Penicillin G acylase producing bacteria isolated from forest soil

Arjun. J K[1], Aneesh. B[2], Kavitha. T[3], Harikrishnan. K[4]

Environmental Biology Laboratory, Rajiv Gandhi Centre for Biotechnology (RGCB), Poojappura, Thycaud P.O., Thiruvananthapuram, Kerala, 695014, India.

Abstract *— The development of resistance by microorganisms to penicillin G and other β lactam antibiotics has prompted the search for new β lactam antibiotics. Microorganisms are an important source of penicillin G acylase (PGA) (EC 3.5.1.11), which hydrolyses penicillins to 6-amino penicillanic acid (6-APA) and 7-amino des-acetoxy cephalosporanic acid (7-ADCA), the β lactam antibiotic intermediates which are widely used by the pharmaceutical industries as the starting material for the manufacture of several semi synthetic antibiotics. In this study penicillin g acylase producing bacterial strains were isolated from the forest soil in Western Ghats. The isolated bacterial strains were screened for PGA production and the positive strain (RG_PGA 269) showing the highest activity was selected for further studies. The bacterial strain showed 2.22 U/ml enzyme activities. The presence of PGA gene in the isolate was confirmed by PCR using the gene specific primers. Molecular identification of the strain was done by 16 S rRNA gene amplification, sequencing and homology analysis of the sequence using NCBI BLAST. The Phylogenetic analysis of the strain was studied along with selected sequences from database and found that the isolate formed cluster with Bacillus megaterium.*

Keywords— *PGA, 6-APA, β lactam antibiotics, 16s rRNA gene, NCBI BLAST.*

I. INTRODUCTION

The enzyme penicillin G acylase (PGA; EC 3.5.1.11) is a heterodimeric protein consisting of a small α subunit and a large β subunit, which are formed by the processing of a single polypeptide precursor [1]. PGA is a hydrolytic enzyme that acts on the side chains of penicillin G, cephalosporin G and related antibiotics to produce the β lactam antibiotic intermediates 6-amino penicillanic acid (6-APA) and 7-amino des-acetoxy cephalosporanic acid (7-ADCA), with phenyl acetic acid (PAA) as a common by-product [2, 3]. These antibiotic intermediates are among the potential building blocks of semi-synthetic penicillins (ampicillin, amoxicillin, cloxacillin, salbactum) and cephalosporins (cephalexin, cephadroxil) [4].

β- Lactam antibiotics, in particular penicillins and cephalosporins, represent one of the world's major biotechnology markets which constitute ~ 65% of the total antibiotics market. β- Lactam antibiotics alone constitute most of the world's antibiotic sales: 3×10^7 kg/year out of a total 5×10^7 kg/year produced worldwide. Therefore, the annual consumption of PGA is estimated to be in the range of 10–30 million tons [5]. The development of resistance by microorganisms to penicillin G and other β lactam antibiotics has prompted the search for new β lactam antibiotics. Due to the rising interest in sustainable development and environmentally friendly practices, microbial enzyme transformation processes are generally preferred over the conventional chemical conversion process. PGA-mediated conversion of β-lactam antibiotics provides a novel direction for antibiotics industries and promotes a safer and cleaner environment. Apart from β-lactam hydrolysis, recent developments have resulted in multiple applications of PGA, including peptide synthesis, resolution of racemic mixture, enantio selective acylation, etc [6].

The characteristics of PGA isolated from different biological and environmental sources were found to be varied in different aspects including, substrate specificity, optimum pH, temperature tolerance etc. Therefore, microorganisms have been extensively screened for isolation of novel penicillin acylases with higher compatibility with industrial deacylation requirements. In recent years, recombinant DNA technology has emerged as a potent technology for high level production of many useful proteins which has potential applications in pharmaceutical industries [7].

Penicillin acylases are widely distributed among many microorganisms, including bacteria, actinomycetes, yeasts, and fungi. Production of PGA by recombinant technology could have several advantages like higher expression level and the expressions of PGA in recombinants can be controlled effectively [8].

