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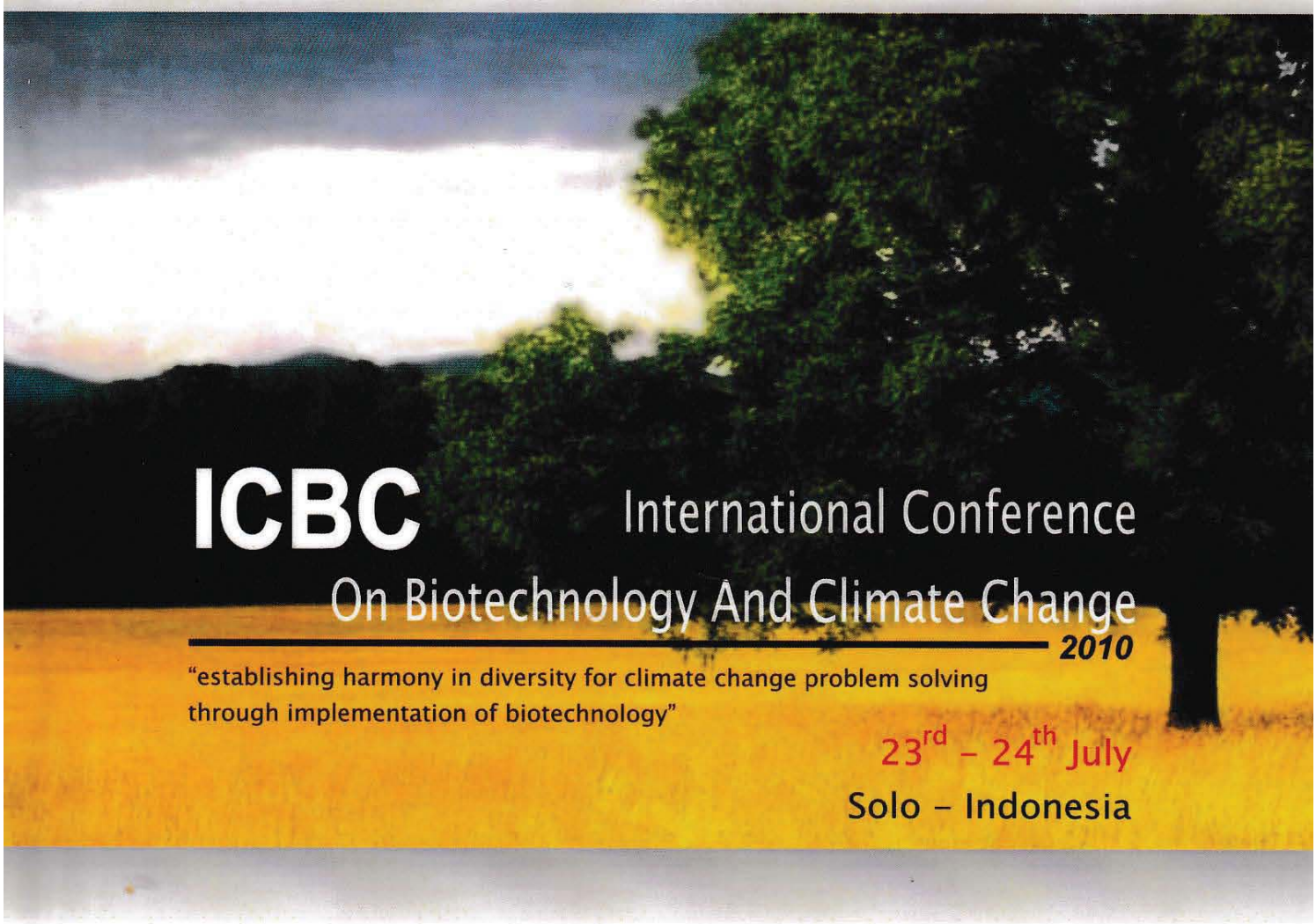

In collaboration with :

- INTERNATIONAL CONFERENCE -
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PROCEEDING

"Establishing Harmony in Diversityy for Climate Change Problem Solving through Implementation of Biotechnology"

