Screening, Isolation and Production of Protease by Marine Actinomycetes

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Abstract

Marine actinomycetes which produce extra-cellular protease were collected from different locations of the Bay of Bengal starting from Pulicate Lake to KanyaKumari and are screened and isolated. A new and simple starch casein double layer agar method was employed for a quick screening of these actinomycetes. Among bacteria, actinomycetes have been found to be a unique source of commercially significant products. Given developments in microbial systematic is now possible to device sophisticated selective isolation strategies and to recognize and characteristic the novel organism. May general or non-selective media were formulated without regard to either the nutrient propertied or tolerances of the target actinomycetes. Many of these medias, Such as colloidal Chitin, half-strength nutrient, starch case in and M_3 agar are widely used through little attempt has been made to evaluate their effectiveness. The differential activity of protease was determined at different optimum pH and temperatures. The production of protease by Phenyl Methyl Sulphonyl Fluoride (PMSF) showed that this enzyme belongs to the serine protease group.

Keywords: Marine actinomycetes; Isolation; Screening; Molecular techniques; Prokaryote.

Introduction

Isolation and screening are rate limiting in the search and discovery of previously unknown, natural products from microorganisms (Goodfellow *et al.,* 1998). It is now apparent that discoveries of important, novel bioactive compounds depends upon the development of objective strategies for the isolation and characterization of novel and rare micro-organisms for existing and new screens (Nolden *et al.,* 2000). Actinomycetes are members of the order actinomycetales belonging to the class actinomycobacteria, containing many readily cultivated and well-known genera, including mycobacterium, *Streptomycetaceae* and *Rhodococus* (Goodfellow *et al.*, 1984).The capacity of *Streptomycetes* to produce new compounds remains unsurpassed through members of other microorganisms like actinomycetes genera are becoming increasingly important as a source of novel products (Nord *et al.,* 1998).The actinomycetes are taken as an example of party because of their unrivalled record as a source of new products but also because the authors are interested in there fascinating prokaryotes (Okamoto *et al.,* 1977).

Actinomycetes are easily isolated from the marine environment, and other, often uncultured. Actinobacteria are detected using molecular techniques. This uncultured diversity has also shown increased success in being cultured. Actinomycetes are a pre-eminent source of bioactive natural products, but in the hypothesis and process-driven field of marine microbial ecology they are not linked to the study of major ecological process nor often recognized as key species (Delong *et al.,* 2005).Thus, it is largely recent “search and discovery”. That is driving a relative explosion in new actinobacterial isolations. Previously, efforts to isolate marine actinomycetes showed poor result because of several factors: a perception of the marine environment as being a 'desert' the conclusion that actinomycetes were inactive spores from terrestrial wash-in 'leading to re-isolation of terrestrial strains and known compounds (Moore *et al.,* 2005).The logistical and technological problems of sampling the marine environment, for example, sampling from the neuston or the deep sea (Fiedler *et al.,* 2005). The near-complete retreat of big pharmaceutical companies from natural-products drug discovery also limited isolation efforts (Barrett *et al.,* 2005).

Nevertheless, the pre-eminence of natural products (Baker *et al.,* 2004). The recognition of increased diversity and the success of marine natural products has prompted. The marine environment as a source of novel chemical diversity for drug discovery (Bull *et al.,* 2005).The similarity of these new natural products to those produced by actinomycetes, and the isolation of novel drug-producing endosymbiolic and host-associated actinomycetes from the marine flora and fauna has lead to an increase in the isolation of marine actinomycetes (Blunt *et al.,* 2004). Equally, more comprehensive molecular techniques and surveys reveal that the actinomycobacteria are a small but significant and uniquitous component of marine communities (Venter *et al.,* 2004). Sometimes dominant in specific habitats (Colwell *et al.,* 2004).

Construction and Use of Taxonomic Data Bases

Poor systematics and a dearth of suitable selective isolation methods accounted for many of the problems experienced by those searching for new and rare actinomycetes. Until recently, actinomycete classifications were derived from a small number of subjectively Weighed morphological and behavioural properties. Strains were assigned to taxa based on colony and micromorphology, staining properties, the presence and absence of spore, the ability to produce acid from sugars, and the

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capacity to grow on different carbon source. Organisms were, for example, assigned to the genus *Nocardia* primarily by an ability to form mycelia that sooner or later fragmented into it is no clear that *Nocardia sensu* encompassed a collection of actinomycetes that have been assigned to nine different genera.

