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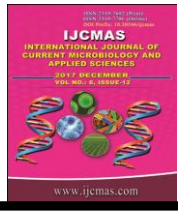

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Pseudomonas and *Proteus*: Partial Purification and Characterization of Antibacterial Protein: A Review

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A B S T R A C T

Marine water samples were collected from a coastal region of Pushpavanam, Nagapattinam District, Tamil Nadu, India. Marine *Pseudomonas* and *Proteus* were isolated and identified according to morphology, biochemical test and selective complex marine growth medium. The identified marine organisms were maintained with LB broth. The cultures were harvested by cooling centrifugation at 9,000x g for 20 min. This suspension was incubated at 28ºC for 24 hours after incubation the cells removed by centrifugation method. The supernatant was filter sterilized and antibacterial activity was zone of inhibition performed by measuring overlay assay technique and drop test assay method. Samples were suspended in sucrose – dye – solution and identification of antibacterial protein band resolved by polyacrylamide gel electrophoresis. The gel was washed with distilled water. The protein fraction was identified by the drop test. All the proteins were assayed with a bicinchoninic acid protein kit. The protein fraction elution was done with phosphate buffer solution. The protein fraction was stored at 20ºC after freeze – drying. The antibacterial protein was detected within SDS-PAGE treatment with Coomassie brilliant blue. The concentration level of antibacterial protein is necessary to result in cell death and to inhibit the growth of marine microorganisms in suitable medium. Bacterial cells were inoculated at time zero and the bacterial growth rates and cell viability were measured by colony forming unit.

Introduction

Marine organisms represent a promising source for natural products of the future due to the incredible diversity of chemical compounds that were isolated. Oceans cover almost 70% of the earth surface and over 90% of volume of its crust (Fenical, 1993). The marine surface environment is a site of intense competition for living space by a wide variety of organisms. Bacteria are generally recognized as primary colonizers of this habitat (Bryers, 1982).

The microbial diversity in the sea is yet to be revealed. In the last few years, marine microorganisms emerged as a new field for the discovery of novel biologically active compounds (Fenical, 1997).

Marine natural products have an exceedingly bright future in the discovery of life saving drugs. The first antibiotic from marine bacterium was identified and characterized (Burkholder *et al.*, 1966).

An increasing number of compounds with antibacterial activity have recently been found to be produced by a variety of organisms present in the marine surface environment. The red alga to produce *Delisea pulchra* has been shown to produce halogenated furanones with activity against representatives of the three major groups of fouling organisms, including invertebrate larvae, macroalgae and bacteria (De Nys *et al.,* 1995).

Bacteria are generally recognized as primary colonizers of this habitat (Bryers and Characklis, 1982) and are able to rapidly form biofilms over freshly exposed surfaces as the prominence of bacteria during early colonization events and their almost universal presence on marine surfaces, including those of benthic marine invertebrates and algae (Provasoli *et al.,* 1980; Walls *et al.,* 1993).

Compared with terrestrial organisms, the secondary metabolites produced by marine organisms have more novel and unique structures owing to the complex living circumstance and diversity of species, and the bioactivities are much stronger (Burgess *et al.,* 1999; Proksch *et al.,* 2002; Schupp *et al.,* 1999).

Furthermore, along with the deep studies of marine natural products biosynthesis, some evidence indicates that many bioactive compounds previously found in marine animals and plants were produced or metabolized by associated microorganisms (Carte *et al.,* 1996; Kohler *et al.,* 1999; Osinga *et al.,* 2001; Rinehart, 2000; Sponga *et al.,* 1999).

Members of the genus *Pseudomonas* show remarkable metabolic and physiological versatility, enabling their colonization of diverse terrestrial and aquatic habitats. *Pseudomonas* are of great interest because of their role in plant and human disease and their growing potential in biotechnological applications (Mena and Gerba, 2009). Stanier *et al.,* 1966 published a comprehensive appraisal of the taxonomy of *Pseudomonas spp.,* which is largely determined by phenotypes and biochemical capabilities (Mena and Gerba, 2009).

The *Pseudomonas* was wide range of abiotic and biotic environments which overlap among the species. *Pseudomonads* are a large group of free- living bacteria that live primarily in soil, seawater, and fresh water. Especially, *Pseudomonas aeruginosa* is particularly prevalent in environments, such as soil, sea water, sewage and associated with some plants. Although commonly isolated from the marine environment, the apparent distribution has been restricted to river outfalls and shorelines (Velammal *et al.,* 1994; Yoshpe – Pure and Golderman, 1987; Kimata *et al.,* 2004).

The genus *Proteus* currently consists of five species: *P. mirabilis, P. vulgaris, P. penneri, P. hauseri and P. myxofaciens,* as well as three unnamed *Proteus genomospecies* (Ohara *et al.,* 2000). *Proteus myxofaciens* is the only *Proteus* species without any significance in the pathogenicity of humans, it has been isolated from living and dead larvae of the gypsy moth *Proteria dispar* (Janda *et al.,* 2006).

Marine organisms

Screening of organic extracts from marine sponges and other marine organisms is a common approach to identify compounds of biomedical importance. Notable exception to this is the study performed by Mebs *et al.,* (1985) and Kristina *et al.,* (1997). Aqueous extracts from sponge species were tested for hemolytic hemagglutinating, cytotoxic, antimicrobial, anticholesterase and lethal activities.

