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Response of *Penicillium Eu-0013* to different growth media for effective antibacterial compound(s) production

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Abstract

Penicllium Eu-0013 is seldom been studied in KPK-Pakistan against both agriculturally and medically important bacteria under different growth media treatments. As bacterial infection is a severe threat to agricultural products and individual healthiness hence the study was conducted to estimate the antibacterial potential of *Penicillium Eu0013* under diverse developmental culture circumstances. Various soil born and endophytic fungi were isolated and preliminary screened for conventional antagonistic bactericidal effect against phytopathogenic bacteria *Xanthomonas campestris* and *Clavibacter michiganensis* followed by optimizing media constituents of the bioactive fungus for maximum antibacterial compounds production. Antibacterial tests using disc diffusion method and microdilution assays were carried out on the ethyl acetate and acetonitrile fraction of potent fungus against the two phytopathogenic bacteria and human pathogenic bacteria *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*. *Penicillium Eu0013* was selected based on its antagonistic property. The Ethyl acetate extract of *Penicillium Eu0013* obtained on Growth Nutrient Broth medium showed maximum zone of inhibition (23.3±0.264mm) against *Clavibacter michiganensis* followed by (19.366±0.351mm) against *Xanthomonas campestris* as compared to the zone of inhibition shown by extracts of other culture media (Glucose Peptone Yeast Broth, Potato Dextrose Broth and Yeast Extract Broth). Also the EtOAc extract and acetonitrile fraction stated the minimal value of MIC (5.208±2.255µg/ml and 10.416±4.510µg/ml) respectively against gram positive *Staphylococcus aureus*. It is revealed from the results that *Penicillium Eu0013* is a potential source of novel antibiotic drugs as it showed a wide range of bioactivity against both phyto and human pathogenic bacteria. Different cultural conditions can yield different bioactive compounds.

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Introduction

Most of the bacterial infections are getting worse in eradication because of the fact that most of the bacteria are developing constant resistance against the existing antibiotics, even though antibiotics are the most significant bioactive compounds against bacterial infections. Scientists around the globe now days therefore are mostly interested to find out novel and successful antibiotics to fight the high load occurrence of multi drug resistant bacteria (Rajasekar *et al.,* 2012). In short novel antibacterial compounds should be discovered to treat the developing resistant pathogens.

In past variety of the diseases were treated with plants only because of the bioactive ingredients found in them and they were the only source but now days great attention has turned towards fungi because of their vast potential in new bioactive metabolites production against recent antimicrobial infections (Strobel, 2003). Mostly fungi produce compounds belong to flavonoids, alkaloids and terpenoids such compounds has antibacterial, antiviral, anti-inflammatory, antitumor, and antifungal properties (Guo *et al.,* 2007; Yu *et al.,* 2010; Aly *et al.,* 2011). These properties obviously proved fungi as a rich cause of biologically effective and chemically exceptional substances (Fenical, 1993; Kerr, 1999). But in practical application as an antimicrobial compounds to be utilized as agrochemical or pharmaceutical agents from fungi still are comparatively less explored organisms (Petrini *et al.,* 1992; Ramasamy *et al.,* 2010). Fungi have a variety of huge habitation, they are eukaryotes and can be found everywhere in nature.

The genus *Penicillium* is possibly the most distinguished filamentous fungi. Most of their species possess antibiotic properties, they have high frequency of bread colonization.

They can be mostly observed on spoiled foods but due to its vast habitat it can be also found in plants, animals and in some cases other fungi. They can be best considered as saprophytes as they grow on organic substrates that include dead plant material (Giguère, 2006). This study was investigated to examine the chemistry of *Penicillium Eu0013*, isolated from soil of Khyber Pakhtunkhwa, Pakistan, to study its antibacterial potential against both phytopathogenic and human pathogenic bacteria under various media culture treatments for maximum production of bioactive antibacterial compounds. Also to test the minimal inhibitory concentration of ethyl acetate extract and its acetonitrile fraction.

