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Research Article

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Isolation of Antimicrobial Compound Producing Fungi from the Rhizospheric Soil of the Medicinal Plant *Azadirachta Indica*

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ABSTRACT

All the microorganisms produce primary and secondary metabolites which have various industrial applications. Metabolites such as antibiotics are mainly produced by the filamentous micro- organisms such as bacteria and fungi. The rhizosphere is a narrow zone surrounding and influenced by plant roots and also a hot spot for several organisms. Organisms found in vicinity of roots include bacteria, fungi, oomycetes, nematodes, protozoa, algae, viruses, archaea, and arthropods. Microorganisms in rhizosphere are different from those residing in the non-rhizosphere surrounding soil, both in numbers of cells and the variety of strains. Rhizosphere microorganisms have been recognized as an important source of a variety of structurally unique and active secondary metabolites. In present study, 6 fungi were isolated form rhizosphere of medicinal plant Azadirachta indica (Neem). Out of 6 isolates, one isolate was selected and identified (Aspergillus niger) as showed maximum antimicrobial activity against test microbe E. coli. Fermentation conditions were optimized such as temperature (25°C), pH (5), carbon source (glucose) and nitrogen source (ammonium nitrate) for maximum production of antimicrobial compound.

Keywords: Antibiotics; Fungi; Secondary metabolites; Optimization; Inhibition zone

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INTRODUCTION

Microbes usually produce several compounds with lots of applications in daily life such as pigments, dyes, health components, antibiotics [1]. Antibiotic means 'against the living organisms' [2]. Antibiotics are the secondary metabolites produced by microorganisms after synthesis of primary metabolites. Secondary metabolites do not play any role in the growth and development of microbes. These are synthesized during the stationary phase and have toxic effect on the microorganism other than the producer one. Antimicrobial compounds are used to kill or retard the growth of the living organisms [3]. Antibiotics are produced by bacteria, fungi, sponges etc. Antibiotics play a very important role in controlling infectious diseases [4]. Penicillin, kanamycin, neomycin are the some examples of antibiotics. Antibiotics are used all over the world. Mainly antibiotic producing microbes are found from soil and water as all growth nutrients are present in the soil and water. It also depends on nature of environment and texture of soil or many other features of soil and water [4]. The large population of the microbes is found in the rhizospheric region of the plants. Rhizosphere is defined as the soil volume present near the roots of the plants [5]. Secretion of organic nutrients from the roots of the plants favors the growth and development of the microorganisms in rhizosphere [6]. The inappropriate use of antimicrobial medicines has prompted the resistant microbes to emerge. Therefore, there is an urgent need to isolate new antibiotic producing microbial strains or new antibiotic molecules to meet the challenges such as emergence of new diseases or resistivity of some pathogens to the existing antibiotics [7-9]. In lieu of the above justification, the objective of present study was to search for the antibiotic producing fungi from rhizosphere that can produce more effective antimicrobial compounds which can kill the life-threatening pathogens.

EXPERIMENTAL SECTION

Collection of Sample

The soil sample was collected from the rhizosphere of medicinal plant '*Azadirachta indica*' (Neem) of Kurukshetra University, Kurukshetra, Haryana, India. The sample was taken in clean and sterilized polythene bags. The sample was taken directly to the laboratory and allowed to air dry. Within 24 hours, the collected sample was used to isolate the fungi [10].

Isolation of Antibiotic Producing Microorganisms

Serial dilution method was used to isolate the fungi. The soil sample was serially diluted in the sterilized water under the sterilized conditions (laminar air flow chamber). Serial dilutions were made upto 10^{-4} and 0.1 ml of each dilution was spread on the Potato Dextrose Agar (PDA) plates having antibacterial agent (50 µg/ml streptomycin) [11]. Plates were incubated at the 30˚C in the incubator for 7 days. Morphologically different fungal colonies were separated by inoculating each colony on the different plate to maintain the pure culture.