This *Nocardial* miscellany included strains labeled Nocardia aerocolonigenes, *Nocardia mediterranei* and *Nocardia orientalis* which produce the antibiotics rebeccamycin, rifampicin and vancomycin. These organisms have been renamed *Saccharothrix aerocolonigenes, Amycolatopsis mediterranei* and *Amycolatopsis orientalis* on data obtained using modern taxonomic methods. The ability to recognise these poorly studied, but commercially significant, strain s ill facilitate the recovery of similar antibiotic-producing isolates from the environment.

Actinomycete systematics has been revolutionised by the application of new and reliable taxonomic methods. Established taxa have been defined with greater precision, new genera have been proposed to accommodate novel isolate s and poorly circum scribed species have been reduced to synonym s of ell-defined taxospecies.

The number of validly described actinomycete genera has risen from four in the sixth edition of *Bergey's Manual of Determinative Bacteriology to over fifty in Bergey's Manual of systematic Bacteriology.* Despite previous success, members of both established and recently described genera continue to be a rich source of new antibiotics.

Isolate	Product	Reference
Actinomadura Oligospora	Polyether antibiotics	Mertz & Yao (1986)
Actinoplanes arizonaensis	Arizonins	Karawowski *et al.,* (1986)
Saccharothrix australiensis	-containing Aminoglyco-side	Labeda *et al.,*(1984)
Streptoalloteichus Hindustanus	Talysomycine A&B	Tomita *et al.,*(1987)
Streptomyces aculeolatus	SF-2415	Shomura *et al.,* (1987)
Streptomyces Libani Subsp.rubropurpurous	Chromo-oxymycin	Iwami *et al.,*(1986)

Table-1. Examples of recently isolated Marine Actinomycetes producing novel secondary metabolites.

Traditional taxonomy, based on a priori weighting, was unable to give stable suprageneric classifications. Indeed, the reliance placed on a small number of morphological and physiological and features resulted in actinomycetes being assigned to families subsequently found to be markedly heterogeneous. In an impressive series of 16S ribosomal RNA sequencing experiments representative actinomycetes ere classified into a number of discrete suprageneric groups.

