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Prevalence of *Penicillium chrysogenum*, its Qualitative, Quantitative Determination and Antibacterial Activity in Indian Soil

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Abstract: The present study was designed to a systematic screening for the potential isolates of *P. chrysogenum* from Indian soils (Haridwar and Dehradun districts of Uttaranchal state) and their antibacterial activity (quantitative and qualitative analysis). Total 329 soil isolates of *P. chrysogenum* were isolated from 120 collected soil samples (Agricultural, Garden and Road soil) from Haridwar (40.12%) and Dehradun (59.87%) districts during the months of May-June and September-October, 2002. All *P. chrysogenum* soil isolates showed remarkable antibacterial activity against *S. epidermidis* (MTCC-435) as test strain. Besides this penicillin G production by *P. chrysogenum* isolates was found with a total positivity from Haridwar (87.39%) and Dehradun (89.78%). Finally, these isolates were confirmed for penicillin G production by HPLC. We observed that agricultural soil is a major source for potential isolates of *P. chrysogenum* as compared to garden and road soils.

Key words: Soil, *Penicillium chrysogenum*, penicillin G, bioassay, HPLC analysis

INTRODUCTION

It is well documented that *Penicillium chrysogenum* is a potential antibiotic producing species of genus, *Penicillium*[1-3]. A number of antibacterial drugs and secondary metabolites have been reported for their sensitivity against broad range of pathogenic microbes[4,5]. Penicillin G produced by *P. chrysogenum* is considered as a member of β-lactam antibiotic family and researchers have reported its antibacterial activity against gram-positive bacteria. Different kind of applications of penicillin G has been documented in the industrial as well as in medical field.

Staphylococcus sp. causes some severe diseases such as suppurative disease, mastitis, arthritis and Urinary Tract Infection (UTI); by introducing numerous virulence factors such as extracellular toxins and enzymes into animal species[6]. For human being, these organisms are important source of food poisoning, pneumonia, wound infections and nosocomial bacteremia[7]. Staphylococcal isolates are frequently resistant to penicillinase-resistant penicillins. These bacteria resist to commonly using antibacterial antibiotics including chloramphenicol, tetracycline, clindamycin, cephalosporins and other β-lactam antibiotics[8-11]. There are several reports on the phenomena of development of resistance in human pathogenic staphylococcal bacterium. It is increasing continuously and it becomes a serious health problem. *P. chrysogenum* has antibacterial activity against *Staphylococcus* bacteria because it is capable of producing penicillin G.

P. chrysogenum is most common and prominent fungus in the soil. Quantitative isolation of *P. chrysogenum* from soil denotes soil as a good source for it[12]. Strain improvement is a natural and continuous process in soil. It was assumed that potential isolates of *P. chrysogenum* may exist in the soil as natural source and penicillin G may be found higher in the quality and quantity than existing isolates of *P. chrysogenum*. Therefore, there is need of a systematic screening program to isolate the potential antibiotic producing strains of *P. chrysogenum* from different soil samples. The present was designed to investigate the potential strains of *P. chrysogenum* from different soil samples and determines the qualitative and quantitative assays for its antibacterial activity.

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MATERIALS AND METHODS

Source and examination of soil samples: Adequate quantity (100 g each) of soil samples collected in sterile containers from two districts (Haridwar and Dehradun) of Uttaranchal state, India. The samples were collected during the period of May to June and September to October 2002. All samples were taken into the laboratory and processed on the same day.

Total 120 soil samples were collected and processed for isolation of *P. chrysogenum* by dilution-plate method[13]. About 10 g of each sample was suspended in 100 mL of sterile physiological saline containing gentamycin (10 µg mL^{-1}). The suspension was stirred for 2 min on a vortex mixer and allowed to settle for 30 min at room temperature. Supernatant was serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilution). Aliquots of 0.1 mL of each serially diluted soil sample were spread on the plates (in duplicate) using bent glass rod over the entire surface of Sabouraud-Dextrose Agar (SDA) medium containing rose Bengal (0.4 µg mL^{-1}), chloramphenicol (0.6 µg mL^{-1}), penicillin (0.4 µg mL^{-1}) and streptomycin (0.6 µg mL^{-1})[14]. All plates were incubated at 25±1°C for 7 days; the plates were examined at daily interval.

