of the isolated nematode-trapping fungi were optimized. Of the 9 different media tested, Corn Meal Agar (CMA) showed luxuriant growth of both the fungi followed by Martinson’s Medium (MM), Jenson’s Medium (JM), Yeast Extract Peptone Soluble Starch Medium (EEPSTM) and Remington’s Medium (RM). *D. flagrans* also grow well on Czapek Dox Agar (CZP) whereas *A. conoides* showed moderate growth on RM as compared to *D. flagrans*. Both isolates showed slow growth on Sabouraud Dextrose Agar (SDA), Potato Dextrose Agar (PDA) and Nutrient Agar (NA) (Figure 3.3).

All the media contain different pure as well as crude carbon and nitrogen sources; still both the fungi were able to grow on all the media tested. This highlighted the saprophytic nature of nematophagous fungi because in soil, different carbon and other nutrients are available at varying proportions and fungi have to manage with this. In case of temperature, the isolates grew optimally at 25 and 28°C. These results are comparable with optimum temperature reported for *A. oligospora* [20, 38].

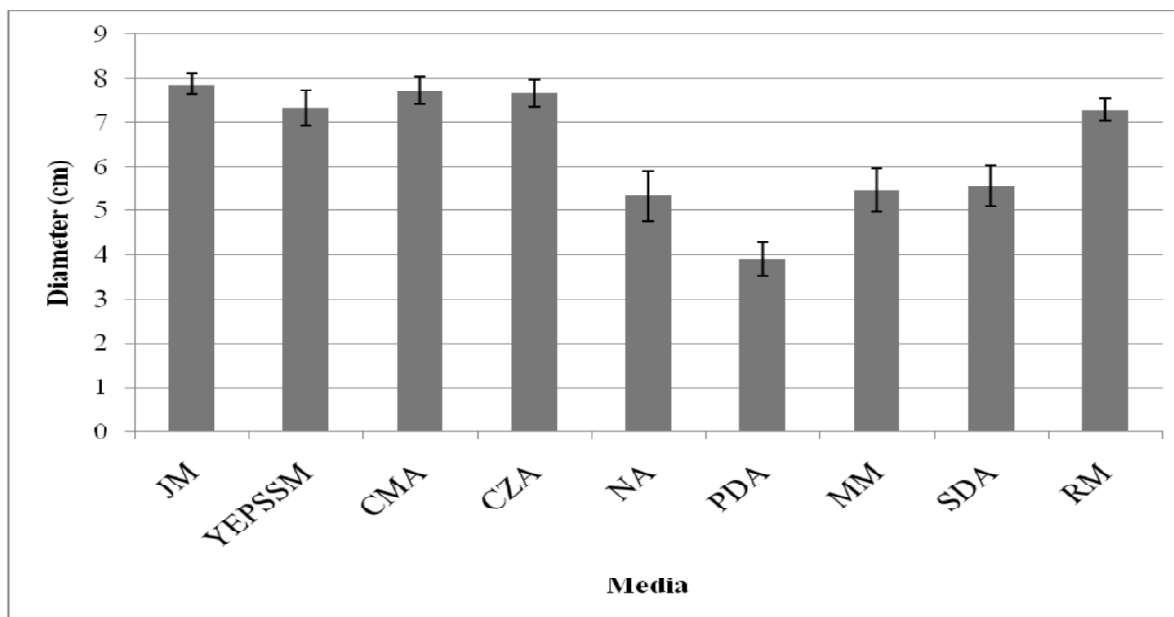


Figure 3.3: Effect of different media on growth of the isolate AO- *Arthrobotrys oligospora*