The genus *Pseudoalteromona*s includes both pigmented and non-pigmented, Gram-negative, rod shaped, heterotrophic marine bacteria that are motile by means of polar flagella. This recently described genus is the result of extensive taxonomic revision on the basis of the phylogenetic relationships among members of the genera *Alteromonas, Shewanella* and *Moritella*. Gauthier *et al.*, (1995) suggested that the genus *Alteromonas* be divided into two genera, with the majority of species being reclassified within the new genus *Pseudoalteromonas*, leaving *Alteromonas macleodii* as the sole species of *Alteromonas*. Species of the genus *Pseudoalteromonas*, the majority of which appear to be associated with eukaryotic hosts (Holmstrom and Kjelleberg, 1999), are isolated frequently from marine waters around the world. Species have been isolated from various animals such as tunicates (Holmstrom *et al.*, 1998), mussels (Ivanova *et al.*, 1996), pufferfish (Simidu *et al.*, 1990) and sponges (Ivanova *et al.*, 1998) and from a range of marine algae (Akagawa - Matsushita *et al.*, 1992; Yoshikawa *et al.*, 1997).

The bacterial strains in this study were isolated from the surface of the marine alga *Ulva lactuca*. Both strains have been shown to inhibit the settlement of larvae of the marine invertebrate *Balanus amphitrite* and the germination of spores of the green alga *U. lactuca* and a species of the red alga *Polysiphonia* (Egan *et al.*, 2000).

Streptomycetes, Gram negative filamentous bacteria, are widely distributed in a variety of natural and man-made environments and constitute a significant component of the microbial population in most soil (Watve *et al.*, 2001). The results of extensive screening have been the discovery of about 4000 antibiotic substances from bacteria and fungi, many of which have found applications in human medicine, veterinary medicine and agriculture. Most of them are produced by Streptomycetes (Demain, 1999). Most streptomycetes and also other actinobacteria produce a diverse array of antibiotics, including aminoglycosides, macrolides, peptides, polyenes, polyether, tetracyclines, etc. In searching for new antibiotics, over 1000 different bacteria (including actinobacteria), fungi and algae have been investigated. To prevent exponential emergence of microorganisms becoming resistant to the clinically available antibiotics already marketed, the periodic replacement of existing antibiotics is necessary.

Marine fungi are an ecological rather than a taxonomic group. However, the *ascomycetes* order *Halosphaeriales* comprises largely marine species with 43 genera and some 133 species (Jones, 1995). This is approximately half of the total number of marine *ascomycetes* (Kohlmeyer and Volkmann – Kohlmeyer, 1991).

Kohlmeyer and Kohlmeyer (1979) stated that "considerable additions of new species in the future are unlikely" and indicated that there were then less than 500 marine fungi. Several recent papers have addressed the issue of the diversity and numbers of marine fungi (Jones, 1995; Jones and Mitchell, 1996; Jones and Alias, 1997). Jones and Mitchell (1996) estimated that there are 1500 species compared with 200,000 marine animals and 20,000 marine plants. The dramatic rise in the numbers of new marine fungi, which has occurred since the statement by Kohlmeyer and Kohlmeyer (1979), is due to the nature of the substrata examined for the occurrence of fungi. Kohlmeyer and Kohlmeyer (1979) listed 42 species of mangrove fungi, while the current number approaches 200 species (Jones and Alias, 1997). Similarly the number of fungi recorded by Kohlmeyer and coworkers from *Junces roemerianus* has also increased (Kohlmeyer *et al.*, 1996).

Actinomycetes are the most widely distributed groups of microorganisms in nature. They are attractive, bodacious and charming filamentous gram-positive bacteria. They make up in many cases, especially under dry alkaline conditions, a large part of the microbial population of the soil (Athalye *et al.,* 1981; Goodfellow and Williams, 1983; Lacey, 1973; Lacey, 1997; Nakayama, 1981; Waksman, 1961). Based on several studies among bacteria, the *actinomycetes* are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are especially prolific (Lacey, 1973; Lechevalier, 1989; Locci, 1989; Saadoun and Gharaibeh, 2003; Waksman, 1961).

Actinomycetes are the most economically and biotechnologically valuable prokaryotes and are responsible for the production of about half of the discovered secondary metabolites. Because of the excellent track record of *actinomycetes* in regard a significant amount of effort has been focused on the successful isolation of novel *actinomycetes* from terrestrial sources for drug screening programmes in the past fifty years. Recently the rate of discovery of new compounds from terrestrial *actinomycetes* has decreased whereas the rate of reisolation of known compounds has increased. Thus, it is excited that new groups of *actinomycetes* from unexplored or under exploited habitals be persued as sources of novel bioactive secondary metabolites (Donia *et al.,* 2003).

The diversity of *actinomycete* secondary metabolites is unrivaled and unmatched in medical significance. Structurally and functionally diverse bioactive compounds have also been isolated from other prokaryotes, including members of the myxobacteria (e.g., *Sorangium)* and cyanobacteria (e.g., *Nostoc*), as antibiotics with antimicrobial, antiviral, and antitumor activities (Patterson *et al.,* 1994; Reichenbach *et al.,* 2001; Schwart *et al.,* 1990; Shimkets *et al.,* 2004). Recently, the rate of discovery of new compounds from existing genera obtained from terrestrial sources has decreased, while the rate of reisolation of known compounds has increased. Moreover, the rise in the number of drug-resistant pathogens and the limited success of strategies such as combinatorial chemistry in providing new agents indicate an uncertain forecast for future antimicrobial therapy (Projan, 2003; Projan *et al.,* 2002). Thus, it is critical that new groups of microbes from unexplored habitats be pursued as sources of novel antibiotics and other small-molecule therapeutic agents (Bull *et al.,* 2000).