Colonies of *P. chrysogenum* isolates were selected and identified on the basis of their cultural and microscopic characteristics[12]. All isolates of *P. chrysogenum* were maintained on SDA medium.

***P. chrysogenum* isolates and its inoculum preparation:** Defined medium was used for growth of *Penicillium chrysogenum* isolates and inoculum was prepared accordingly described by Ariyo *et al.*[2] with slight modifications. Fresh spore suspension (5X10^6 spore mL^{-1}) of *P. chrysogenum* isolates were aseptically inoculated in culture tubes, which was contained in growth medium: 20 g sucrose, 10 g lactose, 5 g mycological peptone, 13 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g Na_2SO_4, 0.55 g EDTA, 0.25 g $MgSO_4.7H_2O$, 0.05 g $CaCl_2.2H_2O$, 0.25 g $FeSO_4.7H_2O$, 0.02 g $MnSO_4.5H_2O$, 0.02 g $ZnSO_4.7H_2O$ and 0.005 g $CuSO_4.5H_2O$ in 1000 mL of distilled water and pH was adjusted to 6.8 with KOH before sterilization. Culture tubes were incubated in benchtop orbital shaker (Thermo forma model 420, Ohio, USA) incubator at 25±1°C for 48 h.

Production of antibacterial substance and its conditions: According to previously described method by Arioyo *et al.*[2], erlenmeyer flasks (500 mL capacity) with growth medium (200 mL quantity with 0.7% phenylacetic acid) were sterilized in autoclave at 121°C for 15 min. Prepared inoculum (48 h old) were inoculated in Erlenmeyer flasks, aseptically and those flasks were incubated in benchtop orbital shaker (Thermo forma model 420, Ohio, USA) incubator (21 rpm min^{-1}) at 25±1°C for 10 days. The growth medium with fungal growth was acidified upto pH-2 with oxalic acid and was filtered immediately. Crude fungal cultural filtrates of *P. chrysogenum* were separated by centrifugation at 2000 rpm for 15 min and kept at 4°C for further analysis.

Bioassay for antibacterial substance of *P. chrysogenum* isolates

Bacterial test strain: *Staphylococcus epidermidis* (MTCC-435) was obtained from MTCC, IMTECH, Chandigarh (India). Bacterial strain was grown on nutrient broth (Difco) at 37°C for 24 h and cell density of test organism was counted by Neubores chamber (Neubauer, Fein-Optik Blankenburg, Germany). Finally, concentration of test organism was adjusted to 1.2 x 10^6 cell mL^{-1} to determine the activity of antibacterial substance in crude fungal cultural filtrates by disk-diffusion method.

Disk-diffusion method: Soil isolates of *Penicillium chrysogenum* were screened for antibacterial activity using crude fungal cultural filtrate by modified version of disk-diffusion method[15,16]. Briefly, autoclaved nutrient agar medium (15 mL $plate^{-1}$) and 100 µL $plate^{-1}$ of *S. epidermidis* MTCC-435 (1.2X10^6 cell mL^{-1}) as a test strain were mixed gently and poured into petriplates. After solidifying the medium at room temperature, soaked paper discs (5 mm in diameter) with crude fungal cultural filtrate (200 µL $disc^{-1}$) were placed on the surface of medium incubated at 37°C for 24 h. Each cultural filtrate of *P. chrysogenum* was tested against gram-positive bacterial test strain (*S. epidermidis)* in triplicates and mean of inhibition zone was calculated.