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- Ministry of Environment - Indonesia
- Association of Indonesian Environmental Expert (IALHI).
- Society for Indonesian Biodiversity **(MBI).**
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PREFACE

The world is facing multiple problem of environment, especially global climate change lingkage with biodiversity utilization and management. They were affected by the accelerated natural resources utilization without an effiently technology. Over-harvest and mismanagement of biodiversity are rising natural degradation and global climate change. Other way the global climate change will be threatening at alarming rate due to the extinction of biological resources from gene to ecosystem. Biotechnology is one of the valuable toot to increase efficiency and effectiveness of biological resource utilization, so can be a benefit to environmental problem solving, especially in global climate change. The quality and quantity of biodiversity-based products can be increased by implementing biotechnology. Every scientific and practice information about biodiversity and environment problematic is necessary to develop an advanced biotechnology. For this purpose, especially on biodiversity, biotechnology, and climate change will be comducted with special theme: "Establishing harmony in diversity for climate change problem solving through implementation of biotechnology.

Solo, July 2010

Sugiyarto

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Marine Actinomycetes as a Potential Source of Bioactive Compounds

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ABSTRACT

The Sea is a complex world which have diverse forms of life including the existence of extreme variation of pressure, salinity and temperature. Marine actinomycetes growing in different habitats than terrestrial actinomycetes, with different stress and obstacles, and become dependent on the marine environment. Adaptation of marine actinomycetes against this condition, allegedly causing microorganisms have the ability to synthesize the metabolite that is unique and not found on the mainland.

Recently, the rate of discovery of new compounds from existing genera obtained from terrestrial sources has decreased. Thus, it is crucial that new groups of actinomycetes from unexplored or underexploited habitats be pursued as sources of novel compounds. The diversity of metabolites produced by marine actinomycetes is a source for the discovery of new bioactive compounds. These efforts can be done through two approaches, namely the molecular detection of genes encoding the enzymes that play a role in the biosynthesis of certain secondary metabolites and the isolation of active compounds by bioassay guided isolation that combines chemical separation techniques with in vitro testing techniques. Identification of marine actinomycetes producing bioactive compounds can be conducted by analyzing its 16S rRNA gene sequences as molecular chronometers. Phylogenetic analysis will show a relationship between the isolates that we found with isolates that have been found previously.

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Keywords: marine actinomycetes, bioassay guided isolation, molecular detection, 16s rRNA.

INTRODUCTION

Nature has provided many things for humankind, including the food and medicine. Until recently, natural products occupy an important role as the main source to the diversity of the chemical structure of bioactive compounds, which can be used as the starting material for the discovery of drug. Despite a recent de-emphasis natural products research by the pharmaceutical industry, no other drug discovery platforms are proven as effective as natural products to contribute a unique chemical structure. In this case, research on natural products can be used directly in treatment or serve as scaffolds in which molecules with enhanced efficacy can be derived (Jensen, *et al.*, 2005).

The existence of natural products as sources of bioactive compounds have been known since 100 years ago. Natural product broadly defined as compounds produced or isolated from living organisms, like plants, animals and microorganisms. This compound is divided into two types, namely primary metabolites and secondary metabolites (Bérdy, 2005; Yoder, 2005; Peraud, 2006). Primary metabolite associated with the normal process of anabolism and

catabolism, whereas the secondary metabolites are compound with unique structure and limited distribution to one particular species or groups of organisms (Dewick, 2002; Yoder, 2005). These compounds were synthesized through secondary metabolic pathway, have a small molecular weight (<3000), no clear in chemically and taxonomy functions, and might not contribute directly to the survival of producing organisms (Bérdy, 2005). These compounds become an important part of the natural products because they show various biological activities, including antitumor, antibiotic, antiparasitic, herbicides, feed additives, as well as several other uniqueness bioactivity in the field of pharmaceutical and medicine.

