JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2011. Vol. **5**(1), p. 293-299

Studies on Antibacterial Efficacy of NTG Treated and UV Irradiated *Penicillium chrysogenum* Strains Isolated from Marsh Soils of Tamilnadu

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(Received: 18 June 2010; accepted: 04 August 2010)

The present study was designed to a systematic screening for the potential isolates of *Penicillium chrysogenum* strains from the marsh soils of Tamilnadu and their antibacterial efficacy by strain improvement. In the present study, antimicrobial tests were conducted for seven species of bacteria (*Staphylococcus aureus, Streptococcus pneumoniae, Bacillus subtilis, Pseudomonas aeurginosa, Salmonella typhii, Escherichia coli and Klebsiella pneumoniae*) and found all are susceptible for penicillin produced by the three isolated strains of *P. chrysogenum* (MPPS1, MPPS2 and MPPS3), under control, NTG treated and UV irradiated conditions. The mean zone diameter for *Staphylococcus aureus* was ranging from 16.20 ± 0.35mm to 20.30±0.45mm in the control, from 19.32±1.17mm to 21.20±0.45mm in the NTG treated cultures and from 18.20±0.22mm to 21.20±0.45mm in the UV treated culture. The susceptibility range of *Streptococcus pneumoniae* was between 10.22±0.35mm and 15.43±0.39mm in the control, 14.40±0.65mm and 17.80±0.39mm in the NTG treated strains and 13.12±1.17mm and 15.36±1.17mm in the UV treated cultures. Larger inhibition zones were observed for *Bacillus subtilis*, (16.36±0.39mm to 22.00±0.40mm) in the NTG treated cultures followed by UV radiated strains (14.10±0.65mm to20.20±0.39mm). The maximum diameter of inhibition zone was recorded for *Pseudomonas aeruginosa* with the UV treated *P. chrysogenum* MPPS2 strains (11.21±0.22mm). *Salmonella typhii* was found more susceptible to *P. chrysogenum* MPPS2 strain treated with NTG (14.30±0.17mm) followed by the same strain, treated with UV radiation (13.30±1.17mm). A low susceptibility rate was observed for *E.coli* in all the antimicrobial effect experiments. Higher susceptibility rates were recorded for *Klebsiella pneumoniae* with all the 3 strains of *P. chrysogenum* treated with NTG (*P.chrysogenum* MPPS1: 9.40±0.72mm;MPPS2: 12.10±0.50mm;MPPS3: 6.30±0.39mm).

Key words: *Penicillium chrysogenum,* UV Irradiated, NTG, Antibacterial efficacy.

The competitive fungal species utilize their toxic metabolites or antibiotics for their establishment in soil, hence providing un-exhausted source of antibiotic (Qureshi, 2003). Since the discovery of penicillin, a number of antibiotics have been discovered mostly from soil inhabiting microorganisms. It is clear that the industrial microbiology field has utilized only a very minor portion of nature's microbial arsenal for the discovery of useful molecules. A number of antibacterial drugs and secondary metabolites have been reported for their sensitivity against broad range of pathogenic microbes (Fernandez *et al.*, 2003). Several penicillin group antibiotics with various chemical structures are widely used to treat infectious diseases in both of humans and animals

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(Puig *et al.,* 2006; Troy, 2006). Penicillin G produced by *Penicillium chrysogenum* is considered as a member of β-Lactam antibiotic family and researchers have reported its antibacterial activity against gram-positive bacteria (Theilagaard *et al.,* 2001). Penicillin works against gram-positive bacteria, such as *Staphylococcus* and *Pneumococcus* by disrupting bacterial cell wall synthesis. Six organisms, coagulase-negative *staphylococci*, *Staphylococcus aureus*, *Enterobacter faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter faecium* are accounted for >80% of human blood culture isolates (Diekema *et al.,* 2000). Induced mutation has been applied for strain improvement in industrial microbiology for many years. To date, the most dramatic practical success of mutagenesis has been in the pharmaceutical industry with specific interest on antibiotic production. Mutation is often suggested as a feasible method for obtaining new and/or improved strains (Jagger, 1961). Ultraviolet light (UV) induced mutagenicity appears to be pertinent to strain improvement. Ultraviolet light (UV) produces various mutagenic effects and is considered a priority mutagen (Hamkalo Swenson, 1969). N- nitro – N-nitrosoguanidine (nitrosoguanidine) (NTG), used widely by bacterial geneticists since an initial report by Mandell and Green berg (1960) as the most potent chemical mutagen .