HPLC analysis for antibacterial substance: Soil isolates of *P. chrysogenum* were used for quantitative determination using crude fungal cultural filtrate by HPLC method[17]. By this procedure, crude fungal cultural filtrate was used for separation of penicillin G on a chemcosorb 300 C column (GynKotecK High precision pump, Germany) with methanol: water: 0.2 M potassium phosphate (pH-5.0) (5:13:1) as mobile phase at a flow rate of 1 mL min^{-1} at room temperature. For determination of penicillin G, 100 µL of sample and standard solutions were injected. Penicillin G was detected by using the UV-detector at 210 nm and data were processed by the C-R6A chromatography system (GynKoteK, Chromatopae, Germany). For confirmation test, an aqueous solution of penicillinase (0.2 mL of 100 U mL^{-1}) was added in cultural filtrate, mixed and incubated at 37°C for 30 min[16]. Then, 100 µL of mixture was subjected to HPLC again for the confirmation of penicillin G chromatogram.

Statistical analysis: Coefficient of Variation (CV) test used for testing the recovery of penicillin G by *P. chrysogenum* isolates from various soil samples (agricultural, garden and road).

RESULTS

Total 329 soil isolates of *P. chrysogenum* were obtained at 10^{-5} dilution and identified from both districts 132 (40.12%) from Haridwar and 197 (59.87%) from Dehradun) of Uttaranchal state (India). Out of 132 soil isolates, 58 (43.93%) isolates were obtained during the period of May to June and 74 (56.06%), during the period of September to October 2002; from sample collection site at Haridwar. A total of 197 isolates 91 (46.19%) from May to June and 106 (53.80%) from September to October 2002, were obtained from Dehradun (Table 1).

Collected samples of soil were found to be significant in relation to recovery of *P. chrysogenum* isolates from both districts of Uttaranchal during the period of May to June and September to October. Collected soil samples from garden and road sites had lesser count of *P. chrysogenum* in comparison to agricultural site from both districts of Uttaranchal in India. Maximum recovery (in number) of *P. chrysogenum* isolates were found in agricultural soil during September to October from both districts (37 isolates from Haridwar and 45 isolates from Dehradun) of Uttaranchal (Table 1). Overall recovery of *P. chrysogenum* from Dehradun has been found higher (19.75%) as compared to Haridwar district.

Determination of antibacterial activity of soil isolates: Total 329 isolates of *P. chrysogenum* were isolated in this study and their antibacterial activity was checked against test strain (*S. epidermidis* MTCC-435). All soil isolates of *P. chrysogenum* were found sensitive against test bacterial strain. Out of 329 *P. chrysogenum* isolates, only 5 soil isolates were selected on the basis of their antibacterial activity (inhibition zone in mm) from each collection site of both districts, which had the maximum inhibition zone and mean of inhibition zones as shown in Table 2.

Soil isolates of *P. chrysogenum* showed qualitatively higher activity, those are originated from agricultural soil of both districts as compared to garden and road soils. There was no significant difference in the antibacterial activity of *P. chrysogenum* isolates during the month of May-June and September-October. Overall data proves that Dehradun district soil isolates of *P. chrysogenum* are potentially higher than Haridwar district soil isolates (Table 2).

Table 1: Prevalence of *Penicillium chrysogenum* at 10^{-5} dilution of uttranchal soil samples

Localities in Uttaranchal state	Soil samples collection sites	Number of *P. chrysogenum* isolates during different period of year, 2002	
		May to June	September to October
Haridwar	Agricultural	28	37
	Garden	19	23
	Road	11	14
Dehradun	Agricultural	39	45
	Garden	34	40
	Road	18	21

Table 2: Antibacterial activity of *Penicillium chrysogenum* against *Staphylococus epidermidis* (MTCC-435) by Disk-diffusion method

Different period (year 2002)	Mean of inhibition zone (in mm) against test organism of *P. chrysogenum* soil isolates from different localities of Uttaranchal state					
	Haridwar			Dehradun		
	Agricultural	Garden	Road	Agricultural	Garden	Road
May to June	17.6	13.3	5.6	20.6	16.6	10.0
September to Octber	19.6	14.0	5.3	21.6	17.0	8.6