In *Escherichia coli*, PGA is a periplasmic heterodimeric protein consists of α and β subunits. The mature protein is synthesized as a single-polypeptide cytoplasmic precursor which consists of an amino acid signal peptide (24 amino acid) and a connector peptide (54-amino acid) that joins the α and β chains. The PGA precursor is auto catalytically processed inside the bacterial periplasm to remove the spacer peptide and produce the mature enzyme [9]. Because PGA expression in *Escherichia coli* is limited

by precursor secretion and maturation, much work has been reported on strategies aimed to improve the expression level of hetero dimeric PGA [10].

The objective of this study was to screen and isolate PGA producing microbial population from the forest top soil of Western Ghats of Kerala. The forests of Kerala are known to be biodiversity hotspots. The microbial resources in these environments are yet to be exploited. Considering the importance of PGA in pharmaceutical industry for the production of novel antibiotics to combat diseases in the ever increasing era of antibiotic resistance among pathogenic bacteria, it is worthwhile to search for novel PGA producers form the environment and to exploit their potential for the welfare of mankind.

II. MATERIALS AND METHODS

A. Collection of soil sample and screening for Penicillin G Acylase

Soil samples were collected in sterile containers, from the forests of Wayanad District, Kerala, India. The samples were aseptically transported at refrigerated condition and permanently stored at -20^{0}C. PGA producing strains were screened from the soil samples as previously described [11]. Soil dilutions (10^{-1} to 10^{-6}) were plated on nutrient agar plates containing 0.15% phenyl acetic acid and incubated approximately for 24 h at 35^{0}C to develop colonies and the plates were overlaid with soft agar containing penicillin G (20 mg/mL) and Serratia marcescens, a bacterial strain resistant to penicillin G but sensitive to 6-APA. The plates were incubated at 35^{0}C for approximately 24 h. PGA-producing strains exhibited a clear zone, since the 6-APA arising from the conversion of penicillin G inhibited the growth of *Serratia marcescens*.

B. Penicillin G acylase enzyme assay

The six bacterial strains which showed clearing zones while screening were inoculated in nutrient medium supplemented with 0.15%phenyl acetic acid and incubated for 24 hrs. Nutrient agar plates supplemented with penicillin G (20 mg/mL) were prepared and plated with *Serratia marcescens* and a well were cut in the centre of the plate. 200µL of the culture supernatant from the 24 h culture was poured in the agar-well plates and incubated at 35^{0}C for 24 h. Clearing zone around the well indicate the presence of PGA in culture filtrate and the 6-APA produced by the hydrolysis of penicillin inhibits the growth of S. marcescens. Among the six bacterial isolates exhibiting PGA activity during the well plate assay, the isolate RG_PGA 269 which was showing the largest clearing zone was selected for further studies. The selected bacterial isolate was inoculated into the standard growth medium along with suitable controls and incubated at 35^{0}C for 14 h. The culture supernatants were collected at 2 h intervals and the PGA activity was assayed using penicillin G (20 mg/ mL) as the substrate and incubation for 30 minutes at 50^{0}C [12]. 6-APA produced was measured after incubation by reacting 0.5 mL of the mixture with 3.5 mL of reagent solution (0.5 mL of 0.5% p-dimethylaminobenzaldehyde in methanol, 2 mL of 20% glacial acetic acid and 1 mL of 0.05 M NaOH). The assay was based on the principle that the PGA enzyme produced by the bacteria hydrolyzes the substrate Penicillin G in the culture medium producing 6-APA which was measured spectrophotometrically at 415 nm [13]. The amount of 6-APA produced was estimated by substituting the Optical density values with the values of 6-APA standard from the standard graph. According to International Union of Biochemistry one enzyme unit equals 1 µM of the substrate hydrolyzed per minute. For penicillin G acylase, the activity was defined as the amount of enzyme required to hydrolyze penicillin G and to release 1µM of 6-APA per hour under the standard assay condition.

C. Thin Layer Chromatography

TLC was performed to detect the hydrolysis products by the PGA activity in silica gel plate (Merck, silica gel 60 F254.). Culture supernatant of RG_PGA 269 after 24 h of incubation was assayed using penicillin G (20mg/mL) as substrate for 30 minutes (at 50^0 C). 25 µL of the reaction mixture was spotted on the TLC plates along with the standards penicillin G and 6-APA and dried. The plates were then kept in the solvent system (acetone: glacial acetic acid: water in the ratio 95:5:10). The resolved spots were identified under UV and the retention factor (Rf) was calculated.