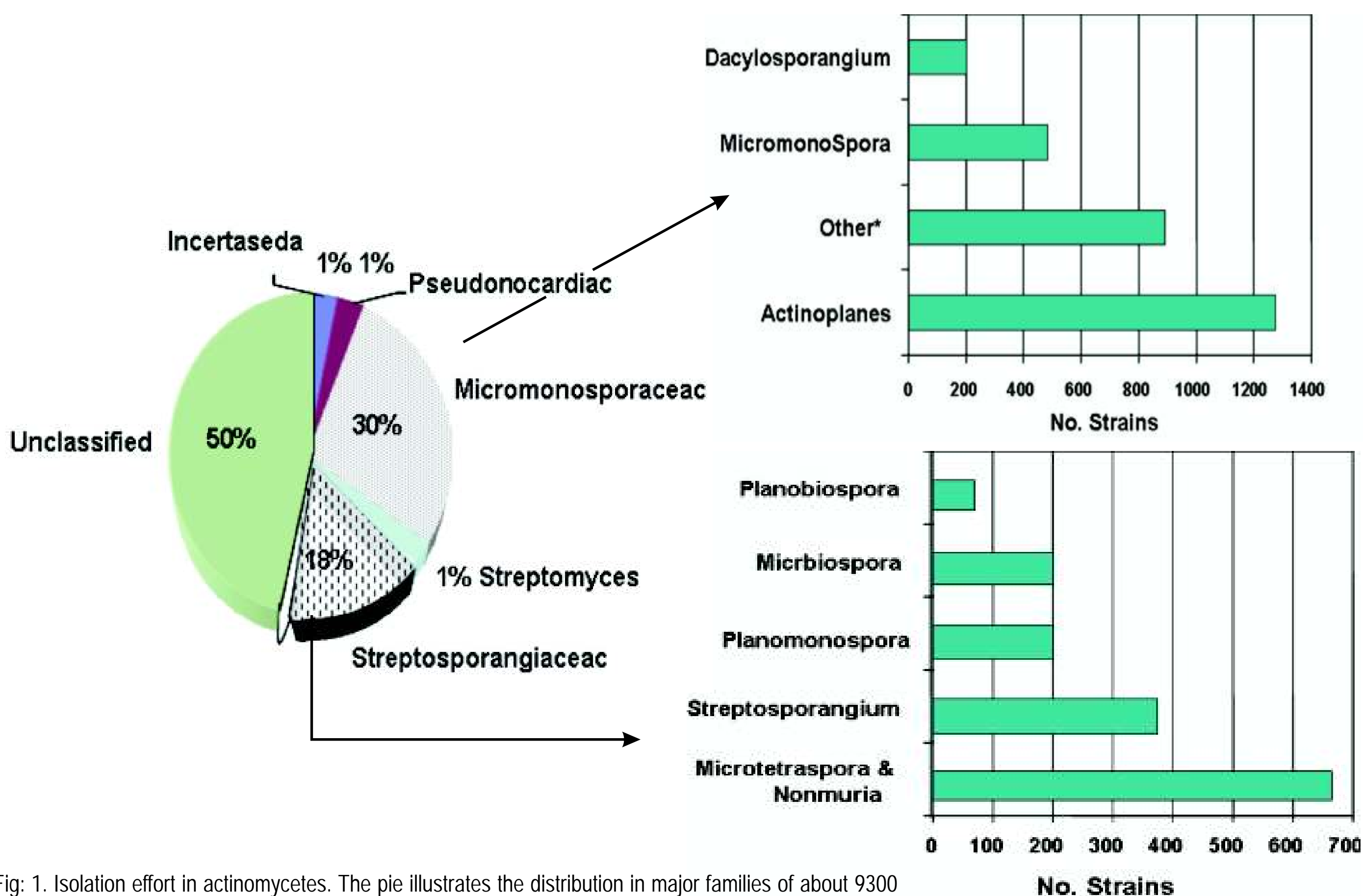

Fig: 1. Isolation effort in actinomycetes. The pie illustrates the distribution in major families of about 9300 strains isolated in the period 2006-2008.The portion unclassified refers to strains for which no classification was made. The enlargements show the actual numbers of strains belonging to various genera in the families *micromonosporaceae and streptosporangiaceae.*

The strain collection and microbial extract bank at Biosearch Italia

Our assumption is that novel metabolites can be discovered by screening unusual and difficult to isolate strain belonging to the to mot prolific groups of producer, the filamentous actinomycetes and the filamentous fungi. This assumption rests on the increased likelihood that they have not been intensively screened in the past and on their promise to be potentially capable of producing secondary metabolites, as demonstrated for some unusual actinomycetes (Sosio *et al.,* 2000a). With this assumption in mind, Biosearch Italia has assembled a large library of processed fermentation broths (>150 000, at the time of this writing) obtained from proprietary collection of over 50,000 strains. These processed fermentation broths are stored in a ready-to-screen format as dried, frozen aliquots in microtiter plates over 90% of them were obtained by fermenting actinomycetes other than *Streptomyces,* and filamentous fungi. Consequently, the strain collection consists mainly of unusual genera of actinomycetes (e.g. the easy-to-isolate *streptomycetes* account for about 1% of the actinomycetes isolated over the last 4 year; Fig.1) and of slow-growing and endophytic fungi, with little emphasis on *pencillium* and *aspergillus* (e.g. about 8% of eukaryotic microbes belong to these two genera; data not shown). The strain collection has been built through the application of isolation methods to a large number of different sources which includes soil samples, plant materials, sediments, marine macro-organisms etc. (Amann *et al.*, 1995). It is continuously enlarged by the addition of newly isolated strains from newly obtained specimens and historical collection of >10,000 dried soil samples.

For isolating such a large number of diverse microbes, tens of different methods are applied, according to the source and the target group of microbes. The objective of an isolation program consist in obtaining, with the minimum effort, as large a number as possible of microbes that are unusual and difficult to isolate.

We have developed a series of methods that can assist in the isolation of unusual groups of microorganisms. We describe here approaches that can be applied for the detection, dereplication and manipulation of unusual groups of actinomycetes.

Isolation effort in actinomycetes: the pie illustrates the distribution in major families of about 9300 strains .The large enlargements show the actual no of strains belonging to various genera in the families Micromonosporaceae and Strepto-sporangiaceae.

Now a days screening is mostly conducted in an automated fashion and is referred to as high throughput screening (HTS). In particular, the ability to produce large number of chemically different secondary metabolites is associated mostly with filamentous actinomycetes, the mycobacteria, the pseudomonas and the cyanobacteria within the prokaryotic world, and mostly to the filamentous fungi for the eukaryotic microbes. These critical factors must all consider when embarking on a screening program for bioactive metabolites from micro-organisms. Different chemical methods for are required at different stages of screening programmer. The first step is to determine whether the objective of including the chemical methods is to assign recent isolates to established taxa or to provide a mechanism by which previously analyzed organisms are excluded from screens Pyrolysis techniques such as pyrolysis mass-spectrometry (Schutiet *et al.,* 1985) and pyrolysis gas chromatography (O' Donnell & Norris 198) is now finding their way into some industrial screening laboratories.