Materials and methods

Isolation of endophytic and soil borne fungi

Soil Samples were collected form rhizosphere soil up to a depth of 6 cm and plant samples (fresh plant parts i-e leaves) were collected randomly from different regions of Khyber Pakhtunkhwa, Pakistan. Samples were transferred into sterile labeled plastic bags to the laboratory of Agricultural Chemistry, University of Agriculture Peshawar. Serial dilution method was used for the isolation of soil born fungi according to the procedure of (Gaddeyya *et al.,* 2012) while for the isolation of endophytic fungi the method of (Dobranic *et al.,* 1995) was followed.

Plant samples were cleaned with running tap water for an hour and then cut into small segments about 1 cm, segments were then surface sterilized by immersing in 70% ethanol for 1 minute, followed by dipping in 4% sodium hypochlorite for 2 minutes and finally rinsed with sterile water followed by drying. Isolates were transferred into PDA plates incubated at 27 °C for 7 days. The isolate so obtained were purified by relocating on PDA media and stored at 4 °C for further analysis.

Morphological characterization

Fungi were identified according to their morphological characterization using the method of (Domsch *et al.,* 1980). According to this method a total of twelve fungal species were examined and identified based on their macroscopic features i-e shape, growth and color of the cultured colonies as well as microscopic features like structure of conidia, hyphae and spore size.

Dual culture assay

Isolated fungal species were tested antagonistically against the phytopathogenic bacterial species (*Xanthamonas campestris* and *Clavibacter michiganensis*) using dual culture technique of (Siameto *et al.,* 2010; Sonawane *et al.,* 2015). According to this method bacterial species adjusted at (1 x 10^8cfu) were streaked on 20 ml sterilized Potato Dextrose Agar (PDA) plate with the help of sterilized wire loop inside Laminar Flow Unit and were put at 37°C for overnight in incubator. Next day 5mm fungal plug was inserted in centre of the petri plate and incubated at 28 °C for 3 days. Plates without fungal inoculums used were as reference plate.

Media optimization for *Penicillium Eu-0013*

Four different types of cultural media: Glucose Peptone Yeast Broth (GPYB), Potato Dextrose Broth (PDB), Yeast Extract Broth (YEB) and Growth Nutrient Broth (GNB) were prepared for the cultivation and fermentation of *Penicillium Eu-0013* (Jain and Pundir, 2011). The main purpose behind was to identify the best medium that result in maximum production of bioactive antibacterial compounds.

Culturing and extraction of secondary metabolites

Extraction of secondary metabolites from *Penicillium Eu 0013* was carried out using the procedure of (Zain *et al.,* 2008).

The prepared sterilized media (1L) was distributed among 5 different sterilized conical flasks of 500 ml capacity (200 ml in each) under aseptic conditions. 5mm pure active inoculum plugs from 8 days fresh culture of *Penicillium Eu 0013* grown on PDA were introduced into these flasks and were put in incubator at 28°C for two weeks, preceding to separation of mycelia through vacuum filtration. The dried mycelia were homogenized in ethyl acetate for 24 hours in order to extract secondary metabolites. Ethyl acetate was then removed with the help of rotary evaporator at 46 ˚C.

Disc diffusion susceptibility test

This test was performed according to (Tong *et al.,* 2011) with minor amendments. Suspensions of *Xanthomonas campestris* and *Clavibacter michiganensis* in sterile nutrient broth was adjusted to approximately 1 × 10^8 cfu/ml, 50µl of the suspension was streaked on Nutrient agar medium plate with the help of sterile cotton swab. 20 µl of test sample (1mg crude extract/1ml 1% dimethyl sulfoxide (DMSO) was added to 6mm sterile whatman filter paper disc, and were positioned on the surface of nutrient agar medium plate comprised test bacteria. Negative control used in this test was 1% dimethyl sulfoxide (DMSO) whereas for positive control Streptomycin was used. Petri plates were incubated at 37 °C for 24 hrs and inhibition zones were measured in millimeters.

