Molecular Characterization of *Penicillium Chrysogenum* Isolated from Soil and Their Antibacterial Activity against Pathogenic Species

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Abstract

Fungi produce a multitude of low-molecular-mass compounds known as secondary metabolites. *Penicillium chrysogenum* is known repositories of secondary metabolites. The interest in the secondary metabolites of *Penicillium chrysogenum* is due to their wide range of its biological activities. The present study was conducted to study the antibacterial activity of the crude extract of *Penicillium chrysogenum* isolated from the rhizospheric soil region of Mint. Samples were collected in a sterilized polythene bag from rhizospheric soil region of Mint. The samples were serially diluted and cultured on fungal media like potato dextrose agarand then purified and identified through colony morphology, microscopy and further molecular characterization through 18s RNA sequencing. The isolates were further cultured using potato dextrose broth media for production of biologically active secondary metabolites. The fungal extract was screened for anti-bacterial activities. The crude ethyl acetate extract of the *Penicillium chrysogenum* was used against five pathogenic bacterial species *E. coli* (22 mm), *K. pneumonae* (30mm), *Pseudomonas* (30mm) *S.epidermitus* (N/A) and *Shigella* (N/A). The ethyl acetate extract of *Penecillium* was tested against all these bacteria and the extract showed significant activity against all the bacteria. It is concluded from current study that *Penicillium chrysogenum* ethylacetate (EtoAc) extract has a great potential

of inhibiting the growth of Pathogenic bacteria.

Keywords: Fungi, secondary metabolites, Soil,Antibacterial Activity and Pathogenic

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Introduction

Soil is highly complex systems with many components playing diverse functions mainly due to the activity of soil organisms [1]. Microorganisms are beneficial in increasing the soil fertility and plant growth as they are involved in several biochemical transformation and mineralization activities in soils. Type of cultivation and crop management practices found to have greater influence on the activity of soil microflora [2]. Soil microflora plays a pivotal role inevaluation of soil conditions and in stimulating plant growth. The quantities of organic and inorganic materials present in the soil have a direct effect on the fungal population of the soil.In addition to chemical fertilizers and wide range of pesticides shows adverse effect on mycoflora which are much useful to maintain soil fertility and eco-balance in the soil atmosphere[2, 3]. The rhizosphere is the narrow region of Soil that is directly influenced by root secretions and associated soil microorganism. The microorganisms plays major role in soil ecosystem. Soil is an oligotrophic medium for the growth of fungi because the fungal growthsare extremely limited for most of the time & readily available are present for short periods in alimited zone [4, 5]. Fungi play significant role in the daily life of human beings besides their utilization in industry, agriculture medicine, food industry, textiles, bioremediation, natural cycling, as bio fertilizers and many other ways. Fungal biotechnology ha¬¬s become an integral part of human welfare. Fungus protects plants by supplying a protective health to supply both water & Phosphorus to the plant roots during droughts [6-8]. A fundamental role is played by fungi in the soil ecosystem. They play a crucial role in many important processes in the forests and agricultural soils, these processes may involve decomposition of organic matter and elemental release by mineralization. Micro fungiregulate the biological activity of soil and play a vital role in nutrient cycling. The quantities of organic and inorganic materials found in soil directly affect the fungal population of the soil. The pesticides also have an adverse effect on the soil mycoflora. The environmental factors which include the pH, temperature, amount, degree of aeration and type of nutrients and moisture are major necessities of the soil microorganisms [9]. Fungi produce a multitude of low-molecular-mass compounds known as secondary metabolites, which have roles in a range of cellular processes such as transcription, development and intercellular communication. Varieties of secondary metabolites are produced by most of the filamentous fungi and are not required for the normal growth of fungi. The identification of biosynthetic genes for metabolites has become easy because of the accessibility of fungal genomic sequences. Secondary metabolites have an incredible effect on society, as some of the metabolites are oppressed for their antibiotic and pharmaceutical activities, while others are involved with the plants or animals

