Available online at: www.cbioresearch.com/journal.html ISSN No. 0973-8207

Characterization of Soil Associated Antimicrobial Agent Producing Microbes (SAAAPM)

Huxley, V. A. J. * G. Prabhakaran and S. Antony Xavier

Biotech Research Laboratory, Department of Zoology,
Thiru. Vi. Ka. Govt. Arts College, Tiruvarur – 3, Tamilnadu, India
*– Corresponding author E. mail: aldobiotech@yahoo.com

Abstract

The isolation and characterization of Antimicrobial Agent Producing Microbes (AAPM) from soil samples were carried out in the diversified soil ecosystem of kanyakumari dist. Based on the results the MS03 location carries more number of bacteria (79 colonies) than the others. Interestingly, the sandy region has least number of bacteria at a rate of 38. The results of crowd plate method also indicated that the MS03 location has more AAPMs at a rate of 2.5%

Key wards: Soil microbes, antibiotics, pour plate, crowd plate

Introduction

Antibiotics are one of the pillars of modern medicine (Ball *et al.,* 2004), but the rate of loss of efficacy of old antibiotics is outstripping their replacement with new ones for many species of pathogenic bacteria (Hancock, 2007). The emergence of antibiotic resistant bacteria is a problem of growing significance in dermatological and surgical wound infections (Giacometti *et al.,* 2000). In general, the most important resistance problems in the management of wounds have been observed with both Gram-positive and Gram-negative species of bacteria (Filius and Gyssens, 2002).

Soil microbial communities are the most complex, diverse and important assemblages of organisms in the biosphere; and they participate in various biological activities. Accordingly, they are an important source for novel antimicrobial agents and molecules with biotechnological importance (Hackl *et al.,* 2004). Considerable research is being done in order to find new chemotherapeutic agents isolated from soil (Rondon *et al.,* 2000; Crowe and Olsson, 2001; Courtis *et al.,* 2003). Among the soil ecosystem, the microbial populations habituated abundance in the rhizosphere. It is a thin layer of soil adhering to a root system which is rich in microbial diversity. The magnitude of this area depends on the plant and the size of the roots that the plant posses (Rondon *et al.,* 2000; Dakora and Phillips, 2002). New attempts were made to isolate potent novel antimicrobial substances from soil ecosystem of Kanyakumari dist of Tamilnadu.

Materials and methods

Study area

Soil samples were collected from the diversified soil ecosystems like detritus soil, clay soil, rain forest soil and river base soil of Kanyakumari District, Tamilnadu. The soil samples were collected from chosen five different collection sites (these locations were chosen after 120 preliminary antimicrobial surveys). The collection sites and their code and descriptions were given in table – 1.

Table – I. The details of collection sites

Sl. No	Collection sites	Code	Descriptions
1	Pachiparai Dam	MS01	Highly fertile top soil with lot of detritus deposition.
2	Tamiraparani River Basin (Mangad)	MS02	Fertile river base wet soil, black in colour
3	Surulode Rubber estate (forest)	MS03	These Forest soil contains rubber and teak plantations. These region get more rain than the other collection site
4	Ethamozhi Coconut soil	MS04	Sand soil with lots of coconut husk ponds
5	Teeraikal Puthoor paddy field	MS05	Clay soil

Journal of Basic and Applied Biology, Vol. 6, No.3&4, 2012, pp. 121-124

Collection of soil samples

Composite soil samples (2-5 cm depth) were taken from each area in sterile plastic bags and transported to the laboratory under ambient conditions and was air dried at room temperature. The rocks and debris were removed aseptically.

Isolation of antimicrobial agent producing microbes (AAPM) Pour plate and Crowded Plate Technique

Serial dilutions were held out as follows: one gram of soil was diluted in 9 ml of 0.85% physiological saline solution 10^{-1}. The soil suspensions were homogenized by shaking at 200 rpm for 15 minutes at 30°C. After that, serial dilutions were done up to 10. Two repetitions of each of the dilutions were inoculated in Tryptic Soy Agar (TSA) and the cultures (or master plates) were incubated at 30°C for 48 hours. After the incubation period, colonies that presented antagonism were designated as Antimicrobial Agent Producing Microbes (AAPM) and were subcultured and purified by streaking them on a TSA plate. After purification, the isolated AAPM's were preserved and stored at -80°C for further tests. Earlier, one separate set was maintained to count the total bacteria present in that area.