The marine environment

The marine environment contains several habitats, from the sea surface micro layer (SML) down through the bulk water column (containing marine organisms and marine snow), which extends from a few millimeters below the surface to > 10,000 meters depth, and further down to the habitats on and under the sea floor (Bull *et al.,* 2000).

The papers reviewed here describe the Actinobacteria found in these various vertically delineated marine habitats, which are spread globally across oceanic realms, separated geographically and influences by varying geophysical parameters of temperature, salinity, underlying geochemistry and ocean currents. Regional ecosystems of salt marshes and wetlands, estuaries, continental shelves, the open ocean and the deep sea all influence the actinobacteria present. Sampling across this geographical distribution is still sparse.

Biogeography

Biogeography has been defined as the “science that attempts to document and understand spatial patterns of biodiversity” (Brown *et al.,* 1988). The concept of prokaryotic biogeography has been debated as a result of the Bass-Becking and Beijerinck theory, which predicts a cosmopolitan microbial world “everything is everywhere, the environment selects” (Hedlund *et al.,* 2004). The size-related distribution and globally distributed populations, we would thus accept a low global diversity of micro-organisms despite high local diversity. This view is supported by the valid description of only ~7000 bacterial species but is challenged by many microbiologists (Horner-Devine *et al.,* 2004). Brown and Lomolino (1998) state that there are “patterns of variation over the earth of numbers and kinds of living things” but for actinobacteria, as for all prokaryotes, we are unable to define these 'kinds' or to count them properly (Papke *et al.,* 2004).

Nevertheless, some biogeography data for prokaryotes, including the marine actinomycetes, is successfully being accumulated (Mincer *et al.,* 2002). This bio-geographic data has practical implications for search and discovery data has practical implications for search and discovery, as well as having ecological and evolutionary implication (Maldonado *et al.,* 2005).

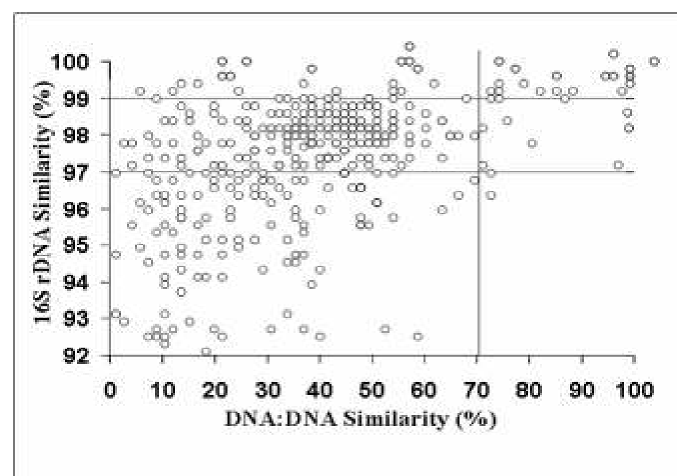

Fig-2: Relationship between 16S rRNA similarity and DNA:DNA similarity for Actinobacteria Using a conservative 70% DNA similarity as the threshold for species definition it is clear that any 16S rRNA sequences which deviate by 97% or more are invariably different species, but a similarity greater than 97% or even greater than 99% is a poor indicator of genomic similarity.

Actinobacteria in the marine environment

the SML and the Neuston

The SML (SeaSurface MicroLayer) is an environment with unique properties, proposed to support a high microbial density and a specific community (the Neuston). Early cultural studies isolated actinobacteria and proteo-bacteria, but the SML has remained poorly characterized (Agogue *et al.,* 2005).The report and results of a recent study in which the 16S rRNA sequences of bacterial isolates from the SML and underlying water (~0.5m) were clustered into five phylogenetic groups: -proteo-bacteria; -proteobacteria; cytophaga-flavobacter-Bacteriodes (CFGS); Actinobacteria (high G+C Gram-positive bacteria); and Firmicutes (low G+C Gram-positive bacteria). In the oligotrophic waters of the Bay of Bengal, the actinobacteria make up a minor fraction of the bacterioneuston, and the numbers and composition of the community in the neuston and in the underlying waters are similar. Actinomycetes are also a minor component in the underlying waters of the Olympic Harbor in Barcelona, but they are the major phylogenetic group (27%) isolated from the SML of this more contaminated site. This study rRNA sequencing of nearly 600 isolates, and Single Strand Conformational Polymorphism (SSCP).