diseases [10]. Saprophytic associations with other organisms are commonly foundin many of these fungi in soil and these molecules provide protection against other inhabitantsof the soil [11, 12]. Studies have estimated the existence of at least 2.2–3.8 million fungal species on Earth, from which only around 10% have been isolated and described. Penicillium chrysogenum, one of the most common fungi in a various range of habitats, has a worldwide distribution and a large economic impact on human life. This genus is of great importance in numerous and diverse fields, such as food spoilage, biotechnology, plant pathology, and medicine, and currently contain 483 accepted species. Several of these species, classified as pre- and post-harvest pathogens, can lead to catastrophic decay in food crops, as described by Frisvad and Samson, Pitt and Hocking, and *Penicillium chrysogenum* can also produce a varied range of secondary metabolites, including several harmful mycotoxins, antibacterial and antifungal compounds, immunosuppressant, and cholesterol-lowering agents [13].The most iconic example of a drug of fungal origin is penicillin, the first antibiotic substance in history. The biosynthesis of several secondary metabolites, such as mycotoxins, depends onseveral environmental cues including the substrate, pH, temperature, water activity, interrelationships with other microorganisms, and the interactions of these different factors in the natural environment[14]. The aim of the study is the secretion of secondary metabolites from fungi and to evaluate its antimicrobial activities on different bacterial species.

Materials and Method

Collection of Soil Samples

The soil samples were collected from rhizosphereic region of Mint from agricultural area of Charsadda. Soil samples were collected from a depth of 3-15cm with the help of a sterilized cork borer pushed horizontally into the ground. The soil was emptied into sterilized polyethylene bags. Each sample bag was labeled appropriately by indicating the siteof collection, time, date and place of collection. The samples were then taken to the laboratoryusing sterilized cellophane bags and kept at 4C for further processing.

Molecular Characterization of the Isolate DNA Extraction

To obtain genomic DNA of the fungal strain, the mycelia was macerated with amortar and pestle in TES lysis buffer (Tris 100 mM; EDTA 10 mM; 2% SDS). First, lysed tissues were incubated at 65°C for 15 min. Then, 140 µL of 5 M NaCl was added, and the mixture was incubated on ice for 30 min. afterwards, 600 µL of chloroform/isoamyl alcohol (24:1) was added and centrifuged at 10,000× g for 10 min at 4°C. The supernatant was isolatedand mixed with 50µL sodium acetate 3M (pH 5.2) and 300µL isopropanol. After the second centrifugation under the same conditions, the supernatant was discarded, and the mixture was washed twice with 600 µL of 70% ethanol following centrifugation steps. After discarding thefinal supernatant, the resulting pellet was diluted in 50µL TE buffer (Tris 10 mM; EDTA 1 mM) and 5µL RNAse (10 mg/mL)

Polymerase Chain Reaction:

Genomic DNA was used to amplify the fungal internal transcribed spacer (ITS) region applying the primer pairs according to the method of Brück *et al.*, (2022). For amplification reactions, a PCR Master Mix Kit was used following the manufacturer's instructions. To visualize the amplification, product electrophoresis was performed on a 1% agarose gel stained with Nancy dye (Sigma–Aldrich, Saint Louis, MO, USA). Next, the amplification products were purified using the Wizard® SV Gel kit and PCR Clean-Up System (Promega) following the kit's instructions. Finally, the PCR product was quantified on a Nano Drop® (Thermo Scientific, Waltham, MA, USA).

DNA Sequencing:

Sequencing reactions was performed with the Terminator Cycle Sequencing Kit following the manufacturer's instructions and analyzed with sequencer system.

Production of Secondary Metabolites:

The procedure used for the production of fungal secondary metabolites is described below. The potato dextrose broth (PDB) media was used as fermentation culture for production of secondary metabolites. For inoculation of fungal mycelia, an 8 mm Cork borer was used. The inoculumwas taken from 7 days old culture of the isolated fungi grown on PDA media.The incubator was set to 150 rpm and 28° C temperature for 10 days. After 10 days of incubation, the flasks were taken out from the incubator. A drop of concentrated HCL was added to the flasks to stop further growth of fungus. After 24 hours of addition of HCL, the cultural broth was filtered through wattman filter. The mycelia of one flask were filtered separately to record its dry weight. For extracting the metabolites, ethyl acetate was used as solvent. To separate secondary metabolites from ethyl acetate, the ethyl acetate was processed in rotary evaporator. The biomass and secondary metabolites was determined using formula:

Average weight of sec. metabolites/ dry mycelia =

Weight of sec. metabolites/ drymycelia in vial – Weight of via

Antibacterial activity

Five identified species of bacteria (*Escherichia coli, Klebsiella pneumonia, Pseudomonas aureginosa, S.epidermites and Staphylococcus aureus*) were grown on nutrient agar media. After that 2 wells were made one for negative control (DMSO) second for fungal crude extract and antibiotic disc (Ciprofloxacin) was placed as positive control. Then plates were kept in bacterial incubator for about 24 hrs, as a result zone of inhibition were shown which is mention in results.

Results And Discussion

Identification of Fungi

The Lactophenol cotton blue staining (LPCB) technique was performed and different structures of the isolate (AU1) were observed. The slide culture technique proved to be more efficient in providing clear image of the fungus as compared to smear and scotch tapemethod. The isolate (AU1) appeared to have a conidiophore, a bunch of spores with single chain and. a philiade from which the spores appeared to be rising upward.

Molecular characterization of the Isolate:

The molecular characterization of the isolate (AU1) was performed by 18S ribosomal RNA gene sequence analysis. The isolate was confirmed to be *penicellium chrysogenum (notatum)* (Accession number MK371712.1). The 18S rRNA sequence and the phylogenetic evolutionary relationship of taxa based on 18S rRNA sequence are shown in thefigures .1 and .2.

>H220816-R01_K08_KTTS1_NS24.ab1 1226
CAATGAAACGGAGTTTGACCAACTTTCCGGCTCTGGGGGGTCGTTGCCAA
CCCTCCTGAGCCAGTCCGAAGGCCTCACTGAGCCATTCAATCGGTAGTAG
CGACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCGGCACGAGCTGATG
ACTCGTGCCTACTAGGCATTCCTCGTTGAAGAGCAATAATTGCAATGCTC
TATCCCCAGCACGACAGGGTTTAACAAGATTACCCAGACCTCTCGGCCAA
GGTGATGTACTCGCTGGCCCTGTCAGTGTAGCGCGCGTGCGGCCCAGAAC
ATCTAAGGGCATCACAGACCTGTTATTGCCGCGCACTTCCATCGGCTTGA
GCCGATAGTCCCCCTAAGAAGCCAGCGGCCCGCAAATGCGGACCGGGCTA
TTTAAGGGCCGAGGTCTCGTTCGTTATCGCAATTAAGCAGACAAATCACT
CCACCAACTAAGAACGGCCATGCACCACCATCCAAAAGATCAAGAAAGAG
CTCTCAATCTGTCAATCCTTATTTTGTCTGGACCTGGTGAGTTTCCCCGT
GTTGAGTCAAATTAAGCCGCAGGCTCCACGCCTTGTGGTGCCCTTCCGTC
AATTTCTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCAGAACCCAAAA
ACTTTGATTTCTCGTAAGGTGCCGAACGGGTCATCATAGAATCCCGTCCG
ATCCCTAGTCGGCATAGTTTATGGTTAAGACTACGACGGTATCTGATCGT
CTTCGATCCCCTAACTTTCGTTCCCTGATTAATGAAAACATCCTTGGCGA
ATGCTTTCGCAGTAGTTAGTCTTCAGCAAATCCAAGAATTTCACCTCTGA
CAGCTGAATACTGACGCCCCCGACTATCCCTATTAATCATTACGGCGGTC
CTAGAAACCAACAAAATAGAACCGCACGTCCTATTCTATTATTCCATGCT
AATGTATTCGAGCAAAGGCCTGCTTTGAACACTCTAATTTTTTCACAGTA
AAAGTCCTGGTTCCCCCACAGCCAGTGAAGGCCATGAGGTTCCCCAGAAG
GAAAGGTCCAGCCGGACAGTACTCGCGGTGAGGCGGACCGGCCAGCCAGA
CCCAAGGTTCAACTACGAGCTTTTTACTGCACAACTTTATATACGCTATT
GGAGCTGGAATTACCGCGGCTGCTGGGACCAGACTTGCCCCCATTGTTCC
CGCTTAAGGGAATAAAATGGTCCCAC