Physico – chemical parameters of marine environment

Analysis of the physico - chemical parameters of the marine water samples collected from different sites. The parameters such as P^H, temperature, electrical conductivity, dissolved solids, salinity, zinc, copper, iron, nickel, cobalt, total mercury, total cyanide, total lead, selenium, total silver, nitrate, nitrite, ammonia, inorganic sulphide and sulphate were analyzed using the standard methods (Strickland and Parsons, 1972).

The collected sediment sample were first air dried at room temperature, then crushed using a porcelain mortar and pestle and then sieved for further analysis. The P^H of the suspension was read using P^H meter (Systronics, India), to find out the soil $P^{H.}$ Electrical conductivity of the soil was determined in the filtrate of the water extract using Conductivity Bridge and Cation Exchange Capacity (CEC) of the soil was determined by using 1 N ammonium acetate solution. The reagents used for the analysis were AR grade and double distilled water was used for preparation of solutions. The analyzed Physico - chemical parameters

were observed by standard methods (Muthuvel and Udayasoorian, 1999).

Bacterial enumeration

Bacteria were enumerated by the acridine orange direct count (AODC) method (Hobbie *et al.,* 1977). Bacteria were sized from enlarged photomicrographs (~ 4300) using a digitizer (Hewlett Packard 9874A) according to Simon (1987). Usually 40 to 120 cells were sized on each filter.

To compute the cell volume we considered the rod-shaped cells to be cylinders with a hemispherical cap and cocci to be spheres. No correction was made for possible cell shrinkage as a result of sample preparation.

The bacterial growth rate was calculated by the equation for exponential growth:

$\mu = (\ln N_2 - \ln N_{1)} / (t_1 - t_{2)}$

Where N_1 and N_2 = bacterial abundance at the beginning and the end of an incubation interval; t_1 and t_2 = the corresponding times.

Fungal Enumeration (Dajoz, 1983)

Total Spore Count (TVC) of aquatic samples was calculated by following pour plate technique. Each sample was serially dilluted with dilutions ranging from 10^{-1} - 10^{-9} and 1ml of each dilution were poured on the nutrient agar plates. Plates were incubated at 37' c for 72 hours in an inverted position and the colonies were counted. The count was expressed as the number of colony forming units in 1 ml of the original sample. The occurrence of fungal species was calculated according to Dajoz (1983), using the formula;

Fo = Ta. 100/ TA

Where,
Ta = number of samples in which a taxon has occurred.

TA = total number of samples.

Values were considered according to the following classification:

<10% = Rare, 10 < 25 % = Low frequency 25 < 35% = Frequent, 35< 50% = Abundant and > 50% Very abundant.

Enumeration of actinomycetes

Isolation and enumeration of *actinomycetes* were performed by soil dilution plate technique using Glycerol-Yeast Extract Agar (Glycerol 1 g, Yeast extract 0.4 g, K2HPO4 0.02 g, Peptone 5.0 g, Agar 3 g, Distilled water 200 ml) complemented with nystatin (50 µg/ml) at 27ºC (Waksman, 1961). One gram of dried soil was taken in 9 ml of distilled water, agitated vigorously and preheated at 50^0 C for 0.5 hours. Different aqueous dilutions, 10^{-3}, 10^{-5}, and 10^{-7} of the suspension were applied onto plates and 20 ml of melted medium at around 50ºC was added to it. After gently rotation, the plates were incubated at 27ºC for 7 to 14 days. Selected colonies (rough, chalky) of *actinomycetes* were transferred from mixed culture of the plates onto respective agar plates and incubated at 27ºC for 7 days. Plates containing pure cultures were stored at 40ºC until further examinations.

Isolation and identification of marine organisms

One gram from each soil sample was placed in 9 ml of asparagine broth enrichment medium consisting of 2g l-1 asparagine L-monohydrate (Fluka, Switzerland) 1g l-1 K_2HPO_4 (BDH, England) and 0.5 g l-1 $MgSO_4 \cdot 7H_2O$ (BDH, UK) in order to enhance *Pseudomonas* growth. The samples were incubated for 48 h at 37ºC with vigorous shaking at 200 rpm to provide aeration for the bacteria. A loopful of the resulting bacterial

suspension was streaked onto asparagine plates containing 1.5% agar (Oxoid, UK) and incubated at 37ºC until colonies developed. The bacteria were then transferred to fresh asparagine plates according to the morphological characteristics of colony colour, shape and size (Projan, 2002).

Actinomycetes were isolated by soil dilution plate technique on starch casein agar medium, starch nitrate agar medium, glycerol glycine agar medium, and chitin agar medium (Haefner *et al.,* 2003). The plates were incubated at 28°C and the number of colonies was determined after 15 days. All the medium containing 50% sea water was supplemented with Nystatin 50µg/ml and Nalidixidic acid 20 µg/ml to inhibit the bacterial and fungal contamination, respectively (Haque *et al.,* 1992).

The selected colonies were picked up and further purified by streak plate technique over starch casein agar slants. The antimicrobial activity was studied preliminarily by cup plate method (Haque *et al.*, 1992) against bacteria and fungi. The test organisms were used are *Bacillus subtilis, Staphylococcus aureus Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger.* After preliminary testing of the isolates for their antimicrobial activities, the most active isolates were selected for further study.

Gram staining

A smear of the isolate was prepared on a clean glass slide and smear was allowed to air dry and then heat fixed. Heat fixed smear was flooded with crystal violet and after one minute, it was washed with water and flooded with mordant Gram's iodine. Smear was decolorized with 95% ethyl alcohol, and then washed with water. The smear was counter stained with safranin for 45 seconds. After washing with water, smear was dried with tissue paper and examined under oil immersion (100x) (Han's Christian Gram, 1884).