Marine snow

Early studies as well as some subsequent ones of marine snow have failed to detect (Delong *et al.,* 1993).The Actinomycetes with the use of molecular methods however, cultured-based methods are isolated (Simon *et al.,* 2002). The significant fraction (10%, but this represents only five from a sample size of 50) of actinomycetes and linked them with expected competitive interaction with in marine Micro-aggregates (marine snow). Four out of the five actinomycetes isolate antagonized the growth of other bacteria strain isolated from the sample. The discrepancy between these studies (the presence (or) absence of actinomycetes in marine snow) could be technique-derived (or) reflect geographical and ecological heterogeneity of marine snow (Grossart *et al.,* 2004).

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Materials and methods

Environmental sampling

The sampling locations are shown in Fig. 5.

Surface sea-water was collected with sterile glass bottles; where as deep-water samples were taken with a J–Z sample or a Niskin bacteriological sampler, which was sterilized before use (Kassell *et al.,* 1970). Many thousands of Actinomycetes have been isolated from the environment, but relatively little is known about the ecological or geo-graphical distributions of even the better known neutrophilic streptomycetes. In practice, samples are often taken at random or from habitats where the microbial community is adapted to relatively extreme conditions (Schlegel & Jannasch, 198). It is also advisable to examine environmental samples using media and incubation conditions that promote the isolation of metabolically diverse strains.

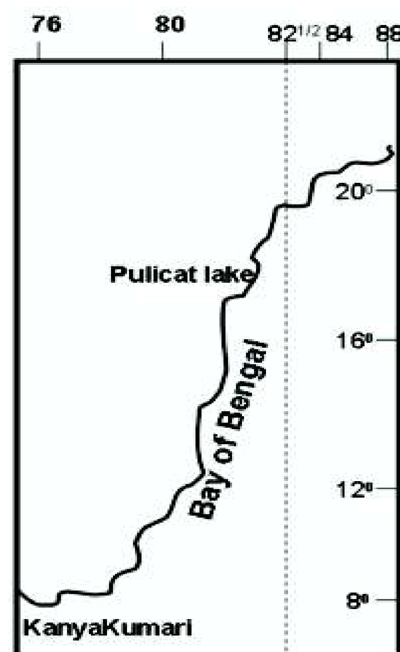

Fig-3. Sampling location at Pulicat Lake to KanyaKumari, Bay of Bengal

Pretreatment of samples

Selectively may be enhanced by chemical or physical pretreatment of environment samples or propagules in suspension prior to plating onto selective media. The pretreatment methods often exert a clear selectively for the isolation of members of particular actinomycetes taxa, but the scientific basis for their action is not always evident. An ingenious addition to pretreatment methods involves the addition of a cocktail of polyvalent streptomycetes on isolation plates (Williams & Vickers, 1988). The method is simple and phage has the advantage of being specific to genera or organisms with the same well chemo type.

Membrane filtration and centrifugation are used to concentrate Actinomycetes propagules in soil, water and sediment samples. Similarly nutrient Enrichment of environmental samples and soil suspensions has been used to increase the number of streptomycetes prior to isolation. A useful departure from the dilution plate procedure involves the isolation of thermophillic Actinomycetes from dry, self-

Selective agent	Target Organisam	Authors
Bruneomycin	Micromonospora	Preobrazhenskaya *et al.* (1975)
Gentamicin	Microsporamono	Ivanitskaya *et al.* (1978)
Kanamycin	Actinomadura	Bibikova et al. (1981)
Novobiocin	Micromonospora	Chrmonova (1978) Sveshnikova *et al.* (1976)
Tellurite Tunicamycin	Actinoplanes Micromonospora	Willough-by (1971) Wakisaka *et al.* (1982)

Table-2. Antibacterial compounds used in selective media for the isolation of actinomycetes

heating plant material. The latter is shaken in a wind tunnel or sedimentation chamber and the spore clouds obtained impacted onto surface-dried isolation plates held in an Andersen (1958) sample. This method has been successfully used to isolate mycolateless wall chemo type IV Actinomycetes which are a source of several important antibodies.

Water can be heated to over 100°C to reduce the number of unwanted bacteria in order to enhance the recovery of rare actinomycetes less extreme heat pre-treatment regimes have been used to isolate micromonosporas Nocardias, Rhodococci and Streptomycetes. It is not known why Actinomycete propagules, both spores (e.g. streptomycetes) and hyphal fragments (e.g. Rhodococci), are more resistant to heat than the vegetative cells of Gram-negative bacteria .It may be possible to derive taxon specific heat pretreatments as there is evidence that Actinomycetes have different heat sensitivity profiles.