Antibiograms Susceptibility Test with Non-filtrated Supernatant

The Antimicrobial agent producing capability of the isolates was tested by a modification of the Kirby-Bauer method described by Boyle *et al.,* (1979). The target microorganisms used were given in table 2. AAPM's were incubated 24 hours at 30°C and the targets were incubated also for 24 hours at 37°C. For the construction of the antibiograms, 500 µl of the broth culture of AAPM was transferred to a sterile 1.5 ml microtube; the microtubes were centrifuged at 13,000 rpm for 20 minutes. The supernatants were transferred to a sterile microtube.

Physiological saline solutions (0.85%) were used in order to prepare cellular suspensions with a 0.08-0.1 absorbance of each target. Absorbance of the target cellular suspensions was determined by using a spectrophotometer set at 625 nm, which is equivalent to a McFarland standard of 0.5. In order to create a bacterial lawn of the targets in TSA, the spread plate technique was employed by using 200 µl of each target. Sterile paper discs were impregnated with 20 µl of the AAPM's supernatant and placed over the bacterial lawns. A negative control was included by using a sterile disk impregnated with the sterile TSB culture media. Antibiograms were incubated 24-48 hours at 37°C. After this period, inhibition zones were measured with a ruler using a mm scale.

Table 2. Bacterial cultures used for the antibacterial screening

No	Test organisms	Source
1	*Bacillus subtilis*	MTCC
2	*Staphylococcus aureus*	MTCC
3	*Proteus vulgaris*	MTCC
4	*Shigella sp*	MTCC
5	*Salmonella typhi*	MTCC

Susceptibility Testing Using Filtered Supernatant

A modification of the procedure described in crowded plate was used in order to determine if the AAPM secreted an antimicrobial substance constitutively to the medium. The same procedure was repeated but this time the centrifuged cultures of the AAPM's were filtered with a low binding protein cellulose filter with 0.2µm pore size (Fisher), and this filtrate was used for the construction of the antibiograms.

Streak Susceptibility Testing

In vitro antimicrobial assays were also done in order to test if the antimicrobial agent produced by the AAPM was secreted to the culture medium. Individual bacterial lawn was inoculated by adding 100 µl of the AAPM TSB culture and dispersing the culture in half of the TSA surface, these cultures were incubated at 30°C for 48 hours.

Results

The diversified soil samples were collected from five different locations of Kanyakumari district of Tamilnadu. From the above five collection spots, the total cultivable bacterial stains were isolated using standard microbiological methods and the strains were shown in table – 3. Based on the results the MS03 location carries more number of bacteria (79 colonies)

than the others. Interestingly, the sandy region has least number of bacteria at a rate of 38. The results of crowd plate method also indicated that the MS03 location has more AAPMs at a rate of 2.5%. Among the 284 isolates, the capability of antimicrobial production of the isolated microbes was determined by using a modified version of the Kirby Bauer susceptibility test, the radial inhibition, and the streak technique, among others.

Table 3.

Source	No of colonies produced	No of antagonistic bacteria	% of occurrence
MS01	42	1	2.38
MS02	62	-	-
MS03	79	2	2.53
MS04	63	1	1.58
MS05	38	-	-
Total	284	4	1.4

The four antagonists were chosen for further microbiological and biochemical characterization. The isolated and characterized bacteria were *Escherichia coli* (Strain Code: SBI092)*, Pseudomonas aeruginosa* (Strain Code: SBI122), *Klebsiella pneumoniae*, (Strain Code: SBI211) and *Staphylococcus epidermidis* (Strain Code: SBI341). The efficacy was compared with gram positive and negative bacteria and the sensitivity results clearly indicated that these bacteria produced considerable amount of activity above 18 mm zone for more than 80% over the tested isolates. The antibiogram of AAPM against common bacterial isolates were shown in table -4. The result indicated that the filtered supernatant produced more susceptible than the non filtered supernatant. Interestingly the SBI122 and SBI241 isolates produced significant zones than the other groups over all tested microorganisms.

Table – 4.

Bacteria	Non filtered				Filtered supernatant				Streak plate testing			
	SBI	SBI	SBI	SBI	SBI	SBI	SBI	SBI	SBI	SBI	SBI	SBI
	92	122	211	241	92	122	211	241	92	122	211	241
B. subtilis	+	+	+	+	++	+	+	+	+	++	++	+
S. aureus	++	+	+	+	++	+++	++	+	+	+++	+++	+
P. vulgaris	+	+	+	-	+	+	+	+	+	+++	+	+
Shigella sp	+	+	+	+	+	++	++	+	+	+	+	+
S. typhi	+	+	-	-	++	+	+	+	+	++	+	+

