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Research Article

Optimization of Fermentation Conditions for Production of Antimicrobial Metabolite from *Talaromyces aurantiacus*

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ABSTRACT
Rhizospheric region of the medicinal plants is the hub of microbes which produces various pharmaceutical active and industrially important compounds such as antibiotics and enzymes. Rhizosphere is the narrow region around the plant roots having maximum quantity of nutrients secreted by the plant roots in the soil which are essential for the growth of the microorganisms. Rhizospheric microbes are different from the non-rhizospheric microbes, both in number of cells and variety of strains. All types of the microbes are found in the rhizospheric region of the soil such as, fungi, actinomycetes, bacteria, actinobateria, protozoa, algae, viruses and archaea. In the present study, the fungus was isolated from the rhizospheric region of the medicinal plant *Ocimum tenuiflorum* 'Tulsi' and screened by using the agar well diffusion method. There were 6 fungal isolates isolated out of which only one fungal isolate N2 exhibited the maximum antimicrobial activity against the two test pathogens (*Staphylococcus aureus* and *Escherichia coli*). On morphological and molecular identification, the fungus was characterized as *Talaromyces aurantiacus*. The fermentation conditions for production of antimicrobial metabolite from the fungus were optimized such as temperature (37°C), pH (3 and 11), carbon source (dextrose and fructose) and nitrogen source (ammonium nitrate and ammonium sulphate).

KEYWORDS: Rhizosphere Soil, *Ocimum tenuiflorum, Talaromyces aurantaicus* Antimicrobial Metabolite

INTRODUCTION

Microorganisms produce various types of industrially important metabolites during their growth phase such as pigments, enzymes, dyes, antimicrobial agents etc. Antimicrobial agents are the chemical compounds which are produced metabolically by the microorganisms such as fungi, actinomycetes and bacteria during the stationary phase of the microbial growth. Antimicrobial agents also known as "Antibiotics" are the secondary metabolites which have no role in the growth and development of living organism. Alexander Fleming observed that the growth of *Staphylococcus aureus* was retarded due to the presence of the other organism *Penicillium notatum*. This was due to the secretion of some antimicrobial agent by the *Penicillium notatum*. After Alexander Fleming accidentally discovered the penicillin the 1940s, antibiotics become a milestone in the modern

medicine with the vast applications in biotechnology. Antibiotics produce the toxic effect against the infectious microorganism such as kill or retard the growth of microorganisms [1]. Antibiotics play a vital role in therapies and medical treatments such as operation and organ transplant etc. [2]. A large population of microbes is present in the soil and water. This happens due to the presence of humidity, nutrients and favorable environment for the optimum growth of the microorganisms. Rhizosphere is the narrow region of the soil nearby the roots of the plants where all types of microorganisms (pathogenic or non-pathogenic) can be isolated. Due to the secretion of various nutrients such as carbohydrates, a large population of microbes is found in rhizospheric soil of plants. The antibiotics such as penicillin, streptomycin and neomycin which are produced by the microorganism are safely used in our routine life. But resistivity of the pathogenic microorganism to the available antibiotics is a major problem [2]. The main aim of present research work was to isolate the more effective antibiotic producing fungi which can kill the life-threatening microbes.

MATERIALS AND METHODS

Collection of sample

The soil sample was collected from the rhizosphere of the *Ocimum teniuflorum* (Tulsi) plant in a sterilized plastic zipper bag [3]. The collected sample was directly taken to the laboratory and air dried. The collected soil sample was used for isolation of fungi within 24 hours.

Isolation of fungi

The fungi were isolated from soil sample by performing serial dilution agar plate method [4]. Serial dilution was carried out under the sterilized conditions (laminar air flow chamber). Potato dextrose agar (PDA) media was used for the isolation of fungi. The 5 test tubes were filled with 9ml sterilized distilled water. 1gm of soil sample was added to the first test tube named as 10^{-1}. 0.1ml from each test tube was spread on PDA plates supplemented with streptomycin (0.2g/l) to avoid the growth of bacteria. The inoculated PDA plates were incubated at 30℃ for 6-7 days. Morphologically different fungal colonies appeared on the PDA plates after 5 days incubation, were sub-cultured separately to obtain pure culture. Pure fungal colonies were inoculated on PDA slants and preserved at 4℃ for further use [5].

Screening for antimicrobial activity

Fungal isolates were screened for antimicrobial activity by agar well diffusion method [6]. 100ml of potato dextrose broth was prepared in 250ml flask. The flasks were covered with tight cotton plug and autoclaved at 121℃ for 20 min. After cooling, the media were inoculated with 2-3 discs from the 6-7 days old culture plate of isolated fungi in sterilized conditions (Air Laminar Flow Chamber). The flasks were incubated at 30℃ for 14-15 days. After completion of incubation period, the broth media were filtered and filtrates were collected by using Whatman No. 1 filter paper. The Mueller Hinton Agar (MHA) Plates were swabbed with 100 µl of test microbes such as *E. coli* and *S. aureus* with the help of sterilized cotton buds under the sterilized conditions[7]. 100µl of filtrate was poured into the well prepared on plates swabbed with test microbes and plates were incubated overnight at 37℃. After the incubation, the growth inhibition zones were observed on the plates around the wells and measured with the help of zone scale [8]. Selection of fungal isolate was done on the basis of maximum diameter of zone of growth inhibition.