Screening for Antimicrobial Activity

Agar well diffusion method was used to screen the fungal isolates for antimicrobial activity [12]. Fungal isolates were grown in 250 ml conical flasks containing 100 ml Potato Dextrose Broth (PDB), autoclaved at 121°C for 20 minutes. After cooling the medium, each flask was inoculated with fungal disc, cut from the 6-7 days old culture of fungi. These flasks were incubated for 15 days at 30°C. After 15 days, fungi were filtered through Whatman No.1 filter paper. The culture filtrates were separated from mycelium. Two test microbes, one gram-negative such as *Escherichia coli* MTCC 40 and one gram-positive such as *Staphylococcus aureus* MTCC 87 were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. Mueller Hinton Agar (MHA) plates were prepared under the sterilized conditions. Pure culture of two test microbes were swabbed on the MHA plates by using sterilized cotton buds. After that, wells were made on the agar plates. To each well, culture filtrate and Ciprofloxacin as positive control were added. Uninoculated broth was added as negative control. The plates were incubated at 37°C for 24 hours. After the incubation, the diameter of growth inhibition zone surrounding the well was measured by using the zone scale [10] and fungal isolate was selected on basis of maximum diameter of zone of growth inhibition against test microbes.

Morphological and Molecular Identification of Fungi

The growth pattern, pigmentation and colony morphology were observed during the incubation period for identification of fungi. Lactophenol cotton blue dye was used for spore staining. A single drop of lactophenol cotton blue was placed on the clean glass slide. With the help of sterilized needle, a small portion of mycelium was taken from the fungal culture and the mycelia was spread on the slide within the drop area [13]. The glass slide was observed under the microscope to see the sporulating structure. The fungal genera were identified by using various manuals and monographs [14-16]. Molecular identification of promising fungal isolate was performed by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune and Maharashtra.

Optimization of Cultural Conditions

Maximum metabolite production as well as growth of the microorganism is influenced by some environmental factors such as pH, temperature, carbon source and nitrogen source [17]. Therefore, the growth conditions and composition of media were optimized for maximum antibiotic production by stepwise modifications in parameters such as pH, temperature, carbon source and nitrogen source [18].

To determine the effective temperature for the growth of fungus and maximum antimicrobial activity, four different temperatures 25°C, 29°C, 33°C, 37°C were adjusted for broths with discs of selected fungus and incubated under stationary conditions for 15 days. After incubation, the filtration was done with sterilized whatman filter paper and culture filtrates were used for testing antimicrobial activity against test microbes by agar well diffusion assay. All experiments were performed in triplicates. Five pH values 3, 5, 7, 9, 11 were taken to determine the optimum pH for the growth of fungal isolate. Broths were autoclaved at 121˚C for 15 minutes. Fungal discs were inoculated into broths and incubated at 30°C for 15 days. The culture filtrates after filtering through whatman filter paper were tested for antimicrobial activity against test microbes by agar well diffusion method. All experiments were performed in triplicates.

To optimize the carbon source, three discs of selected fungus were inoculated into Potato broth added with five different carbon sources dextrose, glucose, lactose, fructose, and sucrose was incubated at 30°C for 15 days under

stationary conditions. After incubation, broths were filtered using whatman filter paper and culture filtrates were tested for antimicrobial activity against test microbes by agar well diffusion method. All experiments were performed in triplicates.
The nitrogen source was optimized by adding five different nitrogen sources that were peptone, urea, ammonium sulfate, ammonium nitrate and ammonium chloride into Potato broth with selected carbon source. Three discs of selected fungal isolate were transferred to these broths and incubated at 30°C for 15 days under stationary conditions. After incubation, broths were filtered using whatman filter paper and culture filtrates were tested for antimicrobial activity against test microbes by agar well diffusion method. All experiments were performed in triplicates.

RESULTS AND DISCUSSION

Isolation of Fungi from Rhizospheric Soil

A total of six fungi were isolated from the rhizospheric soil of medicinal plant *'Azadirachta indica'* (Neem) from Kurukshetra University. All the isolates were sub-cultured on Potato Dextrose Agar (PDA) plates and incubated at 30°C for 7 days. PDA slants were also made for further use in future. The same culture conditions such as growth media (Potato dextrose agar), growth temperature (30°C) and incubation period (7days) were also used by Sharma et al. [19] for *Aspergillus* sp. and *Penicillium* sp.