Motility test

The motility was situated by employing hanging drop method. A drop of culture broth was placed on the centre of the cover slip. The vaseline was applied over each corner slip. Then the slide was observed through the oil immersion microscope (Jordan *et al.,* 1934).

Biochemical characterization

A single colony was selected for biochemical tests. *P. aeruginosa* is oxidase positive, hydrolyses casein, and produces pyocyanin and/or fluorescence. Occasionally a non-pigmented variant of *P. aeruginosa* can occur, therefore a pyocyanin negative, casein hydrolysis positive, fluorescence positive culture can also be regarded as *P. aeruginosa*. Isolated strains were biochemically identified by conventional tests followed by use of API 20 NE identification system (API 20 NE, Biomerieux).

Colony characteristics, including pigment production were determined on Pseudomonas P Agar (Acumedia) and NA (Difco). All strains were characterized by the following classical tests according to Bergey's Manual of Systematic Bacteriology (Palleroni, 1984) Gram staining, cytochrome oxidase production, catalase production and growth on McConkey Agar and 42°C. Isolated strains were biochemically identified by conventional tests followed by use of API 20 NE identification system (API 20 NE, Biomerieux). To examine the enzyme profiles of isolates the API ZYM (Biomerieux) system was used, essentially as recommended by the supplier. The results were obtained in duplicate and analyzed employing the Apilab Plus Software (Biomerieux).

Morphological characterization

Morphological and cultural characters of the selected *actinomycetes* strain was studied by inoculating into sterile International *Streptomycetes* Project ISP 1,3-7,9 media (Matson *et al.,* 1989; Ohshima *et al.,* 1991). The media were sterilized and poured into sterile petridishes. After solidification of the media, culture of the selected strain was streaked on the media surface by simple method aseptically and incubated at 37^{o} C for 7 days (Salle *et al.,* 1948; Shomura *et al.,* 1987). Morphological characters such as colony characteristics, type of aerial hyphae, growth of vegetative hyphae, fragmentation pattern and spore formation were observed.

Molecular characterization

SDS – PAGE (Sodium Dodecyl Sulfate – Poly Acrylamide Gel Electrophoresis)

Gel electrophoresis of proteins with a polyacrylamide matrix, commonly called polyacrylamide gel electrophoresis (PAGE) is undoubtedly one of the most widely used techniques to characterize complex protein mixtures. It is a convenient, fast and expensive method because they require only the order of micrograms quantities of protein. The proteins have a net electrical charge if they are in a medium having a pH different from their isoelectric point and therefore have the ability to move when subjected to an electric field. The migration velocity is proportional to the ratio between the charges of the protein and its mass. The higher charge per unit of mass the faster the migration. Proteins do not have a predictable structure as nucleic acids, and thus their rates of migration are not similar to each other. They can even not migrate when applying an electromotive force (when they are in their isoelectric point). In these cases, the proteins are denatured by adding a detergent such as sodium dodecyl sulfate (SDS) to separate them exclusively according to molecular weight. This technique was firstly introduced by Shapiro *et al.,* (1967). SDS is a reducing agent that breaks disulfide bonds, separating the protein into its sub-units and also gives a net negative charge which allows them to migrate through the gel in direct relation to their size. In addition, denaturation makes them lose their tertiary structure and therefore migration velocity is proportional to the size and not to tertiary structure.

Some highlights of the polyacrylamide gel electrophoresis are:

Gels suppress the thermal convection caused by application of the electric field, and can also act as a sieving medium, retarding the passage of molecules; gels can also simply serve to maintain the finished separation, so that a post electrophoresis stain can be applied

The polyacrylamide gels are formed by polymerization of acrylamide by the action of a cross-linking agent, the bis-acrylamide, in the presence of an initiator and a catalyst. Persulfate ion (S2O8), that is added as ammonium persulfate (APS) is the gelsolidifying initiator and a source of free radicals, while TEMED (N, N, N', N'-tetramethylethylenediamine) catalyzes the polymerization reaction by stabilizingthese free radicals. In some situations, for example, isoelectric focusing the presence of persulfate can interfere with electrophoresis, so ribofavin and TEMED are used instead

Acrylamide solutions are degassed as oxygen is an inhibitor of polymerization. Moreover, the polymerization releases heat that could cause the formation of bubbles within the gel

The rate of polymerization is determined by the concentration of persulfate (catalyst) and TEMED (initiator)

The acrylamide gel electrophoresis systems may be performed using one or more buffers, in these cases of continuous phosphate buffer system (Weber and Osborn, 1968) or discontinuous buffer systems (Ornstein, 1964; Davis, 1964: Laemmli, 1970) adopted the discontinuous electrophoresis method and the term "Laemmli buffer" is often used to describe the tris-glycine buffer system that is utilized during SDS-PAGE. In discontinuous systems the first buffer ensures the migration of all proteins in the front of migration, what causes the accumulation of the entire sample that has been loaded into the well. The separation really begins from the moment when the migration front reaches the boundary of the second buffer. The first gel, "stacking", has larger pore (lower percentage of acrylamide / bisacrylamide) and has a pH more acidic than the second gel which is what really separates proteins. This system is particularly suitable for analyzing samples diluted without losing resolution (Westermeier, 2005).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) The extraction of total cell protein content of the strains studied was determined according to Pot *et al.,* (1994) and separation of proteins based on their molecular weight was achieved through SDS-PAGE technique (Laemmli, 1970).