D. PCR amplification of PGA gene

The PGA gene was amplified by PCR in a thermal cycler (BioRAD, USA) using gene specific primer pair PGAF1 and PGAR1. Primers were selected based on the conserved regions of PGA gene that have already been reported [14]. The amplification reactions were performed in the following steps initial denaturation at 94^{0}C for 4 min, 31 cycles of 94^{0}C for 45s, annealing 47^{0}C for 30s, and extension at 72^{0}C for 2 min and a final extension of at 72^{0}C for 5mins. The samples were subjected to agarose gel electrophoresis and the amplified PGA bands were observed under UV transilluminator and photographed.

E. PCR amplification of 16S rRNA gene

Amplification of approximately 1,500 bp 16S rRNA was also performed using Eubacterial primers 27f and 1492r [15]. The amplification reactions were performed in the following steps initial denaturation at 94^{0}C for 3 min, 30 cycles of 94^{0}C for 1 min, annealing 54^{0}C for 1 min, and extension at 72^{0}C for 2 min and a final extension of 10 min at 72^{0}C. The samples were subjected to agarose gel electrophoresis and the amplified 16S rRNA gene was observed and photographed.

F. Phylogeny analysis

For the identification of the strain, the amplified 16 S rRNA was sequenced using ABI PRISM Big Dye Terminator V 3.1 cycle sequencing Ready Reaction kit, and the primers 27f and 1492r in ABI3730 automated DNA sequencer (USA). The sequences obtained were viewed with ABI sequence scanner v.1, compiled and aligned using Bio Edit version 7.0.9 software [16] and compared with public database (NCBI BLAST). The phylogeny tree was constructed using Mega V.4 software [17].

III. Results and Discussion

The penicillin G acylase producing bacterial isolates were identified using *Serratia marcescens* overlay technique. While screening around 300 environmental isolates, six of them showed PGA activity. The bacterial strains depicted varying ability for penicillin G degradation represented by the difference in the clearing zone. The bacterial isolates producing penicillin G acylase converts the penicillin G present in the medium to 6-aminopenicillanic acid. The penicillin resistant *Serratia marcescens* is sensitive to 6-aminopenicillanic acid produced and in turn inhibited the growth of S. marcescens and developed clearing zones around the colonies of penicillin G acylase producing bacterial isolates. (Fig 1) Among the six isolates, RG_PGA 269 was found to be the more active producer as depicted by a larger clearing zone and was selected for further characterization.

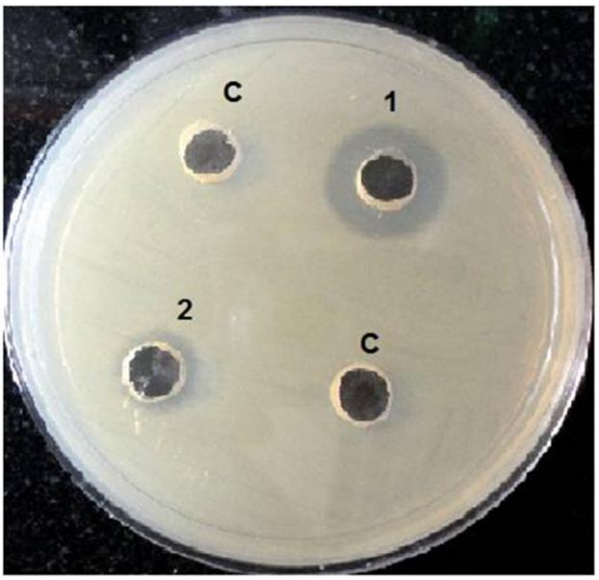

Fig: 1 Serratia overlay method; C- Control, 1-RG_PGA269, 2-RG_PGA69

Penicillin acylase activity was determined by measuring the 6-APA liberated from Penicillin-G. The amount of 6-APA is measured by the intensity of the colour generated by reacting 6-amino penicillanic acid with p-dimethylaminobenzaldehyde to form Schiff's base [18]. The PGA positive isolates were subjected to enzyme activity assay and the results were summarized (Table 1). The enzyme assay of RG_PGA 269 at 2 h interval showed a steady increasing trend during 2 -10 hrs of incubation and reached maximum activity at 10 hrs, 2.22 U/ml. An incubation time beyond 10 hrs showed a steady decline in the enzyme activity during which it reached a value of 1.3 U/ml at 14 hr (Fig 2).