Determination of minimal inhibitory concentration (MIC)

The organic crude extract and acetonitrile fraction of *Penicillium Eu0013* was evaluated for determining the MIC against human pathogenic bacterial strain (two gram negative bacteria (*Escherichia coli* and *Pseudomonas aeuroginosa*) and one gram positive bacteria (*Staphylococcus aureus*) concerning the procedure described by (Reller, 2009). Suspension of bacterial strains were made in 3% Tryptic Soy Broth (TSB) medium at 37°C for 12 hours followed by two times washing with 10mM Tris buffer (pH 7.4) quantified by OD_{600}. 96-well plates (U-shaped, untreated polystyrene) were used in this test for sample preparation using serial dilution with tris buffer and bacterial cells were added to a final volume of 100µl. Plates were incubated in aerobic incubator at 37°C, for 6, 8 and 10 hours according to the growth period of particular bacteria.

Statistical analysis

The experiments were carried out in three replicates and the results of the data are presented as mean ± SD (standard deviation) using statistical software Statistix 8.1.

Results

Morphological characterization

The isolated and purified fungi were identified by their sporulating structures and colony characteristics.

Based on their natural habitation they were divided into two major groups: soil born and endophytes.

Most of the fungal species were of soil origin while some were endophytes. Classes of these fungal species are also determined (Table 1).

Table 1. Classes and habitats of the Isolated Fungi.

Natural habitat	Fungal isolates	Classes
Rhizosphere soil	*Penicillium EU0013*	Eurotiomycetes
	Aspergillus flavus	Eurotiomycetes
	Paecilomyces sp	Eurotiomycetes
	Aspergillus niger	Eurotiomycetes
	Fusarium oxysporum	Sordariomycetes
	Acremonium sp	Sordariomycetes
	Trichoderma harzianum	Sordariomycetes
	Rhizopus stolonifer	Zygomycetes
	Verticillium sp	Incertaesedis
Endophyte	*Pythium* sp	Oomycota
	Alternaria alternate	Dothideomycetes
	Alternaria brasiscicola	Dothideomycetes

Table 2. Zone of inhibition of different media extracts of *Penicillium Eu0013* against *Xanthomonas campestris* and *Clavibacter michiganensis* using disc diffusion method.

Fungus and Standard	Culture Media	Zone of Inhibition (mm)±SD	
		Xanthomonas campestris	*Clavibacter michiganensis*
Penicillium Eu0013	GNB	19.366±0.351	23.3±0.264
	GPYB	13.633±0.404	13.533±0.05
	PDB	8.1±0.2	9.4±0.624
	YEB	0±0.00	0±0.00
Streptomycine		25.266±0.305	28.20±0.360

Dual culture assays/antagonistic assay

Based on conventional antagonistic technique, *Penicillium Eu0013* showed prominent results and was found to be most efficient among other tested fungi. *Penicillium Eu0013* inhibited the growth of tested phytopathogenic bacterial species: *Xanthomonas campestris* and *Clavibacter michiganensis* using dual culture technique and was selected for media optimization (Fig. 1).

Disc diffusion susceptibility test

Zone of inhibitions formed by organic crude extracts of *Penicillium Eu0013* obtained on four different growth media (GNB, GPYB, YEB and PDB) against the two tested microbes (*X. campestris* and *C. michiganensis*) is presented in (Fig. 2).

The maximum zone of inhibition was observed by the organic crude extract obtained on GNB medium which showed diameter of (23.3±0.264mm) against *C. michiganensis* followed by (19.366±0.351mm) against *X. campestris*.

The EtOAc extract of GPYB medium showed (13.633±0.404mm) zone of inhibition against *X. campestris* and (13.533±0.05mm) against C. *michiganensis*. Similarly zone of inhibition shown by PDB medium against these two phytopathogens: *X. campestris* and *C. michiganensis* were (8.1±0.2mm and 9.4±0.624mm) respectively, while no activity was observed from YEB extract.

Streptomycine used as positive control showed (28.20±0.360mm) zone of inhibition against C. *michiganensis* and (25.266±0.305mm) against *X. campestris* as shown in (Table 2).

Microdilution assay

The minimum inhibitory concentration values shown by the organic crude extract and acetonitrile fraction of *Penicillium Eu0013* obtained on GNB medium is presented in (Table 3). The minimal value of MIC (5.208±2.255 µg/ml) was observed by organic crude extract against gram positive *Staphylococcus aureus* followed by (6.510±2.255 µg/ml) against *Pseudomonas aeruginosa* and (10.416±4.510 µg/ml) against *Escherichia coli*. Similarly the acetonitrile fraction of GNB extract showed similar value of MIC (20.833±9.021µg/ml) for *E. coli* and *P. eruginosa,* and (10.416±4.510µg/ml) for *S. aureus*. The MIC value recorded for positive control streptomycine against *Escherichia coli* and *Pseudomonas aeruginosa* was similar (6.510±2.255 µg/ml) while it exhibited the minimal value for the gram positive *Staphylococcus aureus* (3.906±0.00 µg/ml).