The cultures were used as an inoculum (1% v/v) in 10 ml NB, and incubated at 37 ˚C for 24 h.

The pellet washed two or three times with sodium phosphate buffer (0.01M) containing 0.8 % NaCl (pH 7.3; sodium phosphate-buffered saline). The extracts were sonicated on ice during 45 seconds. After lysis, the sonicated extracts were centrifuged at 5000xg for 10 min. The pellet was discarded and the supernatant was supplemented with sample buffer (0.062 M Tris- HCl, 0.75 g; 2-merkapto etanol, 5ml; glycerol, 10ml; bromophenol blue, 1mg) and added 20% SDS, 0.1ml, heated for 10 min at 95˚C. Protein extracts underwent SDS-PAGE on vertical slab gels in an electrophoresis apparatus. Electrophoresis was performed using a power supply operated at a constant current of 20 mA through the stacking gel and 30 mA through the resolving gel. A standard protein solution (MBI Fermentas, SM 0439), containing 7 proteins ranging in size from 14.4 to 116 kDa, was used as a molecular weight marker and for the normalization and interpolation of the protein patterns. The proteins were run on 10% SDS-PAGE and gels were stained with Coomassie blue R250. Afterwards, the gels were distained until protein bands become clearly visible.

Protein separation

Proteins separated on a polyacrylamide gel can be detected by various methods, for instance dyes and silver staining.

Dyes

The Coomassie blue staining allows detecting up to 0.2 to 0.6 µg of protein, and is quantitative (linear) up to 15 to 20 µg. It is often used in methanol-acetic acid solutions and is discolored in isopropanol-acetic acid solutions. For staining of 2-DE gels it is recommended to remove ampholytes by adding trichloroacetic (TCA) to the dye and subsequently discolor with acetic acid.

Silver staining

It is an alternative to routine staining protein gels (as well as nucleic acids and lipopolysaccharides) because its ease use and high sensitivity (50 to 100 times more sensitive than Coomassie blue staining). This staining technique is particularly suitable for two-dimensional gels.

Detection of radioactive proteins by autoradiography

The autoradiography is a detection technique of radioactively labeled molecules that uses photographic emulsions sensitive to radioactive particles or light produced by an intermediate molecule.

The emulsion containing silver is sensitive to particulate radiation (alpha, beta) or electromagnetic radiation (gamma, light...), so that it precipitates as metallic silver. The emulsion will develop as dark precipitates in the region in which radioactive proteins are detected.

Western blot

Western blot is a widely used method in molecular biology and biochemistry to detect proteins in a sample of cell homogenate or extract. The proteins are transferred from the gel to a membrane -made of nitrocellulose, nylon or polyvinylidene difluoride (PVDF), where they are examined using specific antibodies to the protein. As a result, the amount of protein in a sample can be examined and it is also possible to compare levels among various analytical groups.

The method was initiated in the laboratory of George Stark at Stanford. The name "western blot" was given to the technique by Burnette (1981), comparing it with the "southern blot" technique for DNA detection developed by Edwin Southern. The detection of RNA is also called Northern blotting.

The most powerful method is the transference of proteins from the gel to a membrane by applying an electric field perpendicular to the gel. There are however other methods of transferring or applying a protein on the membrane. The simplest is to apply it directly as a small drop of a concentrated solution on the membrane. The absorption of the drop causes the adhesion of the protein to the membrane, leaving it as a spot or "dot" (this is the case of the "dot blot"). There are devices that make possible the application of proteins to membrane directly, using a suction that facilitates the penetration of the solution, and are named "dot blot" or "slot blot" on the basis that the proteins were applied as a circular drop or a line.

Working with proteins bound to a membrane has advantages over employment within the gel:

Staining and discolor are faster

No staining occurs to ampholytes in isoelectric focusing gels

Smaller amounts of proteins are detected as they are concentrated at the surface and not diluted across the thickness of the gel

The membranes are much easier to manipulate than the gel itself

Blotting procedure

It consists of 5 stages:

Immobilization of proteins on the membrane was either by transference (electrophoresis, suction, pressure...) or by direct application. The procedure starts piling a flat sponge on filter paper soaked in transference buffer, the gel, the membrane in direct contact with the gel plus filter paper and finally a flat sponge. This set is included between two layers of perforated plastic and placed in a tank which is a saline solution (transference buffer) and two plate electrodes (designed to achieve a uniform field across the surface of the gel). They are disposed so that the gel is toward the anode (-) and the membrane to the cathode (+).

Saturation of all binding sites of proteins in the membrane not occupied to prevent nonspecific binding of antibodies, which are proteins.

Incubation with primary antibody against the protein of interest.

Incubation with secondary antibodies, or reactives acting as ligands of the primary antibody bound to enzymes or other markers. Enzyme coupled secondary antibodies: an antibody to the specific binding antibody is conjugated to the enzyme peroxidase or alkaline phosphatase (Blake *et al.,* 1984).

Another possibility is the use of an amplifying enzyme which is part of a biotin-avidin-peroxidase complex (Hsu *et al.,* 1981) or a complex with alkaline phospohatase.

Enhanced chemiluminescence (ECL) is other commonly used method for protein detection in western blots (Laing, 1986). ECL is based on the emission of light during the horse radish peroxides (HRP) - and hydrogen peroxide-catalyzed oxidation of luminol. The emitted light is captured on film or by a CCD camera, for qualitative or semi- quantitative analysis. This method allows stripping and re-probing the blot with different antibodies.

Software and database search algorithms to analyze spectral data

A variety of tools and commercially available software exist that allow for protein identification from peptide sequences determined by mass spectrometry.