Among the various sources of secondary metabolites, microorganism is considerable as a potential source. Microoganism can perform a simple but interesting reactions, such as the mechanism of isomerization of n-butyrate isobutirat into the lactone ring-16 which turned out to involve the entry of valine. Synthetic products that previously regarded as non-natural compounds was found in culture broth of microorganisms (Omura, 1986). Various products of secondary metabolites derived from microbial fermentation give a very great benefit for humans, animals, and agriculture. As a producer of secondary metabolites, microorganisms with different strains will usually produce different compounds. So, the search of new bioactive compounds was strongly supported with the diversity of extraordinary strains of microorganisms. However, not all microorganisms have the ability to produce secondary metabolites. This ability is sometimes limited to small groups of bacteria and prokaryotic microorganisms, such as the group actinomycetes, myxobacteria, pseudomonas, and cyanobcteria. While from eukaryotic microorganisms, filament type fungi are sufficient potential producers.

WHAT IS ACTINOMYCETES?

The English word 'actinomycete' means actinomycete - any bacteria (some of which are pathogenic for humans and animals) belonging to the order Actinomycetales. Actinobacteria or actinomycetes are a group of Gram-positive bacteria with high G+C content in their DNA. They are microscopic bacteria that look, and sometimes behave like fungi. They have a tendency to form chains or branching filaments that resemble the mycelia of filamentous fungi. They are sometimes considered as an intermediate group between bacteria and fungi (Ghanem et al., 2000; Mincer et al., 2002).

In general, actinomycetes subspecies have aerobic character, although some are able to live in an anaerobic condition. Actinomycetes can be found at various places, but they prefer alkaline or neutral conditions for growth. Optimal pH range for actinomycetes growth is between 7-8. However, actinomycetes can live under the acidic conditions at pH 4.8 to 5 as a critical condition. Most actinomycetes grow at temperatures 15 - 30°C, but some genus of actinomycetes such as thermophilic actinomycetes can live at a temperature above 60°C (Waksman, 1950; Goodfellow *et al.*, 1 983).

Actinomycetes produce long, spider web-like strands throughout decomposing organic materials. These bacteria are primarily saprophytic and are best known from soils where they contribute significantly to the degradation of complex biopolymers, such as lignocellulose, hemicellulose, pectin, keratin, and chitin (Mincer, *et al.*, 2002). They include some of the most common soil life, playing a vital part in organic matter turnover and carbon cycle. Despite their important role in soil ecology, bacteria belonging to the family Actinomycetaceae are well known for their ability to produce secondary metabolites. The history was take a part on 1940, when Selman Waksman found actinomycin, compound which was recognized as an antibiotic (Magarvey, 2004; Yehuda, 2005).

WHY MARINE ACTINOMYCETES?

The need of new compounds as drug candidates requires some research for searching new sources of natural product. But the data showed that the diversity of new compounds from actinomycetes found in soil until the year 1995 were decreased, while the re-isolation didn't show a novel compound with promising activities. The idea of exploration of marine actinomycetes was started by this reason. Although microorganisms that live on the mainland has incredible diversity, but the greatest biodiversity was found in oceans. More than 70% of the earth's surface is covered by

ocean and life on Earth has its origin in the sea. Experts estimate that in some marine ecosystems such as the deep seabed and coral, there is a higher biological diversity than the tropical rainforest (Haefner, 2003).

Marine environment is a source of new actinomycetes diversity and a source of new metabolites that hasn't been widely used (Lam, 2006). The presence of marine actinomycetes population has been known since 30 years ago, but the distribution of actinomycetes in the sea has not been explored on a large scale. Until now, the existence of indigenous marine actinomycetes remain difficult to comprehend. (Jensen and Fenical, 2004; Lam, 2006). This group of bacterial allegedly found widespread in the whole sea "world" and seabed sediments, intertidal zone and subtidal zone (Magarvey, 2004; Yehuda, 2005). Ecological roles of marine actinomycetes is still an open question, but the bacteria are likely involved in the decomposition of complex organic materials and biopolymers such as chitin, which is present in large numbers in marine waters (Pathom-Aree, 2006, Jensen *et al.*, 2005). Actinomycetes are defined as marine actinomycetes if they require seawater for growth (Yehuda, 2005).