Morphological and molecular identification

For the identification of the fungi microscopically, lactophenol cotton blue dye was used [9,10] for spore staining. A drop of lacto phenol cotton blue was placed on the washed and dried glass slide. A small portion of mycelia was taken and spread within the drop area finely. By using the cover slip, glass slides were placed under the microscope for observation of sporulating structures, spore size, spore shape and structure of conidia. Growth rate, pigmentation and colony morphology of the fungal isolate were observed during growth period. The identification of the fungus was done by using various manuals [9, 11, 12].

Molecular identification of promising fungal isolate was performed by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune (Maharashtra), India.

Optimization of culture conditions

Growth parameters such as nutrition level, incubation time period, growth environment influence the growth of microorganism, hence production of secondary metabolites. Four parameters such as pH, temperature, carbon sources and nitrogen sources were optimized to maximize the yield of metabolite [13]. Five pH values 3, 5, 7, 9, and 11 were adjusted to determine the favorable pH value for the maximum growth of the fungal isolate. Five 250ml flasks were taken to prepare potato dextrose broth. Set the pH of each flask accordingly and autoclaved at 121℃ for 15 min. After cooling the broth, broth was inoculated with 2-3 discs from the 6-7 day old culture plate of isolated fungus. Inoculated broth was incubated for 15 days at 30℃. The broth was filtered by using the whatman filter paper and filtrate was screened for antimicrobial activity by agar well diffusion method against the test microbes. The experiment was done in triplicates.

To determine the optimum incubation temperature for fungal isolate, four values of temperature 25℃, 29℃, 33℃, and 37℃ were adjusted. 100ml potato dextrose broth was inoculated with 2-3 discs from the 6-7 days old culture of isolated fungus and incubated separately at 25℃, 29℃, 33℃ and 37℃ for 15 days. After completion of incubation period, the broth was filtered by using whatman no. 1 filter paper and filtrate was tested for antimicrobial activity by using agar well diffusion method against test microbes. The experiment was done in triplicates.

Five carbon sources such as dextrose, glucose, lactose, fructose and sucrose were selected to determine the best carbon source for the suitable growth of fungal isolate and maximum production of metabolite. The potato broth (have no sugar molecule) was supplemented with five carbon sources and inoculated with 2-3 discs from 6-7 days old culture of fungal isolate. The inoculated broth was incubated for 15 days at 30℃. After completion of incubation period, the broth was filtered and filtrate was tested for antimicrobial activity. The experiment was done in triplicates.

To determine the nitrogen source suitable for the maximum growth of the fungal isolate and maximum yield of antimicrobial metabolite, 5 nitrogen sources such as peptone, urea, ammonium sulfate, ammonium nitrate and ammonium chloride were selected. Potato broth with selected carbon source and five different nitrogen sources were autoclaved at 121℃ for 15 minutes and allowed to cool. The broth were inoculated with 2-3 discs of 6-7 days old culture of fungal isolate and incubated for 15 days at 30 ℃ . The experiment was repeated in triplicates.

RESULTS

Isolation of fungi

A total of 5 morphologically different fungi were isolated from the rhizospheric soil of *Ocimum tenuiferum* (Tulsi) by using serial dilution method. All isolated fungi were sub-cultured on potato dextrose agar (one on each plate) at 30℃ for 6-7 days. Slants were also prepared to preserve the cultures and stored at 4℃.

Screening for antimicrobial activity

The two test microbes *E. coli* and *S. aureus* were used to check the antimicrobial activity of the five morphologically different isolated fungi named as N1, N2, N3, N4 and N5. Three fungal isolates N2, N3 and N5 showed antimicrobial activity against the test microbe *E. coli*. No fungal isolate showed antimicrobial activity against test microbe *S. aureus*. Three isolates N2, N3 and N5 exhibited the growth inhibition zones of 18mm, 16mm and 16mm against test microbe *E. coli* respectively. Un-inoculated broth and ciprofloxacin were used as negative control and positive control respectively and did not inhibit any of test microbe. The results of antimicrobial activity of fungal isolates are shown in Table 1.

Table 1: Antimicrobial activity of fungal isolates

Code of Fungal Isolate	Diameter of Zone of Growth Inhibition (in mm)	
	Gram-negative Bacteria *E. coli*	Gram-positive Bacteria *S. aureus*
N1	NA	NA
N2	18±0.57	NA
N3	16±1.00	NA
N4	NA	NA
N5	16±0.57	NA
Ciprofloxacin (positive control)	23±0.57	NA
Un-inoculated broth (negative control)	NA	NA

NA-no activity, ± standard deviation

Identification of fungal isolate

The fungal isolates were identified as *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp. based on colony characterization (colony color and colony growth) and sporulating structures by following various manuals [9, 11, 12].