Some examples of database search programs and algorithms are:

SEQUEST - identifies collections of tandem mass spectra to peptide sequences that have been generated from databases of protein sequences. It was one of the first, if not the first, database search program. While very successful in terms of sensitivity, it is quite slow to process data and there are concerns against specificity, especially if multiple posttranslational modifications (PTMs) are present (Jimmy *et al.,* 1994).

Mascot - is a powerful search engine that uses mass spectrometry data to identify proteins from primary sequence databases (www.matrixscience.com) (Perkins *et al.,* 1999).

Scaffold 3 – is a software which produces a confidence level for protein identification from one or more Mascot, Sequest, X! Tandem, or Phenyx searches. It can be used in conjunction with a MS/MS search engine in order to validate/visualize data across multiple experimental runs as well as provide a more accurate protein probability

Purification of protein from *Pseudomonas* and *Proteus*

The protein concentrations of the samples recovered in the extraction and purification procedures were estimated by the method of Lowry (Lowry *et al.,* 1951), with bovine serum albumin as standard, and total carbohydrates were determined by the phenol – sulfuric acid procedure of Dubois (Dubois *et al.,* 1956), with glucose as standard.

The antibacterial substance from the active bacteria supernatant was purified by ammonium sulfate precipitation and High – Performance Liquid Chromatography (HPLC). First the purified substance was obtained, and then solid ammonium sulfate was added slowly to 85% and 50% saturation at 4 ºC with constant stirring (10,000 x g for 30 min at 4ºC) and resuspended in H_2O. These samples were then loaded onto Agilent Zorbax 300 SB – C18 column for HPLC. Elution was performed with 95%, 50%, 5%

and 95% acetonitrile as the linear gradient (flow rate: 0.5 mL/min). After elution, the absorbance was monitored at 280 ± 4 nm, and the fractions were collected. These fractions were tested for antibacterial activity against the indicator strain using agar well diffusion to find the fraction containing the active molecule. At each step of the purification, antibacterial activity was assayed against *Bacillus thuringiensis* subsp. *thompsoni* HD542, selected as the indicator bacteria by agar well diffusion assay (Padilla *et al.,* 1996; Chanda *et al.,* 2013; Khan *et al.,* 2013). The antibacterial protein was purified by electroelution using a model 422 Electro – Eluter Module (Bio – Rad). The protein band with antibacterial activity was excised from the gel and placed into a tube connected with membrane caps (molecular mass cut – off 12 – 15 kDa). Electroelution was performed at 10 mA constant current per glass tube for 5 h in elution, buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). After elution, the remaining liquid in the membrane cap was collected. SDS – PAGE and in gel-antibacterial assays were repeated as above to determine the homogenicity and activity of the antibacterial protein (Longeon *et al.,* 2004).

The concentrated protein was applied to gel filtration which was carried out using the column (2.5, 45.0 cm) pre - equilibrated with 50 mM phosphate buffer (p^H – 7). 5ml of concentrated enzyme was loaded to the top of the column. The enzyme was eluted using 50 mM phosphate buffer pH 7 at a flow rate of about 30 ml h^{-1} using a peristaltic pump (Master – flex, Cole – Parmer) and fractions of 5.0ml were collected. The enzyme activity and protein in each fraction were analyzed (Farag and Hassan, 2004).

The extraction of total cell protein content of the strains studied was determined according to (Pot *et al.,* 1994) and separation of proteins based on their molecular weight was achieved through SDS – PAGE technique. The cultures were used as an inoculum (1% v/v) in 10ml NB, and incubated at 37°C for 24 hr. The pellet washed two or three times with sodium phosphate buffer containing 0.8 % NaCl. The extracts were sonicated on ice during 45 seconds. After lysis, the sonicated extracts were centrifuged at 5000xg for 10 min. Protein extracts underwent SDS- PAGE on vertical slab gels in an electrophoresis apparatus. Electrophoresis was performed using a power supply operated at a constant current of 20 mA through the stacking gel and 30mA through the resolving gel. The proteins were run on 10 % SDS – PAGE and gels were stained with Coomassie blue R250. Afterwards, the gels were distained until protein bands become clearly visible (Laemmli, 1970).

Antibacterial protein activity

An overnight broth culture of sample was centrifuged at 6000 rpm for 10 min at 4 ºC. To the cell free supernatant ammonium sulphate was added to achieve 30% saturation. It was stirred well and incubated at 4ºC overnight. The following day, the precipitates were collected by centrifugation at 6000 rpm for 25 min at 4ºC. The precipitates were dissolved in 500µl of sterile distilled water. Antibacterial activity of both the supernatant and the precipitate were tested using agar well diffusion assay (Jayammal and Sivakumar, 2013).

Plant pathogenic bacterial strains were used in both the agar- diffusion method and the agar – spot assay to evaluate the antagonistic activity of *P. syringae subsp. ciccaronei* and the spectrum of antibacterial activity of the bacteriocin. Growth of *P, syringae subsp perrsicae* NCPPB2761 was inhibited by colony and its culture filtrate with the zone of inhibition 7 mm (Lavermicocca *et al.,* 1999).

Similarly, the culture filtrate of *Pseudomonas aeruginosa* inhibited the growth of MRSA with the zone of inhibition 12mm. The active metabolites produced by *Lactobacillus sp* NRRL B-227 exhibited various degrees of activities against *Staphylococcus aureus* with 21 mm zone of inhibition (Atta *et al.,* 2009).