Screening for Antimicrobial Activity

The antimicrobial activity of the six morphologically different isolates (NS1, NS2, NS3, NS4, NS5, NS6) were tested against the two test microbes, gram-positive (*S. aureus*) and gram-negative (*E. coli*). All of six isolates did not inhibit the growth of test microbe *S. aureus*. Three isolates (NS1, NS4 and NS5) showed zone of growth inhibition against test microbe *E. coli*. Isolates NS4, NS5 produced the zone of growth inhibition of 14 mm and 17 mm respectively. The isolate NS1 produced the maximum diameter of zone of growth inhibition against the *E. coli* with 20 mm. Uninoculated broth used as negative control and ciprofloxacin used as positive control did not inhibit the growth of test microbes. The results of antimicrobial activity of fungal isolates are shown in Table 1.

Table 1: Antimicrobial activity of fungal isolates against test microbes

	Diameter of zone of growth inhibition (in mm)	
Code of sample	**Gram-negative bacteria**	**Gram-positive bacteria**
	E. coli	***S. aureus***
NS1	20.33 ± 0.57	-
NS2	-	-
NS3	-	-
NS4	14.66 ± 0.57	-
NS5	17.00 ± 1.00	-
NS6	-	-
Ciprofloxacin (positive control)	-	-
Uninoculated broth	-	-

Identification of Fungal Isolate

The fungi were identified as *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp. based on colony characterization (colony color, colony growth) and sporulating structures and by following various manuals [14-16]. The morphological results are given in Table 2. The fungi *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp. were also isolated from soil by Jahangeer et al. [20] and Raju et al. [21]. Saravanan and Radhakrishnan [1] also reported the same fungi from the soil of Western Ghat. Henerique et al. [22] isolated the fungi *A. niger*, *A. flavus,* and Mucor from the soil sample. Variation in species and genus of fungi from different soils can be happened due to some environmental factors such as pH, temperature of the soil, humidity, salt concentration, nutrient concentration etc.
Fungal isolate (NS1) which showed maximum antimicrobial activity was identified as *Aspergillus niger* based on molecular identification from National Fungal Culture Collection of India (NFCCI), Agahrkar Research Institute, Pune. The results of molecular identification are shown in Figure 1.

Table 2: morphological identification of isolated fungi

Code of sample	Genus name	Colony morphology
NS1	*Aspergillus* sp.	Black, powdery
NS2	*Fusarium* sp.	White pink
NS3	Aspergillus sp	Light green, powdery
NS4	*Aspergillus* sp.	Greenish blue
NS5	*Penicillium* sp.	Dark green
NS6	*Aspergillus* sp.	Light green

Results of Molecular Identification:

- The tested fungal isolate A showed **100%** sequence similarity with ***Aspergillus niger.***
- Sequence analyses with NCBI accession number KT192372, ***Aspergillus niger*** strain 2-00349-1 resulted in following alignment statistics.
- Alignment statistics: Query Length - 525, Score - 948 bits (1050), Expect - 0.0, Identities - 525/525 (100 %), Gaps - 0/525 (0%), Strand - Plus/Minus

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Query  1    GGTTGGAAAACGTCGGCAGGCGCCGGCCAATCCTACAGAGCATGTGACAAAGCCCCATAC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  532  GGTTGGAAAACGTCGGCAGGCGCCGGCCAATCCTACAGAGCATGTGACAAAGCCCCATAC  473

Query  61   GCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGAGAGGGGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  472  GCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGAGAGGGGG  413

Query  121  ACGGCGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  412  ACGGCGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCC  353

Query  181  CCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  352  CCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGC  293

Query  241  AATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  292  AATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCC  233

Query  301  ATTGTTGAAAGTTTTAACTGATTGCATTCAATCAACTCAGACTGCACGCTTTCAGACAGT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  232  ATTGTTGAAAGTTTTAACTGATTGCATTCAATCAACTCAGACTGCACGCTTTCAGACAGT  173

Query  361  GTTCGTGTTGGGTCTCCGGCGGGCACGGCCCGGGGGCAGAGGCGCCCCCCGGCGGC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  172  GTTCGTGTTGGGTCTCCGGCGGGCACGGCCCGGGGGCAGAGGCGCCCCCCGGCGGC  113
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Figure 1: Molecular identification of fungal isolate NS1