A prevailing sentiment expressed in the literature suggest that the vast majority of marine actinomycetes are of terrestrial origin. This opinion arise from the fact that terrestrial actinomycetes can produce resistant spores, which then allegedly carried by water from land to sea, but dormant for several years. This theory persists even there is proved that marine actinomycetes can be obtained from deep-sea sediments and have bioactive compounds as well as having physiological adaptation to sea high salinity water. *Rhodococcus marinonascens* is the first marine actinomycetes species found and accepted as indigenous marine biota (Ghanem et al., 2000; Mincer et al., 2002; Yehuda, 2005; Peraud, 2006; Lam, 2006).

Marine Actinomycetes grow in different habitats than terrestrial Actinomycetes, with different stress and obstacles. They included the high pressure conditions (almost 1100 atm) and anaerobic conditions at temperatures below 0°C on the seabed surface, and very acidic conditions (pH less than 2.8) at a temperature of more than 100°C on near hydrothermal vents at mid ocean. This will appear at genetic and metabolic diversity of marine actinomycetes that have not been widely known. Because they become dependent on the marine environment, it was suspected that this bacterium has been producing unique metabolic and physiological capabilities that are not found in terrestrial actinomycetes. This part makes them an excellent candidate for the synthesis of new secondary metabolites. Marine Actinomycetes character and their adaptation rates that affect the synthesis of secondary metabolites are potential sources for the discovery of new bioactive compounds that eventually lead to the development of new drug compounds (Pela'ez, 2006).

DIVERSITY OF BIOACTIVE COMPOUNDS ISOLATED FROM MARINE ACTINOMYCETES

The ability of marine actinomycetes in producing the active metabolite were shown by their adaptations of inhabiting organic sea aggregates (marine snow), their antagonist

Table 1. Metabolites produced by marine Actinomycetes during the year 2003-2005 (Lam, 2006)

Compound	Source	Activity
Abyssomicins	*Verrucosispora* sp.	Antibacterial
Aureoverticillactam	*Streptomyces aureoverticillatus*	Anticancer
Bonactin	*Streptomyces* sp.	Antibacterial; antifungal
Caprolactones	*Streptomyces* sp.	Anticancer
Chandrananimycins	*Actinomadura* sp.	Antialgt; antibacterial; anticancer; antifungal
Chinikomycins	*Streptomyces* sp.	Anticancer
Chloro-dihydroquinones	*Novel actinomycete*	Antibacterial; anticancer
Diazepinomicin (ECO-4601)	*Micromonospora* sp.	Antibacterial; anticancer; anti-inflammatory
3,6-disubstituted indoles	*Streptomyces* sp.	Anticancer
Frigocyclinone	*Streptomyces griseus*	Antibacterial
Glaciapyrroles	*Streptomyces* sp.	Antibacterial
Gutinglmycin	*Streptomyces* sp.	Antibacterial
Heiquinoline	*Jambacter limosus*	Antibacterial
Himalomycins	*Streptomyces* sp.	Antibacterial
IB-00208	*Actinomadura* sp.	Anticancer
Komodoquinone A	*Streptomyces* sp.	Neutilogenic activity
Lajollamycin	*Streptomyces nodosus*	Antibacterial
Marinomycins	*'Marinispora'*	Antibacterial; anticancer
Mechercharmycins	*Thermoactinomyces* sp.	Anticancer
MKH-349A	*Nocardiopsis* sp.	Unknown biological activity
Salinosporamide A (NPI-0052)	*Salinispora tropica*	Anticancer
Sporolides	*Salinispora tropica*	Unknown biological activity
Trioxacarcins	*Streptomyces* sp.	Antibacterial; anticancer; antimalarial

activities to sustain life, and their role in decomposition and mineralization of organic compounds. Marine actinomycetes form a stable population, persistent in many different habitats in marine ecosystems, indicate the existence of indigenous marine actinomycetes in the marine environment.