Bacterial colonies were grown for 7 days. Target cells were grown to stationary phase and mixed with 0.6% VNSS agar plates at 45℃ at a density of 0.4 ml of target culture per 3ml of agar. Each 7 day culture plate was overlaid with 3ml of this mixture and incubated overnight (James *et al.,* 1996).

Concentrated bacterial supernatant was prepared as described above. An overnight broth culture of the target strain was prepared, and 100 ml of the culture was spread over the surface of an agar plate of the growth medium appropriate to that strain. Drops containing 20 µl of bacterial supernatant were placed on the target plates, which were then incubated overnight. Live cells could not be revived from the inhibition zones formed in sensitive strains when swabs were taken from this region and reinoculated onto fresh agar. No cells were recovered from the inhibition zones of all assays taken as positive for bacteriocidal activity. Relative sensitivities of different strains to the bacteriocidal effect of the protein were estimated with serial dilutions (James *et al.,* 1996).

Identification of antibacterial protein by native gel electrophoresis

Samples with antibacterial activity were suspended in sucrose – dye –solution (5% sucrose, 0.01 % bromophenol blue) and resolved by polyacrylamide gel electrophoresis (PAGE) with an 8% gel. The gels were washed for 20 min in distilled water prior to being placed on VNSS agar plates, after which stationary phase target cells were spread over the gel surfaces (James *et al.,* 1996).

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS – PAGE) was performed in vertical gels by standard protocols using Tris – glycine buffer (Laemmli, 1970). Duplicate samples of purified antibacterial substance and molecular mass markers were subjected to 15% SDS-PAGE. Molecular weight markers for peptides ranging from 10 to 225 kDa were used as a molecular marker standard. After electrophoresis, the gel was sliced vertically. The first part, consisting of samples of purified antibacterial substance and protein standards, was stained with Coomassie Brilliant blue R-250. The other part of the gel was assayed for direct detection of antibactertial substance activity according to Cherif method (Cherif *et al.,* 2001).

Antibacterial protein purification

The supernatant are high – density bacterial suspension was prepared as described for use in the drop test assay, after the ammonium sulfate was added to a concentrated of 60%.The precipitated fraction was collected by centrifugation (15,000 x g for 30 min), resuspended in 20 mM Tris buffer (p^H 7.5), and dialyzed against this buffer overnight. The sample was passed through a 5- ml Econo – pac Q anion – exchange cartridge from which the bacteriocidal fraction was eluted at between 0.1 and 0.2 M N aCl at p^H - 7.5. The bacteriocidal fraction was identified at each step by the drop test. All protein assays are carried out with a bicinchoninic acid protein assay kit (James *et al.,* 1996).

The bacteriocidal fraction eluted from the ion – exchange column was dialyzed overnight against 0.001 M NaCl and passed through a 5-ml Econo – pac hydroxyapatite cartridge which was then washed with 1 M NaCl and

0.01 M phosphate buffer prior to elution of the protein with 0.4 M phosphate buffer. The purified fraction was stored at 20℃ after freeze –drying (James *et al.,* 1996).

The purified antimicrobial compound displayed a single protein peak at 225nm in the UV spectrum, and the infrared spectroscopy showed two characteristic absorption bands at 1,650 and 1,075 cm^{-1}, which corresponded to peptide bonds and polysaccharide residues, respectively. A wide band indicative of the presence of polar groups in the molecule appeared at 3,350 cm^{-1} (Stewart, 1965).

Bioactive compounds

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body and these chemical substances are called phytochemicals. These phytochemicals were used to cure the disease in herbal and homeopathic medicines (Chitravadivu *et al.,* 2009). These are non-nutritive substances, have protective or disease preventive property (Ahmed and Urooj, 2009). There arises a need and therefore to screen medicinal plants for bioactive compounds as a basis for further pharmacological studies. With advances in phytochemical techniques, several active principles of many medicinal plants have been isolated and introduced as valuable drug in modern systems of medicine. The most important of these bioactive compounds are alkaloids, flavonoids, tannins and phenolic compounds (Purkayastha and Dahiya, 2012). These are the important raw materials for drug production (Tullanithi *et al.,* 2010). Most plants contain several compounds with antimicrobial properties for protection against aggressor agents, especially microorganisms (Silva *et al.,* 2010). Medicinal and aromatic plants are used on a large scale in medicine lack of success of treatment in infectious diseases. Medicinal plants are the major sources of new medicines and may constitute an alternative to the usual drugs (Tepe *et al.,* 2004). Aromatic oils are used in many industries, including food preservation, pharmacy, and medicine (Elgayyar *et al.,* 2001; Singh *et al.,* 2012). They are expected to form new sources of antimicrobial drugs, especially against bacteria (Nraskin *et al.,* 2002). The antibacterial effectiveness of aromatic oils has been divided into a good, medium, or bad (Bankole *et al.,* 2007; Shan *et al.,* 2007). These oils can also produce some defense products against several natural enemies. In addition, and in order to continue their natural growth and development, aromatic oils may produce some secondary metabolites in response to some external stress (Prabuseenivasan *et al.,* 2006). The antibacterial activity of ethanol extracts was determined by agar well diffusion method. The plant extracts were more active against gram-positive bacteria than against gram negative bacteria among all the pathogens, all gram - positive bacteria were inhibited by all four plant extract. All gram - negative bacteria i.e. *Pseudomonas spp*, *Proteus spp*, *Escherichia coli*, *Shigella dysenteriae*, *Klebsiella pneumonia* and *Salmonella typhi* were showed zone of inhibition against extract of *Ocimum sanctum* (Park *et al.,* 2003). In vitro microbicidal activity of the methanol extract of *Origanum marjorana* was tested against six bacteria (*Bacillus subtilis*, *B. megaterium*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus aureus).* The methanol extract of *O. marjorana* can be used as an effective herbal protectant against different pathogenic bacteria (Joshi *et al.,* 2009; Hameed *et al.,* 2015). The inhibitory activity was highly significant in the aqueous extracts of *Oxalis corniculata*. Most of the plant extracts showed significant antibacterial activity than bacitracin. MIC of aqueous extract of twelve plants varied between 4-50 µl. Results