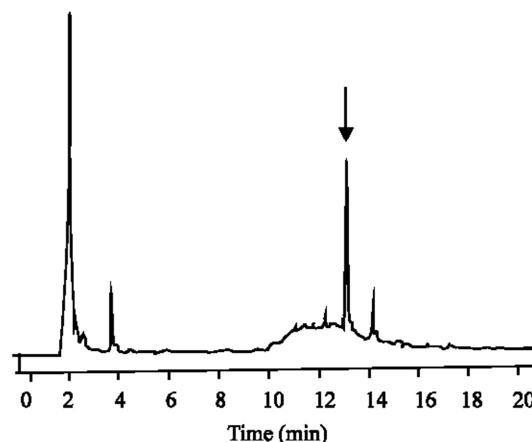

Fig. 1: HPLC analysis is showing penicillin G peak (retention time-13.2 min) in the crude fungal cultural filtrate of *P. chrysogenum* soil isolates

Identification and confirmation of antibacterial substance of *P. chrysogenum*: HPLC analysis of the crude fungal cultural filtrate of *P. chrysogenum* isolates was performed to confirm the identity of the antibacterial metabolite as Penicillin G. A peak with retention time of 13.2 min corresponds to the control sample of penicillin G was observed in the crude fungal cultural filtrates (Fig. 1). Therefore, it was concluded that hat *P. chrysogenum* produces authentic penicillin G antibiotic and it's been confirmed by penicillinase activity test, as well.

Quantitative determination of penicillin G: It is well recognized that HPLC is the most recommended and highly sensitive technique for the quantitative

Table 3: HPLC analysis for penicillin G production by *Penicillium chrysogenum* isolates

	Positive isolates of *P. chrysogenum* from different soil for penicillin G production during different period of year, 2002						Total positive isolates of *P. chrysogenum*	
	Agricultural		Garden		Road			
	Hrd	Dhr	Hrd	Dhr	Hrd	Dhr	Hrd	Dhr
May to June								
Number	26.00	37.00	17.00	32.00	09.00	12.00	52.00	81.00
Percentage	92.85	94.87	89.47	94.11	81.81	66.66	89.65	89.01
September to October								
Number	34.00	43.00	19.00	37.00	10.00	16.00	63.00	96.00
Percentage	91.89	95.55	82.60	92.50	71.42	76.19	89.13	90.56

Hrd – Haridwar, Dhr – Dehradun

Table 4: Recovery of penicillin G from different soil isolates of *P. chrysogenum*

	Penicillin G production (in %) in different period from both districts							
	May to June				September to October			
	Haridwar		Dehradun		Haridwar		Dehradun	
Types of soils	Recovery	CV*	Recovery	CV*	Recovery	CV*	Recovery	CV*
Agricultural	92.96	1.89	95.15	1.89	94.56	1.14	96.44	1.06
Garden	87.80	1.64	91.53	1.22	89.04	1.28	92.10	1.20
Road	80.51	1.56	84.60	1.90	80.44	1.41	83.44	1.23

*Coefficient of variation (in %)

determination of the penicillin G in different natural sources.

Table 3 shows that maximum isolates of *P. chrysogenum* able to produce penicillin G i.e. 91.89 to 95.55% from agriculture, 82.60 to 94.11% from garden and 66.66 to 81.81% from road soil. Agricultural soil shows higher percentage of penicillin G producing isolates as compared to garden and road soil samples. In the month of May-June, there is no significant difference between Haridwar (89.65%) and Dehradun (89.01%) soil isolates of *P. chrysogenum* for the production of penicillin G. Overall Dehradun district shows maximum percentage (90.56%) of *P. chrysogenum* isolates, which are able to produce the penicillin G in the month of September-October (Table 3). Potential isolates of *P. chrysogenum* were prevalent in agriculture soil of both district and duration.