PGA positive strains	Enzyme activity (U/ml)
RG_PGA 16	1.56
RG_PGA 45	1.23
RG_PGA 69	0.86
RG_PGA 116	1.73
RG_PGA 236	1.48
RG_PGA 269	2.22

Table 1: PGA activity of positive strains at 10 hr after incubation

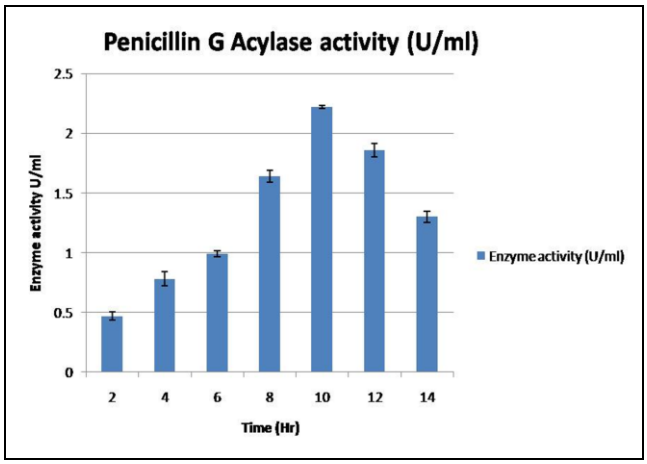

Fig: 2 PGA activity of RG_PGA 269 at different time intervals

The decrease in the enzyme activity may be due to the proteolysis which was reported previously [19]. The presence of the gene responsible for the synthesis of PGA in RG_PGA 269 was further confirmed by PCR which yielded an amplicon of 2.35kb (Fig 3). Similar observations on amplified PGA gene from Bacillus, E coli etc were reported earlier [20].

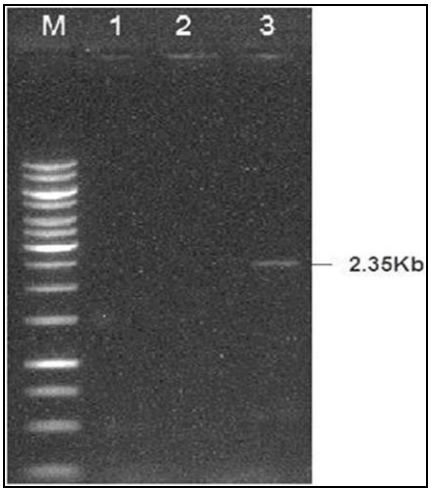

Fig: 3 Lane M -1kb DNA ladder (Fermentas) Lane 1, 2-negative control; lane 3- Full length PGA gene amplified from RG_PGA269

The 6-APA produced during the hydrolysis of the substrate Penicillin G by the enzyme PGA produced by the RG_PGA 269 was detected by Thin Layer Chromatography The spots developed by the standard 6-APA as well as the sample indicated an Rf value of 0.87 and the spot of penicillin G resolved to a higher Rf value of 0.98 (Fig 4). Thin-layer chromatography permits the simple and rapid separation and detection of different spontaneous, chemical and enzymatic degradation products of penicillins and cephalosporin [21]. One dimensional thin-layer chromatography (TLC) detects 6-APA in the presence of intact penicillins and other degradation products and substrate of penicillins have higher Rf values than 6-APA as observed in the present study [22]. The observation on bacterial culture growth and activity of PGA was found to be in perfect synchrony as indicated by increased activity with the increase in culture growth.