Choice of isolation media

The selectively of an isolation medium is controlles by its nutrient com position, pH the addition of selective inhibitors and by incubation conditions. Large numbers of media formulations have been recommended for the isolation of actinomycetes in general and for selected genera in particular. Surprisingly, many 'general' or 'non-selective' media were formulated without regard to either , the nutrient properties or tolerances of the target actinomycetes .Many of these media, such as colloidal Chitin, half- strength nutrient, Starch casein and M_3 agars (Row Bothan & Cross, 1977) are widely used, through little attempt has been made to evaluate their effectiveness. This point was clearly demonstrated when the use of various objectively formulated selective media resulted in qualitative differences in streptomycetes isolated from the same soil sample. It is now apparent that general isolation media can be selective for streptomycetes, notably those belonging to the streptomycetes albidoflavus (griseous) group.

Antimicrobial agents, notably anti-biotic, have been effectively used to improve media selectively. It is now standard practice to eliminate fungal contamination by supplementing isolation media with antifungal antibiotics, such as actidione and nystatin, which do not inhibit actinomycetes (e.g. Gregory & Lacey 1963; Williams & Davies, 1965).

Incubation

The temperature and length of incubation also contribute to selectivity. Incubation at 25°–35°C favours meso-philic bacteria, and incubation at 55°C enhances the chances of isolating thermo-tolerant and thermophilic actino-mycetes.

Nonomura and Ohara (1971) succeeded in isolating several new species of less common actinomycete genera when they incubated isolation was also one of then factors contributing to the isolation of the slow-growing mutualistic symbiotic Frankia. Little attention has been paid to the selective isolation of psychophilic, anaerobic or autotropic Actinomycetes. Falconer (1988) isolated facultative autotrophic Actinomycetes using gaseous C_1 compounds and showed that they formed a distinct and diverse group.

Selection of colonies

This is still the most time-consuming step in the isolation procedure. When selective isolation media are used the target organisms can often be presumptively identified using a high-power lens with a long working distance objective. The ability to recognize members of actinomycete taxa on primary isolation plates require a blend of skill and experience that will be difficult, if not impossible, to automate. However, with image analysis and the application of sophisticated software, if may be possible, to design systems capable of recognizing certain colony types directly on isolation plates. Thus the majority of streptomycete soil isolates, grouped by easily determines pigmentation characteristics, are identified to corresponding clusters in a probability matrix (Williams & Vickers, 1988). Surface spread plates, dried thoroughly before and after inoculation, provide the most satisfactory way of recognizing enumerating and isolating streptomycete colonies (Vickers & Williams, 1987).

Taxonomic approaches to selective isolation

Selective media can be prepared on objective criteria given the advances in actinomycete systematics that have been described. Indeed, nutricat taxonomic data bases, which contain extensive information on then biochemical, nutritional, physiological and antimicrobial sensitivity profiles of the constituent taxa, are ideal resources for the formulation of isolation media deemed selective diagnostic methods allow for industrially significant actinomycete taxa. Further, improved diagnostic methods allow selective media to be evaluated as representative colonies can be identified with confidence.

Formulation and evaluation of new selective media

The discovery that diagnostic sensitivity test agar supplemented with tetracyclines was selective for *Nocardia asteroides* was based on antibiotic sensitivity data and the product of an earlier numerical phonetic survey. This observation raised the possibility of reducing the number of *streptomycetes* and other unwanted bacteria on isolation plates, with a view to isolating *Strepto verticillium*, a commercially significant group of Actinomycetes rarely recovered on 'genera' isolation media. Hanka, Ruekert & Cross (1985) were able to raise the proportion of *Strepto verticillium* colonies on isolation plates using an agar medium supplemented with oxy-tetracycline and then membrane filter stripping method of (Papke R.T ward D.M., 2004).

The most extensive studies have been carried out by Williams and his colleagues (Simon.M., Grossart, H.P., Schweitzer B.,Ploug,H.,2002). who used particular combinations of carbohydrate and amino acid, with and without antibacterial antibiotics in order to favour the growth of uncommon *streptomycetes* previously shown to be promising source antibiotically active metabolites or to discourage the growth of the ubiquitous Actinomycetes. The selective agents were objectively chosen by examine the neutrophilic *Streptomycete* data base (Williams *et al.,* 1983b) using the DIACHAR program (Sneath, 1980) which selects the most diagnostic characters for individual clusters. The highest diagnostic scores are given by characters which are all positive or negative strains in one cluster when compared with all of the other numerically defined taxon.