Optimization of Growth Conditions

Temperature is an important parameter that influences the overall growth and development of the microorganism [23]. The growth and development of the microorganisms could be decreased at much higher as well as much lower temperature. In present study, the favourable temperature for the growth of fungus *A. niger* and maximum antimicrobial activity was found 25°C with maximum zone of growth inhibition of 18 mm in diameter. At 29°C, 33°C and 37°C, the sizes of zone of growth inhibition by culture filtrate were lower as 13 mm, 17 mm and 17 mm respectively compared to diameter of zone of growth inhibition obtained at 25°C. The temperature 25°C was reported as the optimum temperature for maximum bioactive metabolite production from *Aspergillus sp.* by Mathan et al. [24]. Parmar et al. [25] also reported 25°C as optimum temperature for the production of highest biomass and fusaric acid by Fusarium oxysporum. The optimum pH value for the proper growth of the fungus *A. nige*r and maximum antibiotic production was found 5. The largest zone of growth inhibition was obtained at the pH 5 with 20 mm zone in diameter. Culture filtrate at pH values 3 and 7 showed zone of growth inhibition of 14mm and 19 mm respectively. No zone of growth inhibition was obtained by culture filtrate kept at pH 9 and 11. Compared to results of present study with previous studies, Mathan et al. [24] and Boonyapranai et al. [26] reported the 5.5 pH for Aspergillus sp. The optimized parameters are not same for the microorganisms due to variations in the growth conditions or the strain difference. Mainly the maximum metabolites are produced around the pH 5-6 such as fungus *Fusarium solani* which produce large volume of metabolite at pH 6 [27]. During the microbial fermentations, the carbon source not only acts as a major constituent for building material of cells but also used in synthesis of polysaccharides and as energy source [28]. In present study, various carbon sources (glucose, lactose, fructose, dextrose. Sucrose) were added individually to the potato infusion 2 g per 100 ml. The maximum activity was shown by strain *A. niger* in the broth having glucose with maximum zone of growth inhibition (20 mm) against *E. coli*. Culture filtrates of *A. niger* in medium amended with dextrose, fructose, lactose and sucrose produced the zone of growth inhibition of 16 mm, 18 mm, 18 mm and 17 mm respectively. Therefore, the favourable carbon source for fungus *A. niger* for maximum antimicrobial activity was glucose which is in accordance with results of Bhavana et

al. [29] who observed Glucose as favourable carbon source for maximum antimicrobial compound and mycelium growth of *Streptomyces carpaticus* MTCC 11062.
Five nitrogen sources, peptone, ammonium nitrate, ammonium sulfate, ammonium chloride and urea, were taken to optimize the nitrogen source for maximum antibiotic production by fungus *A. niger*. Culture filtrate in medium supplemented with peptone, ammonium sulfate and ammonium chloride showed the zone of growth inhibition of 25 mm, 26 mm and 27 mm in diameter. Addition of ammonium nitrate increased the antimicrobial production by strain *A. niger* with 28 mm zone of growth inhibition. The results of present study demonstrate the fact that strain *A. niger* can effectively utilize inorganic nitrogen sources containing nitrate. Ammonium sulphate as promised nitrogen source was reported by the Padmavathi et al. [30]. Ismaiel et al. [31] reported sodium nitrate as best nitrogen source for the production of metabolite by fungus *Fusarium roseum*. The results of the optimization of fermentation conditions are in Table 3.

Table 3: Optimization of fermentation conditions for maximum antibiotic production

Parameters		Diameter of zone of growth inhibition (mm)	Parameters		Diameter of zone of growth inhibition (mm)
Carbon sources	Dextrose	16.33 ± 0.57	pH	3	14.66 ± 0.57
	Fructose	17.66 ± 0.57		5	20.00 ± 0.00
	Lactose	17.33 ±0.57		7	18.00 ± 0.00
	Glucose	20.66 ± 0.57		9	-
	Sucrose	18.33 ± 0.57		11	-
Nitrogen sources	Peptone	25.00 ± 1.00	Temperature	25°C	18.00 ± 0.00
	Ammonium nitrate	28.33 ± 0.57		30°C	13.66 ± 0.57
	Ammonium sulfate	26.33 ± 0.57		33°C	16.66 ± 0.57
	Ammonium chloride	26.66 ± 0.57		37°C	17.33 ± 0.57
	Urea	-			

CONCLUSION

Optimization of temperature, pH, carbon source and nitrogen source increased the yield of antimicrobial compound by fungus *A. niger*. Work is needed to optimize the other parameters also like inoculum size, vessel size, concentration of selected carbon source and nitrogen source etc. to further increase the yield of antimicrobial metabolite. Purification and characterization of antimicrobial metabolite will be performed.

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