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.* The coastal soils of India (Kumari *et al.,* 2005), especially Tamilnadu (Kathiresan and Manivannan, 2006) potentially have enormous biodiversity of *Penicillium* sp. However, they have not been extensively explored to identify the commercially viable species of *P. chrysogenum* strains and to evaluate their ability to form penicillin (Jayashree *et al.,* 1999). 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 study has been designed to investigate the potential strains *P. chrysogenum* from different soil samples and determines the qualitative and quantitative assays for its antibacterial activity by strain improvement.

MATERIAL AND METHODS

Isolation of *P. chrysogenum*

Total 236 soil isolates of *Penicillium chrysogenum* are obtained at 10^{-5} dilution and identified from 3 different soil types collected from coastal mangrove Marshes 82(34.74%), Off-shore mangrove marshes 78(33.05%), and agricultural habitat 76 (32.20%) of Muthupettai mangrove forests occurring on the fringes of shallow lagoons of brackish water of Bay of Bengal in Tamil Nadu. All samples are taken into the laboratory and processed on the same day. Total 50 soil samples are collected and processed for isolation of *Penicillium chrysogenum* by dilution-plate method. Aliquots of 0.1ml of each serially diluted soil sample are spread on the plates (in duplicate) using bent glass rod over the entire surface of Potato Dextrose Agar (PDA) containing streptomycin. All plates were incubated at 25±1°C for 7 days; the plates are examined at daily interval. Colonies of *P. chrysogenum* isolates are selected, identified and maintained on PDA medium.

UV Mutation

The source of ultraviolet (UV) radiation (253 nm) is a low-pressure mercury arc lamp, model PCQ-XI. Obtained from ultraviolet products, a dose rate of 300 to 400 UV cmn^2 Sec^{-1} was used for all UV-radiation experiments. The intensity measurements were determined with the aid of a UV intensity meter obtained from UV products. Inc. Mutagenesis by ultraviolet irradiation (Saha and Bhattacharyya, 1990).

NTG Mutation

The mutation protocol is adapted from those used by Muthurayar *et al., (*2006). N^1-nitro-N-nitrosoguanidine (NTG) is obtained from Chemical Company. The routine technique used a concentration of 0.025M NTG and an exposure of the culture for periods ranging from 0 to 15 min. *P. chrysogenum* strains (MPPS1, MPPS2 and MPPS3) are grown in submerged culture for 7 days. Nine ml of cultured broth is transferred to a sterile centrifuge tube and added 1ml of mutagen solution. The mutagen solution is freshly prepared by dissolving 5mg of N^1-nitro-N^1-nitrosoguanidine (NTG) in 20ml of sterile distilled water. The final concentration of the mutagen solution is 25µg/ml of solution in the centrifuge tube. The reaction tube is incubated at 24°C and 220 rpm for 5 min.

Using a pin device, the mutagenized culture is transferred to NL-406 agar plates, which are incubated at 24°C until colonies appeared.

Bioassay for antibacterial substance of *P. chrysogenum* isolates

Bacterial test strain

Staphylococcus aureus was obtained from MTCC, IMTECH, Chandigarh (India). Bacterial strain was grown on nutrient broth at 37°C for 24 h. Then the test organisms to determine the activity of antibacterial substance in crude fungal cultural filtrates by Agar-diffusion method.

Cup Plate Method

The antimicrobial activity of the crude penicillin against 7 bacterial species was test tested using cup plate method. In this method sterilized petriplates were prepared with an equal thickness of Muller Hinton Agar (MAA). Test organisms were grown at 37°C in MH broth to achieve turbidity equivalent to a 0.5 Mac Farland standard (approximately 1-1.5 x 10^8 organism g/ml). This broth culture was used for seeding the agar plates. Then cups with 6mm in diameter were created in seeded agar with appropriate spaces (2cm) (Qaziasgar and Kermanshahi, 2008). Ten µl of the pure antimicrobial agent were added to each cup respectively, then plates were placed in 4°C for 1-2 hrs and finally after 24 hrs incubation at 37°C, the zone of inhibition were measured (Forbes *et al.*, 2002).