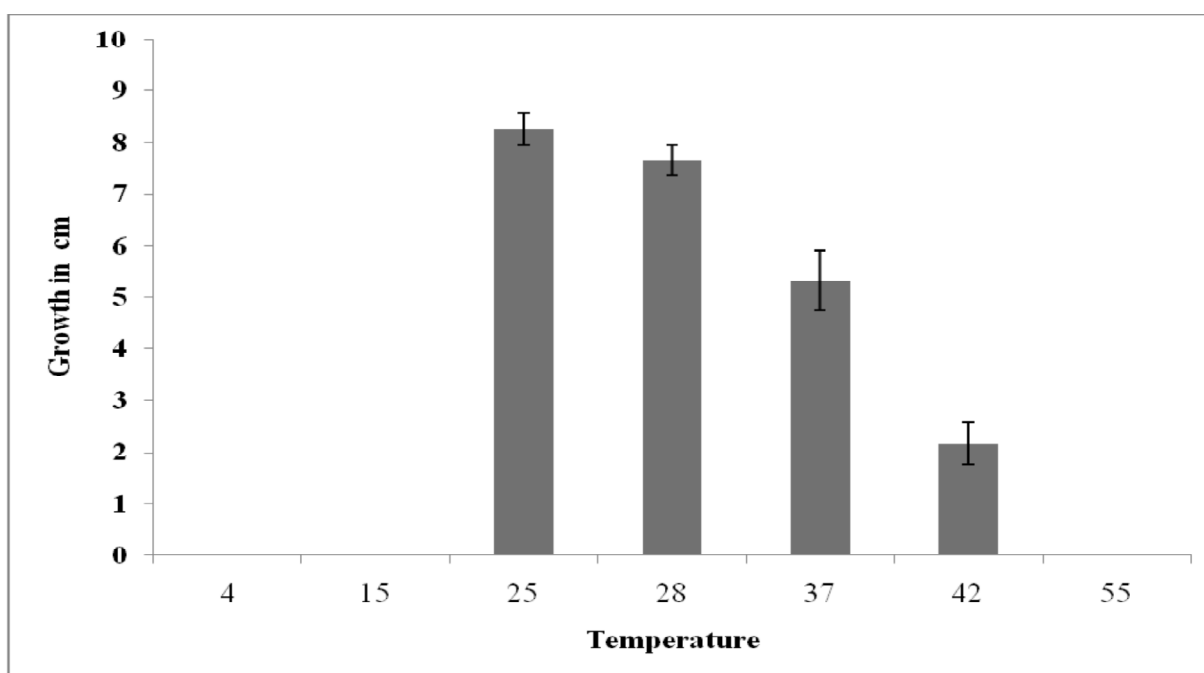


Figure 3.4: Effect of temperature on growth of the isolates

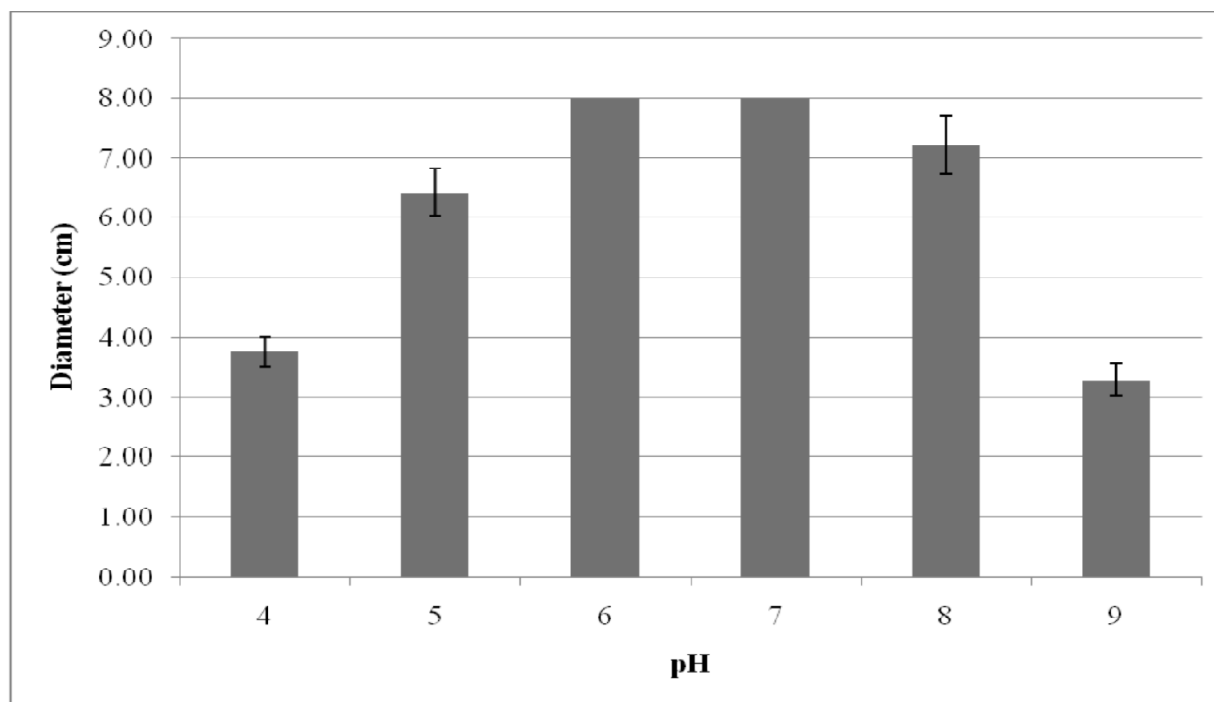


Figure 3.5: Effect of pH on growth of the isolates

Stunted growth of the fungi was observed at 42°C and at 4°C, isolates were failed to grow. This outcome clearly explained that very high and low temperatures is inhibitory for the growth of the fungi. In case of pH, healthy growth of the isolates was observed between pH 6 to 8. These results are comparable with

the optimum pH reported for *A. oligospora* [44] and *Pochonia chlamydosporia* [43].