Table 3. Minimal Inhibitory Concentration of ethyl acetate and acetonitrile fraction of GNB medium of *Penicillium Eu0013* against human pathogenic bacterial strains using dilution method ELISA plate method.

Fungus and Standard	Fractions	MIC (µg/ml)		
		Escherichia coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Penicillium Eu0013	Crude Extract	10.416±4.510	6.510±2.255	5.208±2.255
	Acetonitrile	20.833±9.021	20.833±9.021	10.416±4.510
Streptomycine		6.510±2.255	6.510±2.255	3.906±0.00

Discussion

Penicillium Eu0013 possess antibacterial properties and hence produced bioactive antibacterial compounds that inhibited the growth of phytopathogenic bacterial species and therefore showed efficient antagonistic effect in dual culture technique based on which this fungus was selected for further studies among other isolated fungal species. (Georgakopoulos *et al.,* 2002) reported that with antagonism one can easily control the pathogenicity from phytopathogens.

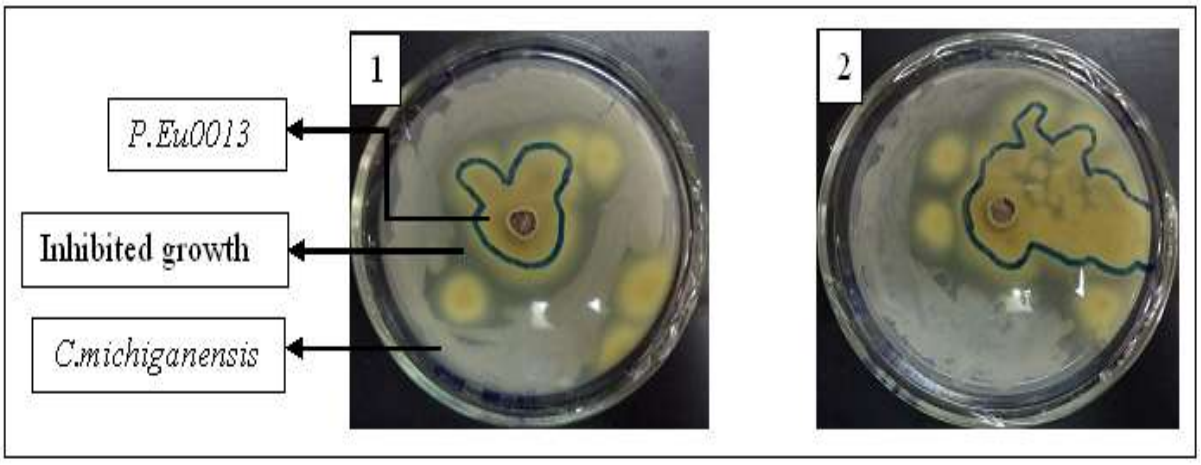

Fig. 1. *Penicillium Eu0013* antagonistically inhibited the growth of *Clavibacter michiganensis* (1) and *Xanthomonas campestris* (2).

My results accomplished the selection of suitable potent media for bioactivity and antibacterial compounds production, my findings also revealed that the organic extract obtained from growth nutrient broth (GNB) medium exhibited maximum inhibition zone against the phytopathogenic bacteria *X. Campestris* and *C. Michiganensis* as compared to the organic extracts obtained on other culture media: glucose peptone yeast broth (GPYB), yeast extract broth (YEB) and potato dextrose broth (PDB) media which suggested that this medium (GNB) can improve the antibacterial compounds production. The effect of media on antibacterial compounds production was confirmed from the results of (Slininger and Shea-Wilbur, 1995) who described that changing the composition of growth media, changes in pH and variation in temperature has greater influence on the secondary metabolite production in broth media. While Ramos and Said, 2011 reported that antibacterial activity of the fungus can be improved by changing the constituents of culture broth media. (Vijaykumar *et al.,* 2013) described that maximum activity of the culture medium may be due to the production of bioactive compound in high concentration in that specific growth media. Also surrounding environment can improve yield and bioassay.