Statistical Analysis

Random sampling was used for the entire test in triplicates. Calculations were carried out in triplicate with their mean values and standard error by using the formula given by Gupta (1977), Jayachandran *et al.*, (2008). Positivity index was calculated by comparing the zone of inhibition of Test organisms with standard antibiotics.

$$\text{Activity Index} = \frac{\text{Inhibition area of test sample}}{\text{Inhibition area of standard antibiotic}}$$

RESULTS AND DISCUSSION

The antimicrobial effect of *P. chrysogenum* treated with UV radiation and N-nitro-N-nitrosoguamidine are given as Ranges and means of zone of diameters for each of the *P. chrysogenum* strain and bacteria species are presented in Tables 1, 2, and 3 and the percent sensitivity of bacterial species to different strains of *P.chrysogenum* are shown in Figures 1, 2, and 3. Bacterial species were susceptible to penicillin, as indicated by the zone of inhibition (diameter mm). All the bacterial species tested (*Staphylococcus aureus, Streptococcus pneumoniae, Bacillus subtilis, Pseudomonas aeuroginosa, Salmonella typhii, Escherichia coli* and *Klebsiella pneumonia)* were susceptible to penicillin.

Penicillin from all the *P. chrysogenum* strains was more effective against *Staphylococcus aureus.* The mean zone diameter was ranging from 16.20 ± 0.35mm to 20.30±0.45mm in the control, from 19.32±1.17mm to 21.20±0.45mm in the NTG treated cultures and from 18.20±0.22mm to 21.20±0.45mm in the UV treated culture. NTG treated showed cultured high sensitivity (96.3%) towards *Staphylococcus aureus*.

The susceptibility range of *Streptococcus pneumoniae* was between 10.22±0.35mm and 15.43±0.39mm in the control, 14.40±0.65mm and 17.80±0.39mm in the NTG treated strains of *P. chrysogenum* and 13.12±1.17mm and 15.36±1.17mm in the UV treated cultures. Sensitivity was higher (98.1%) in the NTG treated *P. chrysogenum* MPPS2 strains.

Bacillus subtilis was more susceptible to penicillin with an increase in the diameter of inhibition zones produced in all the 3 strains of *P. chrysogenum*. Larger inhibition zones were observed, which were ranging from 16.36±0.39mm to 22.00±0.40mm in the NTG treated cultures, followed by UV radiated *P. chrysogenum* strains (range: 14.10±0.65mm to20.20±0.39mm) and control culture (range: 11.30±0.22mm to 16.32±0.47mm). NTG treated *P. chrysogenum* MPPS2 strains exhibited higher sensitivity to *Bacillus subtilis* (97.3%).

Invariably, all the 3 strains of *P. chrysogenum* showed declined level of antimicrobial susceptibility to *Pseudomonas aeruginosa*. The maximum diameter of inhibition zone was recorded with the UV treated *P. chrysogenum* MPPS2 strains (11.21±0.22mm) and the minimum was found with the *P. chrysogenum* MPPS1 strain cultured in the control (6.10±0.17mm). A moderate sensitivity level (66.3%) was observed in the UV treated *P. chrysogenum* MPPS2 strain.

Salmonella typhii was found more

susceptible to *P. chrysogenum* MPPS2 strain treated with NTG (Zone diameter: 14.30±0.17mm) followed by the same strain, treated with UV radiation (zone diameter: 13.30±1.17mm) and the control culture (zone diameter:10.30±0.25mm). Lowest susceptibility was observed in the control

Table 1. Antimicrobial activity of the culture filtrate of *P. chrysogenum* strains, determined by agar well diffusion method

Bacteria	Inhibition zone (mm)*			
	MPPS 1	MPPS 2	MPPS3	Penicillin G
Staphylococcus aureus	18.10 ± 0.25	20.30 ± 0.45	16.20 ± 0.35	24.40 ± 0.35
Streptococcus pneumoniae	12.36 ± 0.50	15.43 ± 0.39	10.22 ± 0.35	20.80 ± 0.45
Bacillus subtilis	14.50 ± 1.17	16.32 ± 0.47	11.30 ± 0.22	22.60 ± 0.40
Pseudomonas aeuroginosa	6.10 ± 0.17	8.20 ± 0.39	6.20 ± 0.50	16.90 ± 0.47
Salmonella typhii	7.26 ± 0.06	10.30 ± 0.25	6.10 ± 0.50	19.70 ± 0.35
Escherichia coli	6.10 ± 0.17	9.20 ± 0.25	6.20 ± 0.67	10.10 ± 0.25
Klebsiella pneumoniae	6.36 ± 0.39	9.10 ± 0.72	6.10 ± 0.17	17.60 ± 0.71

* Values are the mean of 3 replicates; values are shown in mean ± SD

Table 2. Antimicrobial activity of the culture filtrate of *P. chrysogenum* mutant (NTG mutated), determined by agar well diffusion method