Penicillin G recovery from *P. chrysogenum* isolates: During both periods, there is no significant difference in the overall recovery of penicillin G from both districts soil isolates. While Dehradun isolates *of P. chrysogenum* are showing better recovery of penicillin G than Haridwar isolates. Agricultural soil isolates of both districts indicates more recovery of penicillin G, as compared to garden and road soil during both periods I. e. May-June, 02 (92.96% in Haridwar and 95.15% in Dehradun) and September-October (94.56% in Haridwar and 96.44% in Dehradun) (Table 4).

DISCUSSION

P. chrysogenum is present in abundance and it is commonly isolated from soil, as a natural source[6]. *P. chrysogenum* is a well-known penicillin producing fungus and has medical and industrial importance[18,19]. In present study, we isolated and screened potential *P. chrysogenum* from different soils of Uttaranchal State, India. Antibacterial activity of 329 isolates of *P. chrysogenum* was tested qualitatively by disk-diffusion method. Quantitative measurement and confirmation of penicillin G production was done by HPLC.

P. chrysogenum is not only species of genus *Penicillium*, which is capable to produce the penicillin antibiotic. Several researchers have reported that other species of *Penicillium* are also able to produce penicillin such as *P. notatum*, *P. nalgiovense*, *P. dipodomys*, *P. griseofulvum* and *P. flavigenum*[20-22]. Besides these, few species of *Penicillium* have been reported that does not produce penicillin such as *P. verrucosum*. However, the antibacterial activity observed with *P. verrucosum,* which may be due to the production of secondary metabolites such as patulin and penicillic acid.[23]. Laich *et al.*[22] reported that three genes (*pcb*AB, *pcb*C and *pen*DE) are responsible for the production of penicillin antibiotic in the genus of *Penicillium*[22]. In this study, all isolates of *P. chrysogenum* are not able to produce the penicillin G because those isolates could not have penicillin G producing gene. On the basis of previous reports, *P. chrysogenum*, *P. nalgiovense* and *P. griseofulvum* are most common penicillin producing strains. *P. chrysogenum* is one of them, which is used for the bulk production of penicillin in different industries[19,24].

P. chrysogenum is able to synthesize penicillin with specific hydrophobic side chains, when the appropriate precursor is fed to the production medium[25]. Due to the development of drug-resistance in bacteria, several studies have been conducted with different concentrations of various chemicals to improve the quality of penicillin antibiotic. Several workers tried to improve the potentiality of penicillin regarding its quality and quantity and got some success to solve this problem upto certain level. Some species of *Penicillium* have been frequently isolated from food and food products[26]. *P. chrysogenum* and *P. nalgiovense* are known penicillin producers; the latter has the ability to produce penicillin when it grows on the surface of meat products and secrete it into the medium[27]. Besides that, several workers are working on recombinant strains to enhance the production of penicillin[3,22,28]. The results of the present study indicate that there is no need for the major changes in penicillin producing medium and strains of

P. chrysogenum. The present study shows that soil is a tremendous approach for improvement in the quality of penicillin G producing fungus, *P. chrysogenum.* On the basis of geographical distribution, we found that higher altitude affects the potentiality of penicillin production. Dehradun district soil has higher number of potential isolates of *P. chrysogenum* than Haridwar soil, as the former one is located at higher altitude.

Recently, HPLC technique for the analysis of antibiotics have been developed and also applied to the determination of residual antibiotics in foods[3,17,22,29]. In previous studies, workers established HPLC method for determination of residual penicillin G in animal tissues as an on-line concentration and purification system and successfully applied it to analysis cattle liver, kidney and muscle tissues[22]. A method for the HPLC determination of various penicillin's have been well stabilized that has several advantages and HPLC system is a rapid, sensitive and selective method for determination of penicillin antibiotic. We used HPLC in this study for quantitative analysis and confirmation of penicillin G, which was produced by soil isolates of *P. chrysogenum.*

The present study was designed for the isolation of potential isolates of *P. chrysogenum* from natural source. Different soils of Haridwar and Dehradun districts were selected as sample sites for the isolation of *P. chrysogenum* from Uttaranchal State of India. This study indicates that soil is the most suitable and appropriate natural source for the potential strains of *P. chrysogenum*.

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