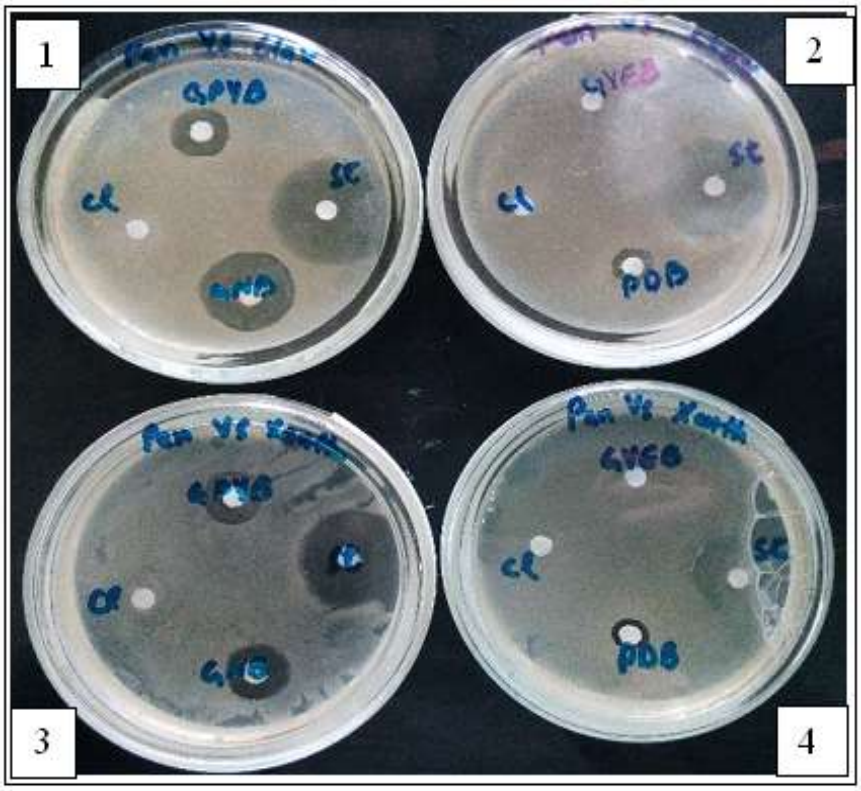

Fig. 2. Zone of inhibition shown by ethyl acetate crude extract of *Penicillium Eu0013* grown on four different media (GPYB, GNB,YEB and PDB) against *Clavibacter michiganensis* (1,2) and *Xanthomonas campestris* (3,4).

Both ethyl acetate extract and acetonitrile fraction of *P. Eu0013* demonstrated the growth inhibition of gram positive and gram negative human pathogenic bacterial strains in 96 well microtitre plate and proved to be a medically important fungus.

The minimum inhibitory concentration values shown by *P. Eu0013* in 96 well microtitre plate drop within the range of strong potency by comparing with the cut off values mentioned by (Kuete, 2010): ("the cut-off value for MIC is as follows: significant (MIC ≤ 100 µg/ml), moderate (100 µg/ml - 625 µg/ml) and weak (MIC ≥ 625 µg/ ml)").

The MIC values of *P. Eu0013* in my findings were quite promising when compared with the MIC values of *Penicillium* species against soil borne bacteria described by (Ali *et al.,* 2011).

Conclusion

Penicillium Eu0013 is possibly the most distinguished filamentous fungi having antibiotic properties. This fungus inhibited both human and plant pathogenic bacteria and therefore is a potential source of antibacterial compounds and could be an effective cause against medically and agriculturally important pathogens. The fungus produced improved bioactive antibacterial compounds by changing the composition of its growth media which suggest that novel antibiotics of interest can be discovered which are of pharmaceutical importance. As there is a great need for new antibiotics of natural origin which are less toxic and environment friendly, it is therefore concluded that this fungus can be used as a rich source for novel antibiotics.

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