Similarly, acidophilic actinomycetes belonging to cluster 25 (Lonsdale, 1985) can be instantly detected as they produce characteristic pink pigmented colonies on starch casein agar supplemented with rifampicin (Simpson, 1987). Members of this taxon form a homogeneous group on the basis of chemical data. There seems to be no reason why a strategy developed for actinomycetes should not be extended to include other industrially important bacteria, such as aerobic, endospore-forming bacilli. Extensive data bases are already available on *bacillus* (Priest, Goodfellow & Todd, 1988).

The medium, glucose yeast extract agar supplemented with rifampicin, had been seeded with dilutions of diverse, heat pretreatmented soils using a procedure designed for the selective isolation of Actinomadura strains (Athalge *et al,* 1981).

Recognizing novel taxon

The choice of actinomycetes for industrial screens, especially those with allow throughout is essentially a problem of distinguishing between known Actinomycetes and recognizing new ones (O' Donnel, 1986;1988).This problem is partly historical, the need to identify unknown actinomycetes having rarely been recognized as a relevant task for industrial microbiologists, Consequently, current procedures for the selection of actinomycetes for screening owe more to custom and practice than to any rational design for practical purposes, novel actinomycetes can be regarded as those organisms which can't be assigned to establishment taxa although, for those working with an "in house" screening system and associated data base, it may be better to describe a novel organism as one which has not been screened previously. The latter definition is particularly important with respect to 'target-directed screening' in which the objective of the analysis is to provide more representative of known or target organism. Since these vary widely, so do the criteria used to justify the descriptions of novel taxa (Williams *et al.* 1984a).

The reorganization of novel actinomycetes taxa is complicated by the fact that many classifications and identification systems are based on type strains which have been isolated from soil using classical enrichment techniques. Consequently, the majority of strains show low substrate specificity and are selected on the basis of their maximum growth rate. Similarly, sample pre-treatment selects for those organisms resistant to the physical and chemical agents used prior to inoculation making it unlikely that the resultant classification will truly reflect the natural diversity of strains (O' Donnell, 1988).

Unfortunately, such schemes are not available for all microbial groups and those which are waiting through testing with strains from a range of natural habits. The characterization and identification of novel Actinomycetes is essentially a two-fold process. Reliable criteria are needed to assign an organism to a genus and family prior to the selection of diagnostic tests for identification to specific or sub specific levels. Identification of genus level and above can usually be achieved using a combination of morphological and chemical properties (Lecheralier, 1988; Goodfellow & Cross, 1984; Good fellow, 1989) but few reliable and well tested schemes are available for the differentiation of species & biotypes. Those which are available still involve considerably more effort than can easily be accommodated in the majority of industrial screening laboratories.

Screening

It is possible to view all systematics as chemosystematics, since morphology, pigmentation, serology and the biochemical properties of micro organisms reflect their chemical composition, but it is useful to restrict the definition to the study of the distribution of specific chemical components such as lipids, wall amino acids, sugars and proteins amongst microbial taxa. Such information can be used at all taxonomic levels and for some Actinomycetes taxa details of their lipid and wall di-amino acid composition are already included as past of the species description (Schaal, 1986; Wayne & Kubica,1986; Williams, 1989).

It is now apparent that the discriminating power of chemical criteria rises between taxa. Different chemical methods are required at different stages of screening programmes. The first step to determine whether the objective of including the chemical methods is to assign recent isolates to established taxa or to provide a mechanism by which previously analyzed organisms are excluded from screens. By analyzing key chemical markers, the situation in which meso-diamino pimelic acid containing sporo actinomycetes are subjected to the 41 biochemical tests described by Williams *et al.* (1983b), and identified as novel strains of streptomyces can be avoided.

Pyrolysis techniques such as pyrolysis mass spectrometry (Shute *et al,* 1985) and pyrolysis gas chromotogaphy (O 'Donnell & Norris, 1981) are now finding their way into some industrial screening laboratories. The data produced are complex and need to be analyzed using suitable data analysis routines. Fortunately, these statistical routines are readily available with many of the packages suitable for personal computers (Bratchell, MacFie & O' Donnell, in press).

Probabilistic identification systems are widely used to identify unknown strains such schemes are more reliable than monthetic sequential keys and diagnostic tables which are susceptible to test rank and test error (Sneath, 1974). Several investigations have emphasized that the quality of identification matrices is critically important. Ideally, taxa should be homogeneous and well separated by the discriminatory tests. It is now good practice to check that strains known to belong to a taxon identify with a high probability and those taxa do not show any significant overlap. Theoretically sound, workable computer-assisted producers are available for the identification of slow growing mycobacteria

(Wayn *et al.*, 1980), *Strepto verticillia* (Williams *et al.,* 1985b) and neutrophilic actinomycetes (Williams *et al.* 1983b). More recently, a probability matrix has been assembled for the identification of unknown acidophilic Actinomycetes (Saddler).