+ - megre, ++ - moderate and +++ - good

Discussion

Many groups of soil microorganisms have the ability of synthesizing antimicrobial agents. Pandey *et al*. (2002) states that the top cultivable antimicrobial agent producers present in soils are the actinomycetes. The actinomycetes are a group of gram-positive bacteria that exhibit characteristics of both bacteria and fungi. These microbes produce filamentous structures which agglomerate forming pseudo-mycelia. Another group of gram-positive bacteria present in soil and responsible for the production of antimicrobial agents with clinical and agricultural importance is the genus *Bacillus*. This genera is characterized by being gram-positive, spore forming rods. It has been demonstrated that these microbes produce antimicrobial agents in various stages of their growth curve. For example, *B. subtilis* 168 can produce non ribosomal oligopeptides with antifungal and antimicrobial properties such as surfactins, inturinics and bacilysin (Oskay et *al.,* 2004). *Pseudomonas* encompasses gram-negative rods that have the ability of producing antimicrobial agents in soil. Most of the biomolecules that they produce are of agricultural importance. The antibiotics pyoluteorin (Plt), pyrrolnitrin (Prn), phenazine-1-carboxylic acid (PCA), and 2, 4-diacetylphloroglucinol (Phl) all of them are currently a major focus of research in biological control (Gardener *et al.,* 2000). Strains of *Pseudomonas* have been isolated from soils that exhibited suppressive effects against plant diseases such as take-all of wheat, black root rot of tobacco, *Fusarium* wilt of tomato and damping off of tomatoes caused by *Rhizoctonia solani* (Gardener *et al.,* 2000).

Acknowledgements

The authors are thankful to Prof. Pamela, former principal and Dr. Arivoli for the facilities and encouragement. Help from rendered by Shri. E. Vanakamudi, Museum Keeper. Thiru. Vi. Ka. Govt. arts college, Tiruvarur, during the collection of specimens is thankfully acknowledged.

References

Ball AP, Bartlett JG, Craig WA, Drusano GL, Felmingham D, Garau JA, Klugman K P, Low DE, Mandell LA, Rubinstein E, Tillotson GS (2004). Future trends in antimicrobial chemotherapy: expert opinion on the 43rd ICAAC. J. Chemother.; 16: 419-436.

Courtis S., C. Cappellano, M. Ball, F. Francois, G. Helynck, A. Martizez, S. Kolvek, J. Hopke, M. Osburne, P. August, R. Nalin, M. Guerineau, P. Jeannin, P. Simonet, and J. Prenodet. 2003. Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. Applied and Environmental Microbiology .69:49-55.

Crowe J., and S. Olsson. 2001. Induction of laccase activity in R. solani by antagonistic Pseudomonas flouescens strains and a range of chemical treatments. Applied and Environmental Microbiology.67:2088-2094.

Dakora F., and D. Phillips. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant and Soil. 245:35-47.

Filius PMG and Gyssens IC (2002). Impact of increasing antimicrobial resistance on wound management. Am. J. Clin. Dermatol.; 3 (1): 1-7.

Gardener B., K. Schroeder, S. Kalloger, J. Raaijmakers, L. Thomashow, and D. Weller. 2000. Genotypic and phenotypic diversity of phID-containing Pseudomonas strains isolated form the rhizosphere of wheat. Applied and Environmental Microbiology. 66:1939-1946.

Giacometti A, Cirioni O, Schimizzi AM, Del Prete MS, Barchiesi F, D'Errico MM, Petrelli E, Scalise G (2000). Epidemiology and microbiology of surgical wound infections. J. Clin. Microbiol.; 38 (2): 918-922.

Hackl E., S. Boltenstern, L. Bodrossy, and A. Sessitsch. 2004. Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Applied and Environmental Microbiology. 7:5057-5065

Hancock REW (2007).The end of an era? Nat. Rev. Drug. Discov.; 6: 28.

Oskay M., Ü. Tramer, and C. Azeri. 2004. Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. African Journal of Biotechnology. 9:441-446.

Pandey B., P. Ghimirel, V. Prasad, M. Thomas, Y. Chan, and S. Ozanick. 2002. Studies of the antimicrobial activity of the actinomycetes isolated from the Khumby region of Nepal. Department of Bacteriology, University of Wisconsin— Madison, Madison, Wisconsin

Rondon M., P. August, A. Bettermann, S. Brady, T. MacNeil, C. Minor, C. Tiong, M. Osborne, J. Clardy, J. Handelsman, and R. Goodman. 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Applied and Environmental Microbiology.66:2541-2547.

* Author for correspondence