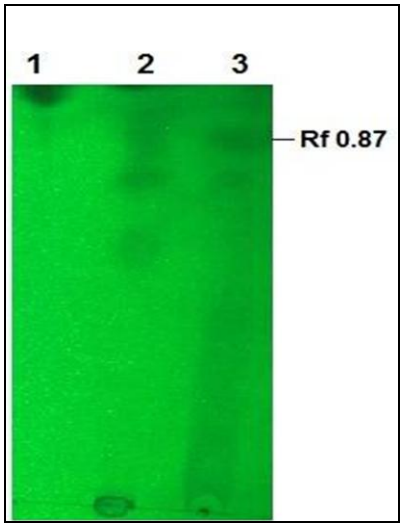

Fig: 4 Thin layer chromatography, Lane 1: Penicillin G (Rf-0.98), Lane 2: 6-APA produced by RG_PGA269, Lane 3: Standard 6-APA (Rf-0.87)

The amplification of 16S rRNA gene of RG_PGA 269 yielded amplicon size of 1538 bp (Fig 5). The 16S rRNA amplicon was sequenced and compared with the sequences in the public database for homology by NCBI BLAST. Phylogenetic tree was constructed using Mega V.4 software and the analysis revealed that the isolate RG_PGA 269 form cluster with Bacillus megaterium. (Fig 6) The Bacillus sp. are known to be an potent source of PGA and there are several reports suggesting that the extracellular production of the enzyme by Bacillus megaterium makes the downstream process much easier and cost effective[23]. The penicillin acylase from Bacillus megaterium is one of the most massive and widely used enzymes in the β lactam antibiotics industry [24]. The results observed that the strain isolated in the resent study from the forest environment can be used s a potent source of the enzyme PGA.

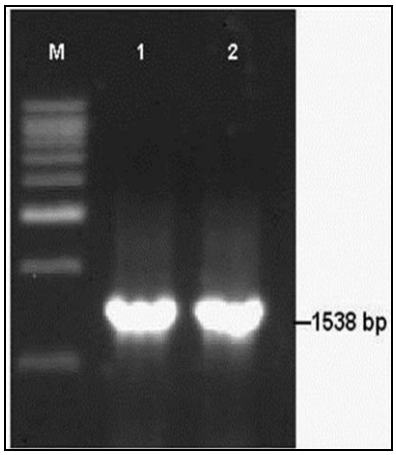

Fig: 3 Lane M -1kb DNA ladder (Genei) Lane 1, 2- 16S rRNA gene amplified from RG_PGA269

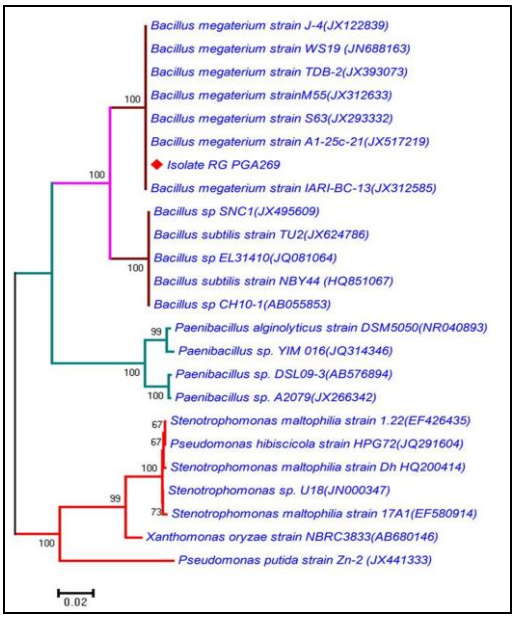

Fig: 6 Phylogeny tree of RG_PGA269 constructed with MEGA V 4.0

IV. CONCLUSION

Biological screening methods have been used for the isolation and identification of PGA producing bacteria from different environments. From our studies it was found that conventional screening methods in conjunction with PCR amplification of gene responsible for production may aid in more accurate and precise screening of PGA producing environmental isolates. PGA is one of the key pharmaceutical enzymes in the production of polysynthetic β lactam antibiotics. To conclude, continuous improvement in PGA production is needed for the desired success of the β lactam antibiotic industry. As the microbial antibiotic resistance continues to increase, it is essential to explore the new sources of PGA residing in the environmental pools, which are still unexplored.

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