Figure .1: 18S rRNA sequence of the Isolated Fungus

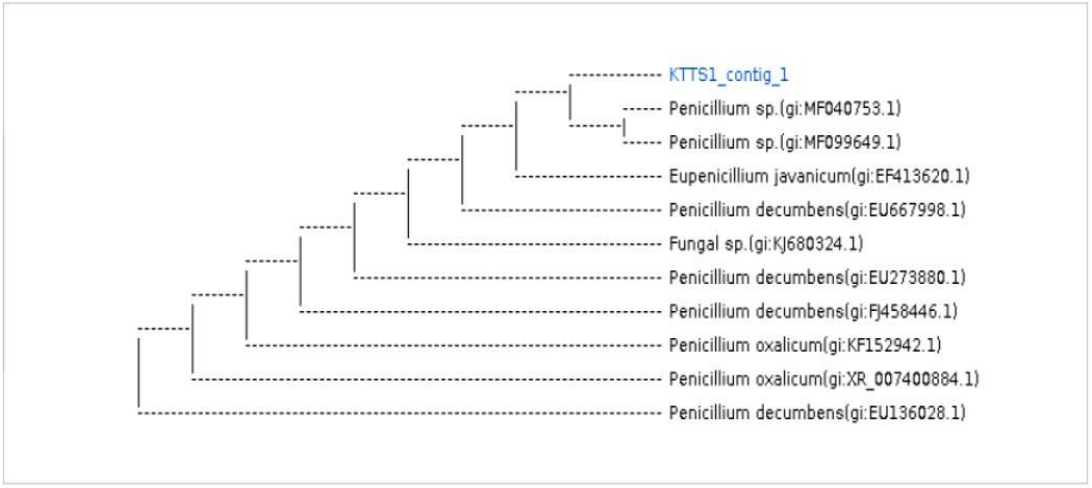

Figure .2.Phylogenetic tree

Secondary Metabolites Production and Extraction:

After incubating the fungus in Potato dextrose broth for 10 days, the secondary metabolites produced in the flask were isolated through Ethyl acetate. The rotary evaporator was used to separate the Ethyl acetate from metabolites. The mycelia in the flask was dried and weighed and the metabolites produced were also weighed and their values were recorded. The biomass and metabolites were determined by the formula:

Average weight of sec. metabolites/ dry mycelia =

Weight of sec. metabolites/ dry mycelia invial – Weight of vial

1) Weight of dry mycelia per flask (250 ml PDB)=2.90 grams
2) Weight of Secondary metabolites produced per flask (250ml PDB)=0.06 grams
3) Weight of dry mycelia from 2500 ml PDB=29.00 grams
4) Weight of Secondary metabolites from 2500 ml PDB=0.6 grams

Antibiotic Susceptibility Test:

For antibiotic susceptibility test, a total of eleven antibiotics were used, which are shown in the table. All of the eight bacterial species showed multi-drug resistance (MDR) ability. The CLSI standard values of resistance, susceptibility and sensitivity of each antibiotic used are shown in the table. The resistance, sensitivity and susceptibility of the isolated bacteria to the antibiotics used, are shown in the table 1.

Table.1. Antibiotic Susceptibility Pattern of Isolated Bacteria

		Antibiotics Susceptibility Pattern (mm)				
Isolate	**Bacteria**	**FOX**	**FOS**	**DO**	**CTX**	**OFX**
1	*E.coli*	20	18	25	24	11
2	*P.aeruginosa*	25	24	N/A	27	20
3	*K.pneumoniae*	18	19	21	22	17
4	*Shigella*	30	R	20	21	R
5	*S.epidermites*	21	16	15	20	18

Antibacterial Activity of Crude Extract:

The antibacterial activity of the ethyl acetate crude extract of *Penicellium chrysogenum* was tested against five Multi-Drug Resistant (MDR) bacteria i.e *E. coli, P. aeruginosa, Shigella, Klebsiella pneumonia,* and *S. epidermidis.* The crude extract at 0.4 gram per 1 ml DMSO and CRO were used to determine the activities at concentration rate of 100µl, 75µl and 50µl. The extract showed remarkable activity against the tested bacteria. The result of antibacterial activity at different concentration of extract is described below.