Fungal isolate N2, which showed maximum antimicrobial activity, was identified as *Talaromyces aurantiacus* based on molecular identification from National Fungal Culture Collection of India (NFCCI), Agahrkar Research Institute, Pune. The molecular identification results revealed that the tested fungal isolate N2 showed 99% sequence similarity with *Talaromyces aurantiacus*.

Optimization of fermentation conditions

Various parameters such as temperature, pH, carbon source, nitrogen source affect the growth of the microbes and metabolite productivity. These growth parameters were optimized for the promising fungal isolate *Talaromyces aurantiacus*. Temperature is a most common factor which influences the growth of microbes. Microbes never grow at higher temperature nor at very low temperature therefore, is optimum temperature required for the proper growth of microorganisms. Results of optimized fermentation conditions are given below in Table 2.

Table 2: Optimization of fermentation conditions for production of antimicrobial metabolite by *Talaromyces aurentiacus*

Parameters		Diameter of Zone of Growth Inhibition (mm)	Parameters		Diameter of Zone of Growth Inhibition (mm)
Carbon Sources	Dextrose	24±0.57	pH	11	16±0.23
	Glucose	14±0.57		9	NA
	Fructose	24±0.35		7	NA
	Lactose	19±1.00		5	NA
	Sucrose	18±0.23		3	21±0.57
Nitrogen Sources	Peptone	27±1.00	Temperature	25°C	NA
	Ammonium Nitrate	29±0.57		30°C	17±0.23
	Ammonium Sulphate	29±0.73		33°C	19±0.35
	Ammonium Chloride	26±0.57		37°C	22±0.23
	Urea	NA			

NA-no activity, ± standard deviation

DISCUSSION

Soil is a major reservoir of the bioactive substance producing microbes. Root exudates in rhizospheric region of the soil, helps to grow the microbes. Hence, the large population of bioactive substance producing microbes is

present in rhizospheric region of the soil than non- rhizospheric region. In the present study, the fungal isolates were morphologically identified and found to belong to genera of *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp. from the rhizospheric soil of *Ocimum tenuiflorum* 'Tulsi' plant. Most of the antibiotics, industrially important compounds and other bioactive compounds are isolated from the soil microflora such as actinomycetes, fungi or bacteria. Soil microflora varies from place to place and according to environmental factors. Hussain *et al.*, [14] isolated *Penicillium* sp., *Fusarium oxysporum*, *Rhizopus* sp., *Aspergillus flavus*, *Aspergillus niger*, *Alternaria* and *Paecilomyces* sp. form the rhizospheric region of the two medicinal plants i.e. *Aloe vera* (Ghee Kuwar) and *Punica granatum* (Pomegranate) and the two fungi i.e. *Fusarium solani* and *Helminthosporium* are used as the test pathogen. In the present study, two bacterial test pathogens (*Staphylococcus aureus* and *Escherichia coli*) were used to evaluate the antimicrobial activity of the fungal metabolite same as used by Makut *et al*. [1]. The optimum growth of *T. aurantiacus* isolated in the present study was recorded at 37ºC which is accordance with studies of Cao *et al*. [15] who reported that *Penicillium* sp. could grow from 8ºC-39.8ºC but growth was dramatically inhibited at 40ºC. According to Pandey *et al.*, [16], the maximum growth of the fungus *Penicillium* sp. (GBPI_P155), isolated from soil of Indian Himalayan region was at 25 ºC and pH 3. According to the results of the present study, the maximum metabolite production from the fungus was obtained at 3 and 11 pH value. Fructose was recorded as the carbon source and yeast extract was recorded as the nitrogen source for the maximum growth of fungi by Pandey *et al.* [16]. But according the present research work, Fructose and Dextrose were recorded as the favourable carbon source and ammonium sulphate and ammonium nitrate were found to be favourable nitrogen source for the fungi. This difference of the carbon and nitrogen may be due to the difference in species of the *Penicillium*. Rani *et al.* [17] optimized the fermentation conditions such as temperature (25°C), pH (5), carbon source (glucose) and nitrogen source (ammonium nitrate) for maximum production of antimicrobial compound from *Aspergillus niger* isolated from the rhizospheric soil of the medicinal plant. Goyari *et al.* [18] found the optimum 3.5 pH value for the growth of the *Talaromyces* sp. which is more or less similar to the result of present study. But according to the Chadni *et al.*, [19], pH 6.5 was found to be optimum for the growth of *Talaromyces verruculosus*. According to Goyari *et al.* [18], the best growth of fungi was obtained when the temperature was maintained at 30°C. Devi *et al*. [20], reported the dextrose as a favourable carbon source for the growth of fungi.

CONCLUSION

In the present study, *Talaromyces aurantiacus* isolated from rhizosphere soil of *Ocimum tenuiflorum* plant was found to exhibit antimicrobial potential. Optimization of temperature, pH, carbon source and nitrogen source increased the yield of antimicrobial compound produced by fungus *T. aurantiacus*. Further research is required to optimize the other parameters 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 to determine the bioactive component is also required to be studied for its commercial applications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in this research article.

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