At pH 5 and 9 moderate growth was observed and no growth was observed at pH 4 (Fig. 3.5). *A. oligospora* showed relatively fair growth at pH 5 and pH 9 than *D. flagrans*. This result showed that very

acidic and alkaline conditions are not conducive for the growth of both the fungi. These results are analogous with results of AO [28].

Optimization of the growth conditions for *D. flagrans* was carried out similarly as for *A. oligospora*, and results showed that the optimum temperature for its growth is 25-28 °C, pH 6-7, and YSA medium showed appropriate results after 5 days of inoculation.

Thus temperature 25-28 °C and pH 6-9 was found to suitable for both the isolates. These parameters should be kept in mind together with other abiotic and biotic factors at the time of field application otherwise it may lead to unanticipated results.

### MOLECULAR CHARACTERIZATION OF THE ISOLATES

Nematode-trapping fungi produced different types of trapping devices to catch and kill nematodes. They make three fundamental types of trapping devices adhesive knobs, constricting rings and adhesive networks [58]. Previously nematophagous fungi were identified and classified solely based on the type of trapping devices and morphology of conidia. However, only morphological base identification does not lead to concluding results. Knowledge of DNA sequence has become very essential in any biology study. For the very first time, Ahren and their colleagues make use of 18S rDNA sequences to study the phylogeny of nematode-trapping fungi [1]. They have concluded that 18S rDNA based phylogeny is supported with the type of trapping structure produced by fungi. Afterward [33] proved the same theory. Yet identification of the nematophagous fungi using small and large ribosome coding gene sequences is considered to be more accurate than exclusively morphology based [83,84].

A good quality and quantity of genomic DNA (Figure 3.6) was isolated from both the fungi as described in the methodology.

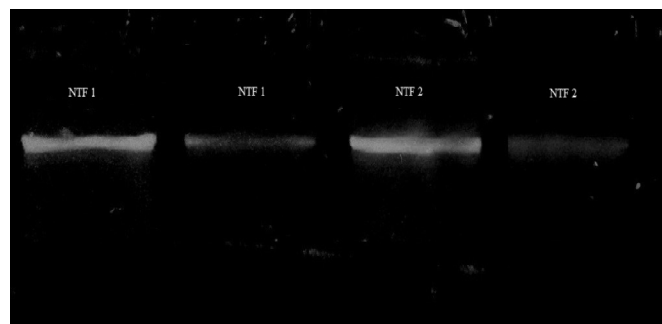


Figure 3.6: Genomic DNA isolated from DF- *D. flagrans* (NTF 1) and AO- *A. oligospora* (NTF2) on 1.0 % agarose gel

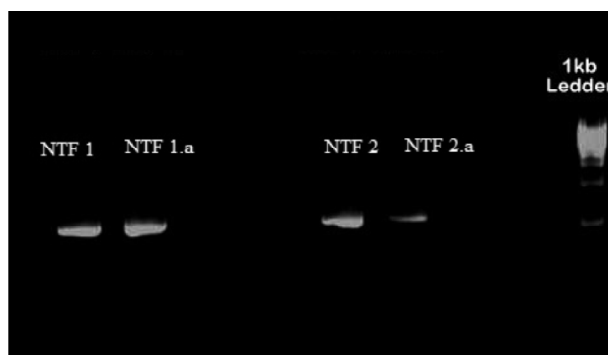


Figure 3.7: Amplified 18s rDNA encoding gene partial sequence with 100bp ladder, DF- *D. flagrans* (NTF 1) and AO- *A. oligospora* (NTF2)

**Identification of fungal isolates:** The amplified 18S rRNA sample was sent for sequencing. The sequence obtained were BLAST in order to know the identity of the fungal isolates. Based upon these information the isolates were identified as below in Table 3:

- **NTF1:** Sequence shows 99% similarity with *Duddingtonia flagrans*.
- **NTF2:** Sequence shows 99% similarity with *Duddingtonia flagrans*.

Table 3

Sample ID	Organism identified	Sequence length (bp)	% similarity with NCBI Subject sequence
NTF1	<i>Duddingtonia flagrans</i>	547	99%
NTF2	<i>Arthrobotrys oligospora</i>	549	99%

- ✓ Thus, after performing 18S rRNA, the fungal isolates were identified. Fungi NTF1 has 99% sequence similarity with *Duddingtonia flagrans*. *Duddingtonia* is a genus of fungi in the family *Orbiliaceae* comprising only the species *Duddingtonia flagrans*.
- ✓ NTF2 has the sequence similarity with *Arthrobotrys oligospora*.

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