Salinospora, it found some compounds with bicyclic framework of ie salinosporamide A which possess activity as an inhibitor of proteolytic activity of proteasome. These compounds suspected of having the basic halogenated framework. Salinosporamide B has a structure similar to salinosporamide A, but the loss of Cl from the structure salinosporamide A causing its biological activity decreased 500 times. This means that the substitution of sea water element affects the biological activity in the total structure of a molecule (Mincer, 2002; Jensen *et al.*, 2005; Lam, 2006).

Salinispora also produce other compounds, sporolides A and B, which is a polyketides compound. Both of them have similar structures, differ one to another only in the position of chlorine atoms, but does not indicate a specific biological activity (Lam, 2006). Marinomicyn A and B which were isolated from the genus Marinophilus are compound with a stereocenter, the structure of these compounds is almost the same. Although their basic framework is a makrolida polyenes, but they didn't show antifungal activity, such as polyene antibiotics. Marinomicyn A and B are cytotoxic against NCI 60 panel cell and antibacterial activity against two antibiotic-resistant bacteria (Jensen and Fenical, 2004).

Streptomyces sp. CNB-091 has six Salinamides, five of them have been characterized and each Salinamides was named as A, B, C, D, and E. The sixth Salinamides is reported in only small quantities, making it difficult to identify. Salinamides A was reported to have potential as anti-inflammatory and antibiotic against gram-positive bacteria. Moore *et al.* (1999) was written down that the basic framework on Salinamide A is possible a peptides structure derived from hexapeptide,involving NRPS biosynthetic pathways.

Streptomyces strain B8005 from the Gulf of Mexico lagoon produces resistomycin and its derivatives, 1-hidroxy-1-noresistimycin. They are quinone compounds which has dekaketide basic framework. These two compounds differ only in one position substitution, suspected that both of them have the same biosynthetic pathways but differ in the last step of biosynthetic pathways. Resistomycin, one-hidroxy-1-noresistimycin and tetracenomycin have antibacterial and antitumor activity, whereas resistomycin and resistoflavin have antiviral activity and antiprotozoals, as well as antioxidants on testing of DPPH method (Kock, et al., 2005).

Lam (2006) has reviewed various studies on secondary metabolites of actinomycetes isolated from the sea and the spectrum of their activities, as shown in table 1 below. This indicates that marine actinomycetes produce compounds with different structures and various activities.

HOW TO DISCOVER NOVEL BIOACTIVE COMPOUND FROM MARINE ACTINOMYCETES

The efforts to find a novel compound from marine actinomycetes can be done through two approaches, 1) detection of genes encoding the enzymes that play a role in the biosynthesis of certain secondary metabolites and 2) isolation of active compounds by bioassay guided isolation that combines chemical separation techniques with in vitro testing techniques. The first approach emphasizes the role of molecular biology as one of the techniques used in exploration of natural products. Secondary metabolites are not essential for growth and reproduction, but mainly used as a defense mechanism for Actinomycetes to compete in nature.

Biosynthetic pathways of secondary metabolism usually involve a series of reactions catalyzed by various enzymes. Because of its specificity, each enzyme will catalyze a particular stage of the biosynthesis mechanism. Through this variety of secondary metabolic pathways, a various actinomycetes can produce a variety of bioactive compounds. Genes detection in this regard can be done by designing specific primers for amplification of the specific gene sequences that play a role in the biosynthesis of targeted secondary metabolites. Targeted gene sequence amplification technique can be conducted using Polymerase Chain Reaction (PCR), while its visualization can be done by electrophoresis. One example is research by Genilloud and Ayuso (2004). They have succeeded in designing primers for gene amplification of PKS/NRPS which showed that NRPS gene amplification

using A3F/A7R primers generate DNA fragments with a size of 700-800 bp. In other side, the PKS-I gene amplification using K1F/M6R primers generate DNA fragments with a size of 1200 - 1400 bp. PKS and NRPS is an enzyme involved in the biosynthesis of bioactive compounds produced by microorganisms, including actinomycetes. Based on their structure, the PKS and NRPS are multifunctional polypeptides arranged into several modules with different enzymatic activity. Each module consists of three minimum domains, namely β-ketoacyl synthase, acyltransferase, and acyl carrier protein. PKS-I gene or NRPS was not shared by all the actinomycetes, but there are some actinomycetes species that have both of them.