Bacteria	Inhibition zone (mm)*			
	MPPS 1	MPPS 2	MPPS3	Penicillin G
Staphylococcus aureus	21.20 ± 0.45	23.50 ± 0.50	19.32 ± 1.17	24.40 ± 0.35
Streptococcus pneumoniae	17.30 ± 0.39	20.40 ± 0.89	14.40 ± 0.65	20.80 ± 0.45
Bacillus subtilis	19.20 ± 0.72	20.00 ± 0.40	16.36 ± 0.39	22.60 ± 0.40
Pseudomonas aeuroginosa	8.33 ± 0.39	10.43 ± 0.22	9.20 ± 0.50	16.90 ± 0.47
Salmonella typhii	10.52 ± 0.87	14.30 ± 0.17	8.26 ± 0.39	19.70 ± 0.35
Escherichia coli	8.60 ± 0.39	9.00 ± 0.50	6.10 ± 0.06	10.10 ± 0.25
Klebsiella pneumoniae	9.40 ± 0.72	12.10 ± 0.50	6.30 ± 0.39	17.60 ± 0.71

* Values are the mean of 3 replicates; values are shown in mean ± SD

Table 3. Antimicrobial activity of the culture filtrate of *P. chrysogenum* mutant (U.V. rays mutated), determined by agar well diffusion method

Bacteria	Inhibition zone (mm)*			
	MPPS 1	MPPS 2	MPPS3	Penicillin G
Staphylococcus aureus	21.20 ± 0.45	23.10 ± 0.50	18.20 ± 0.22	24.40 ± 0.35
Streptococcus pneumoniae	15.36 ± 1.17	18.30 ± 0.22	13.12 ± 1.17	20.80 ± 0.45
Bacillus subtilis	17.30 ± 0.39	20.20 ± 0.39	14.10 ± 0.65	22.60 ± 0.40
Pseudomonas aeuroginosa	8.10 ± 0.39	11.21 ± 0.22	8.33 ± 0.39	16.90 ± 0.47
Salmonella typhii	10.20 ± 0.22	13.30 ± 1.17	8.10 ± 0.39	19.70 ± 0.35
Escherichia coli	7.23 ± 0.06	9.20 ± 0.50	6.23 ± 0.50	10.10 ± 0.25
Klebsiella pneumoniae	7.10 ± 0.17	11.10 ± 0.17	6.20 ± 0.50	17.60 ± 0.71

* Values are the mean of 3 replicates; values are shown in mean ± SD

culture of *P. chrysogenum* MPPS1 strain (7.26±0.06mm). The perent sensitivity (72.5%) was higher with the *P. chrysogenum* MPPS2 strain.

A low susceptibility rate was observed for *E.coli* by the standard penicillin G antibiotic in a controlled experiment (zone diameter: 10.10±0.25mm). The same trend is followed in all the antimicrobial effect experiments carried out in the present study, with all the experimental *P. chrysogenum* strains under all experimental conditions. The maximum size of inhibition zone diameter was recorded (9.60±0.50mm) with the *P. chrysogenum* MPPS2 strain treated with NTG and the minimum in *P.chrysogenum* MPPS1 strain under control culture (6.10±0.17mm). A higher sensitivity (89.1%) for *E. coli* was observed in *P. chrysogenum*

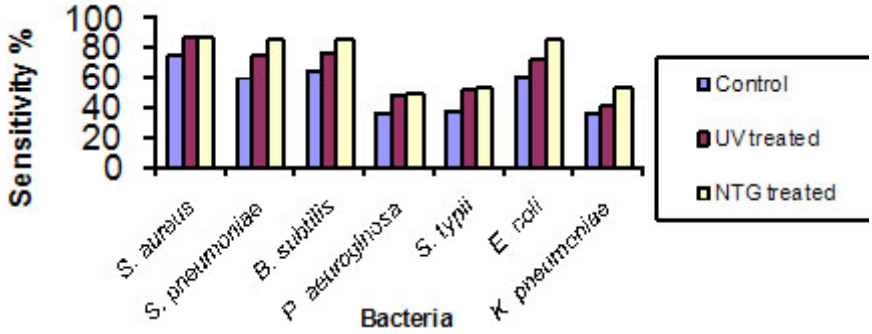

Fig. 1. The percent sensitivity of bacterial species to different strains of *P. chrysogenum* MPPS 1