Activity at 0.4 gram per 1 ml DMSO

The antibacterial activity at 0.4grams per 1ml DMSO at 100µl, 75µl, 50µl concentrations is described below.

Concentration rate of 100µl, 75 µl and 50 µl:

The crude extract at 0.4 grams per 1 ml DMSO at 100µl concentration showed significant activity against *Shigella* (17mm), *P.aeruginosa* (21mm), while at 75µl concentration it showed significant activity against *Pseudomonas* (20mm), and at 50µl it showed significant activity against *Pseudomonas* (17mm) and *E.coli* (15mm).The detailed results are shown below in table 2.

Table.2.Antibacterial Activity of EtoAc extract and CRO at 0.4 gram per 1 ml DMSO

	Zone of Inhibition (mm)	-ve control	CRO

S.NO	Isolate	100 µl	75 µl	50 µl		
1	*E.coli*	19	17	15	-ve	22mm
2	*P.aeruginosa*	21	20	17	-ve	37mm
3	*K.pneumoniae*	15	13	0	-ve	30mm
4	*Shigella*	17	15	10	-ve	R
5	*S.epidermites*	10	0	0	-ve	R

Soils are highly complex systems with many components playing diverse functions mainly due to the activity of soil organisms. Microorganisms are beneficial in increasing the soil fertility and plant growth as they are involved in several biochemical transformation and mineralization activities in soils. Stefani *et al., (2013)* concluded that the rhizosphere is the narrow region of soil that is directly influenced by root secretions and associated soil microorganism. The microorganisms play major role in soil ecosystem. Kumar *et al., (2015)* stated that Soil is an oligotrophic medium for the growth of fungibecause the fungal growths are extremely limited for most of the time & readily available are present for short periods in a limited zone. Raja *et al., (2017)* stated that Soil samples (approximately 5g) were collected with clean dry and sterile polythene bags along with sterile spatula. The collected samples brought to the laboratory and preserved for further studies. The soil samples were collected from the month of September 2015 to March 2016 in Loyola college campus at various locations. The soil samples collected from twenty different zones of Loyola college campus. In the current study the samples were collected and brought to the laboratory in similar way from the month of Jun 2022 to September 2022 from agricultural area of Charsaddha. Sara *et al.* (2020) conducted a study that involve the antimicrobial activity of the *Aspergillus oryzae* strain isolated from saline soil (El-Baida marsh in Algeria). Whereas, in current study, we isolated the fungus *Penicillium chrysogenum* from the rhizospheric soil of the Mint. Ababutain *et al.* (2021) in their study identified the fungus through morphological characteristics and 18s rRNA gene sequencing method. Similarly, in this study, we utilized LPCB staining and SEM analysis to observe the morphology of the *Penicillium chrysogenum* and 18s rRNA gene sequencing to identify the isolated fungus. Mohammed *et al.* (2021) investigated biological activities and metabolic profile of three fungal strains identified from different desert sites in Saudi Arabia and their antibacterialactivity was investigated against *Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumonia*, and *Escherichia coli* by using nutrient agar. The result showed that Identified fungal isolates, *Chaetomium sp. Bipolaris sp.* and *Fusarium venenatum* showed different inhibitory activity against tested bacteria. In

current study *Penicillium chrysogenum* fungus was identified from rhizospheric soil by using serial dilution method and their secondary metabolites were used against five different clinical bacterial isolates. The fungus active compound showed highest inhibitory activity against all tested bacterial isolates.

Conclusion

In the present study *Penicillium chrysogenum* were isolated from therhizospheric region of the mint show a great antibacterial activity against 5 human pathogenicMDR bacteria. *Penicillium chrysogenum* showed maximum zone of inhibition against *E. coli, K. pneumonia, P.aureginosa, S.epidermites, Shegella*. Therefore, there is a need of further in depth studies of these isolated Fungi. Further growing those on large scale, modifying cultureconditions and supplying some stimulants might help in getting better production of particularbioactive compound.

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