indicated the potential of these plants for further work on isolation and characterization of the active principle responsible for antibacterial activity and its exploitation as whereas *Oxalis Acacia nilotica* varied between 9 - 35.5 mm. Whereas *Oxalis corniculata* was effective against all the tested bacteria in case of *Lawsonia inermis* it varied between 9 to except *Shigella sonnei* and *Proteus mirabilis* (Leeja and Thoppil, 2007). Effectiveness of organic extracts of *Piper nigrum* fruit against pathogenic strains of *Escherichia coli* (MTCC 723), *Staphylococcus aureus* (MTSS 96), *Streptococcus pyogenes* (MTSCC 442), *Proteus mirabilis* (MTCC 1429) by tube dilution method. The study revealed that 70% alcoholic hot extract had higher antibacterial activity as compared to chloroform hot and petroleum ether cold extracts (Patil, 2007). The aqueous extract was found to be antibacterial and it was studied against various gram positive and gram-negative bacterial strains by using MIC, agar well diffusion method to find zone of inhibition. The MIC results of aqueous extract of *Plectranthus amboinicus* indicated that *Proteus vulgaris, Bacillus subtilis* and *Staphylococcus aureus* were least susceptible among the organisms tested and *Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa* are not shown any inhibition to aqueous extract of *Plectranthus amboinicus* (Kumar *et al.*, 2008).

Protein subunit structure

The following treatments were carried out upon samples of purified antibacterial protein to determine whether it contains subunits. The effects of treatments with β −mercapto ethanol and heat upon the subunit structure of the protein were visualized by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS – PAGE) (Laemmli, 1970).

The purified protein was dissolved in SDS-PAGE treatment buffer (0.062M Tris- Cl (P^H -6.8) 4% SDS, 20% glycerol) and divided into four samples. The first sample was exposed to 5% β −mercaptoethanol, the second was heated in a boiling water bath for 90 s and the third was exposed to both treatments. The fourth sample was left untreated as a control. The antibacterial protein was detected within these samples by SDS-PAGE on 8% 1.5-mm polyacrylamide gels stained with Coomassie brilliant blue (Bryers *et al.*, 1982).

The N- terminal amino acid sequence of both of the subunits of the antibacterial protein was determined. The purified protein fraction was treated with both 5% β −mercaptoethanol and heat as described below and the bands were resolved by SDS – PAGE on 8% 1.5 - mm polyacrylamide gels an electroblotted to problott polyvinylidene difluroride membrane with a CAPS (3 – (cyclohexylamino) – 1 - propanesulfonic acid) buffer system. The blotted proteins were stained on the membrane with amido black (James *et al.*, 1996).

Protein release during the growth curve of bacterial cells

The release of antibacterial protein by bacterial cells into its growth medium at different stages of the growth curve was determined as follows. One liter of VNSS growth medium was inoculated with 10 ml of precultured bacterial cells and incubated as a shaking batch culture at 28 ºC. Cell density was measured throughout the growth curve. Samples of growth medium were taken at 4.5, 6.5, 7.5, 8.5 and 25 hours and filter sterilized, and the antibacterial protein from these samples was concentrated onto an ion-exchange column as described in the purification protocol. The antibacterial protein was detected within these samples by SDS – PAGE on 8 % 1.5 – ml polyacrylamide gels

stained with Coomassie brilliant blue (James *et al.,* 1996).

MBCs and minimum growth inhibitory concentrations

The concentrations of antibacterial protein necessary to result in cell death and to inhibit the growth of a variety of marine bacterial isolates in solution was determined as follows. Purified protein in sterile VNSS was added to the wells of a 96 – well plate at a series of dilutions ranging from 0 to 800 x. Bacterial cells were inoculated at time zero, and the growth rates were measured by absorbance. Cell viability was checked at 24 hours by streaking samples onto VNSS agar plates (Egan *et al.,* 1996).

The metals and antibiotics used in this study are as follows: $HgCl_2$, AgCl, $CaCl_2$, $ZnSO_4$, ampicillin, kanamycin, chloramphenicol and tetracycline. The ability of SPI and SPIM to grow in media containing metals, except $HgCl_2$ or antibiotics was tested by adding these chemicals at the concentrations.

The growth of bacteria was monitored after SPI was cultured at 28ºC for 48 h. The minimal inhibitory concentrations of metals and antibiotics were defined as the lowest concentrations that caused no visible bacterial growth (Murtaza *et al.,* 2002).

The potency of the marine organisms *Pseudomonas* and *Proteus* to produce a highly active protein has an antibacterial activity. Purification and characterization of the protein showed good stability of the microbial compound preparation and its relatively wide range of gel filtration and SDS- PAGE method. This study revealed also that the isolated protein has potent antibacterial activity.

In conclusion, an antibacterial protein and compounds from *Pseudomonas* and *Proteus* showed inhibitory activity against the bacterial pathogen *Staphylococcus* and *Bacillus.* The antibacterial protein and the compounds may have a synergistic antibacterial effect in connection with bioactive compounds.

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