If the targeted gene was successfully amplified, the next steps are carried out to purify and sequencing to determine the base sequences of targeted gene. The sequences were then compared with the NCBI data bank. Here we will show a certain percentage of homology, which indicates the similarities of our isolated compounds compared to the known secondary metabolites. Through bioinformatics analysis, it can be known whether we have been isolated the new compounds or only rediscovery of known compounds previously (Ansari et al., 2004).

The second approach is a combination of chemical separation techniques with in vitro testing techniques. Secondary metabolites were synthesized through a pathway which associated with primary metabolism and influenced by them. Intermediates compounds of primary metabolism metabolites serve as precursors for the biosynthesis of secondary metabolites. In addition, the composition of culture medium was also related to the ability of organisms to produce metabolites, which would affect the bioactive compounds produced. The same single strain is likely to produce different secondary metabolites when cultured on different medium (Pelaez, 2005). These active compounds are usually produced by extracellular fermentation. The isolation of compounds from fermentation medium involve a combination of various separation techniques such as extraction, precipitation, column chromatography, HPLC and other separation techniques. The screening process was guided by biology testing of many targeted activities such as antibiotics, antifungal, anticancer, antiparasitic, etc. Only the extracts which showed potential activities would be prioritized for follow up studies.

The next step after screening process is the purification and identification of compounds responsible for the biological activity detected in the extract. At this point it is critical to have an efficient system to identify uninteresting or already known compounds as early as possible. Here we can use many spectroscopic methods for elucidate the chemical structure of targeted compounds.

In addition to elucidation of bioactive compounds, other factors are quite important in the exploration of natural product from marine Actinomycetes is the identification of its metabolites-producing strains. The part of the DNA now most commonly used for taxonomic purposes for bacteria is the 16S rRNA gene. The 16S rRNA gene is also designated 16S rDNA, and the terms have been used interchangeably: current ASM policy is that "16S rRNA gene" be used. The 16S rRNA gene can be compared not only among all bacteria but also with the 16S rRNA gene of archeobacteria and the 18S rRNA gene of eucaryotes.

This gene was choosen base on the fact that it seems to behave as a molecular chronometer. The degree of conservation is assumed to result from the importance of the 16S rRNA as a critical component of cell function. This is in contrast to the genes needed to make enzymes. Mutations in these genes can usually be tolerated more frequently since they may affect structures not as unique and essential as rRNA (if a bacterium does not have the gene to make the enzymes needed to utilize lactose, it can use an alternative sugar or protein as an energy source). Thus, few other genes are as highly conserved as the 16S rRNA gene. Although the absolute rate of change in the 16S rRNA gene sequence is not known, it does mark evolutionary distance and relatedness of organisms. Although it is generally recognized that the 16S rRNA analysis yields satisfactory results at delineating prokaryotic taxa down to family and genus level, but a severe limitation occurs in its incapability to provide sharp resolution at the species level and lower.

CONCLUTIONS

Secondary metabolites from Actinomycetes, whether generated directly from the fermentation and its derivatives, or compounds which are obtained from chemical modification, are potential sources of bioactive compounds. They give a huge benefit for both humans,

animals, and agriculture. Because of these secondary metabolites produced by fermentation of microorganisms, the factor that plays an important role is the biological screening that includes a) the proper use of biological materials, such as bacteria, animal cells, enzymes and the other for the detection of specified activities, 2) testing capability with small quantity of compounds that are easily and quickly, and 3) the ability to modify the conditions of isolation and cultivation of microorganisms

Efforts to find a novel bioactive compounds from marine actinomycetes is still open widely and it will be very useful to isolate strains of new species or genera. Rare actinomycetes are usually quite interesting, because it has the possibility to produce undiscovered bioactive substances. There are several factors critical to the discovery of novel bioactive compounds, among others a) the number of strains that were screened and the level of diversity, 2) the uniqueness of each strain, 3) its potential for producing secondary metabolites.

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This is to certify that

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