Processes

Micro organisms producing alkaline protease were screened using a casein double layer agar plate method. The upper layer was composed of MP and with 1% Hammarsten casein, while the lower layer contained only MP. The purified isolates were stab inoculated on the plates (90 mm) and incubated at 20°C for 5 days. Five strains were examined on one plate. When this method was employed, as many as 300 strains could be screened in a day. After incubation, 1ml alkaline protease subtilisin (Nagase Sangyo Co. Ltd. Japan) (500 ug/ml) was added to the plates and they further incubated for 6 h at 25°C. Isolates that formed a white, opaque inhibitory zone (halo) were considered to be strains that produce alkaline protease.

A total of 300 strains i.e marine samples were collected from different locations of the Bay of Bengal starting from Pulicat lake to Kanyakumari and 208 isolates of marine actinomycetes were isolated using starch casein agar medium. The growth pattern, mycelial collaboration, production of exo-polysaccharides and diffusible pigment and abundance of *Streptomyces* spp. were documented . Among marine actinomycetes *steptomycetes* spp. were present in large proportion (88%).

Isolation

The natural-product search and discovery as a major driving force in the detection of Actinomycetes diversity. Many studies are culture based, sometimes in complement with molecular techniques (Magarvey *et al.,* 2004). Identification raises from fully charac-terization and valid description to identify phenotype characterized Actinomycetes (Papincau *et al.*, 2005)

Alkaliphilic actinomycetes isolated from sediment samples of the Bay of Bengal were studied for the production of protease activity. Strain MA1-1 was selected as a good alkaline protease producer as measured by the clear zone diameter by the hydrolysis of skim-milk and casein. The alkaline protease production from the marine alkaliphillic actinomycetes MA1-1 was studied by using different carbon and nitrogen source in

medium containing glycerol, peptone, KCl, $MgSo_{42}$, K_2HPO_4, and trace elements at 30^0C for 72 h. Among the different carbon and nitrogen sources, fructose, starch, maltose, D(+) glucose, yeast extract, malt extract, beef extract, and peptone provided higher production of protease. Starch was also found to be effective for growth and enzyme production with highest specific activity at 699 U mg^{-1}.

Result and Discussion

Culture based and molecular based approaches provide way different views of actinobacterial diversity. Two seminal lines of research promise more integration and broader picture. First there is the screening and isolation of Actinomycetes which produce protease. This not only uses classical marine molecular microbial ecology terminology for Actinomycetes isolates but also has isolated unequally widespread, persistent marine Actinomycetes with a distinct bio-geography.

This argues more persuasively for the presence of marine Actinomycetes than does the complex picture of the isolation of terrestrially related actinomycetes from Bay of Bengal (Jensen *et al.*, 2005).

Result of screening test

Three hundred marine samples were collected from different locations of Bay of Bengal from Pulicat lake to KanyaKumari. Among them 208 isolates of marine Actinomycetes were isolated using differential media such as starch casein agar medium. Among 208 marine Actinomycetes, 111 isolates exhibited antimicrobial activity against human pathogens and 151 showed antifungal activities against two plant pathogens. Among 208 isolates, 183, 157, 116, 72 & 68 isolates produces lipase, protease caseinase, gelatinate, cellulose & amylase respectively. The results of diversity, antimicrobial activity and enzyme production here increased the scope of finding industrially important marine Actinomycetes from the Bay of Bengal and these organisms would be vital sources for the discovery of industrially useful molecules enzymes.

Result of isolation techniques

Alkaliphilic actinomycetes isolated from water samples were studied for the production of protease activity. Strain MA1-1 was selected as a good alkaline protease producer. Starch was also found to be effective for the growth and enzyme production with highest specific activity at 699 U mg-1.Purification was achieved by adsorption on Diaion HP 20 which resulted in a recovery rate of 68% with a specific activity of 7618 U mg^{-1} protein and 40-fold purification.

The optimum pH and temperature of the partially purified protease were determined as pH 9.0 and 50°C, but high activity was also observed at pH 8.0–13.0 at 35°–50°C. The inhibition profile exhibited by Phenyl Methyl Sulphonyl Fluoride (PMSF) showed that this enzyme belongs to the serine-protease group.

Conclusion

The objective of this study is to produce enzymes of distinguished characters with special properties and in addition which produce lipase, cellulose, amylase in the process of isolation techniques. This method of protease production by isolation and screening techniques from marine Actinomycetes is helpful for the production of desired or qualified protease from marine water.

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