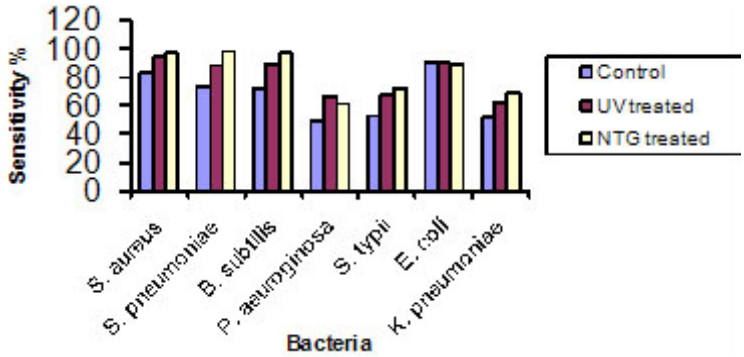

Fig. 2. The percent sensitivity of bacterial species to different strains of *P. chrysogenum* MPPS 2

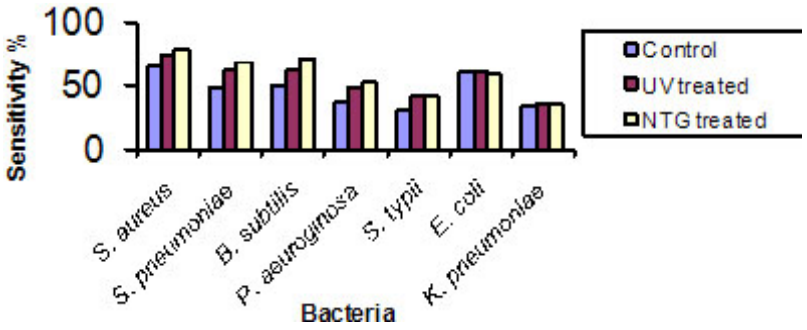

Fig. 3. The percent sensitivity of bacterial species to different strains of *P. hrysogenum*MPPS 3

MPPS2 strain treated with NTG.

Higher susceptibility rates were recorded for *Klebsiella pneumoniae* with all the 3 strains of *P. chrysogenum* treated with NTG (*P.chrysogenum* MPPS1: 9.40±0.72mm;MPPS2: 12.10±0.50 mm; MPPS3: 6.30±0.39mm). Low susceptibility was observed in the control cultures (*P. chrysogenum* MPPS1:6.36±0.39mm; MPPS2:9.10±0.72; MPPS3:6.10±0.17mm). NTG treated *P. chrysogenum* MPPS2 strain expressed higher sensitivity (68.7%) to *K. pneumoniae*.

Thus, the present research confirms the enhanced antibacterial activity of the *P.chrysogenum* strains, after strain improvement, against both Gram-positive and Gram-negative Bacterial species. The methicillin resistant *Staphylococcus aureus* (MRSA) is found susceptible to penicillin produced by the newly isolated *P.chrysogenum* strains. Both the gram-negative rods, including *Klebsiella pneumoniae* and *Escherichia coli*, which are producing extended-spectrum β-lactamases (ESBLs), are also found susceptible to the penicillium. *P. aeruginosa*, which is known as resistant to a wide range of antimicrobial drugs (Senda *et al.*, 1996) is also observed susceptible (68.3%) to penicillin produced by *P.chrysogenum* MPPS2. In view of the problems of antimicrobial resistance of several pathogens encountered globally, laboratories should now screen such pathogenic organisms at least for penicillin susceptibility. Among the most clinically important gram-positive species including pneumococci, and gram negative bacilli such as *E. coli*, susceptibility to penicillin appears to have changed little (Karlowsky, 2002). Recently, susceptibilities of pathogens to some classes of antimicrobials are decreasing. In Europe and the United States, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE) have been widely disseminated in many medical institutions; gram-negative rods, including *Klebsiella pneumoniae* and *Escherichia coli* producing extended-spectrum ß-lactamases (ESBLs), are also becoming a clinical threat. *Pseudomonas aeruginosa* is resistant to a wide range of antimicrobial drugs (Senda *et al.*, 1996). The overall isolation frequency of penicillin-intermediate *Streptococcus pneumoniae* and penicillin-resistant *Streptococcus pneumoniae* is estimated to be approximately 50%, according to reports from individual hospitals and health districts in Japan (Oguri *et al.*, 2000).

ACKNOWLEDGMENTS

The authors are thankful to the management of A.V.V.M. Sri Pushpam College (Autonomous), Poondi – 613 503, for providing the necessary facilities for my research programme in the college of excellence.

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