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## Bioactive Compounds from Bacteria Associated to Marine Algae

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### 1. Introduction

Since ancient times, humans have sought to satisfy their needs, one of which is, without a doubt, to stay alive. The fear of getting sick and dying, led man to study the organisms that surround him, discovering that the chemicals compounds present in some of them could be beneficial for treating illness. Thus; began the chemistry of the natural products; biotechnology area for human welfare. Several of these organisms produce secondary metabolites, which are part of a wide variety of natural compounds used by humans to combat diseases. Secondary metabolites are defined as organic compounds formed as bio products in organisms, not directly related to growth, development and normal reproduction of thereof. Some examples are fibers (cotton, silk, wool); fuels (oil and natural gas), and medicines (antibiotics, hormones, vaccines).

The importance of finding and using these secondary metabolites can be justified in two ways (1) to know the natural substances that can be beneficial for man and (2) to identify the organisms that produce these substances in order to make a rational exploitation of them, because they may be the only carriers of useful compounds to combat pathogenic microbes.

Marine organisms possess an inexhaustible source of useful chemical substances for the development of new drugs; among these organisms we find marine algae that are capable of biosynthesizing a broad variety of secondary metabolites and bacteria that live in the oceans and that are crucial organisms used in biotechnology in the discovery of new compounds from marine origin.

The discovery of new bioactive compounds necessarily involves previously diversity studies, because by knowing the type of microorganisms that reside in a certain environment, it is possible to design cultivation techniques adapted for all the microbial communities present in a certain ambience. That is why it is very important to identify the organisms that produce bioactive secondary metabolites, and to be able to structure a plan of use and preservation of those species that represent a potential source for new drug development, especially those obtained from bacteria, because of their own cultivation

characteristics, have attracted attention on either a big quantity of investigators on a global scale in the search of new natural products with anticancer and antibiotic activity principally.

## 2. Anticancer activity

Cancer is an illness that comprises more than hundred types. This disease appears when old cells are not replaced by new cells and are accumulated in a mass of tissue known as tumor (Figure 1).

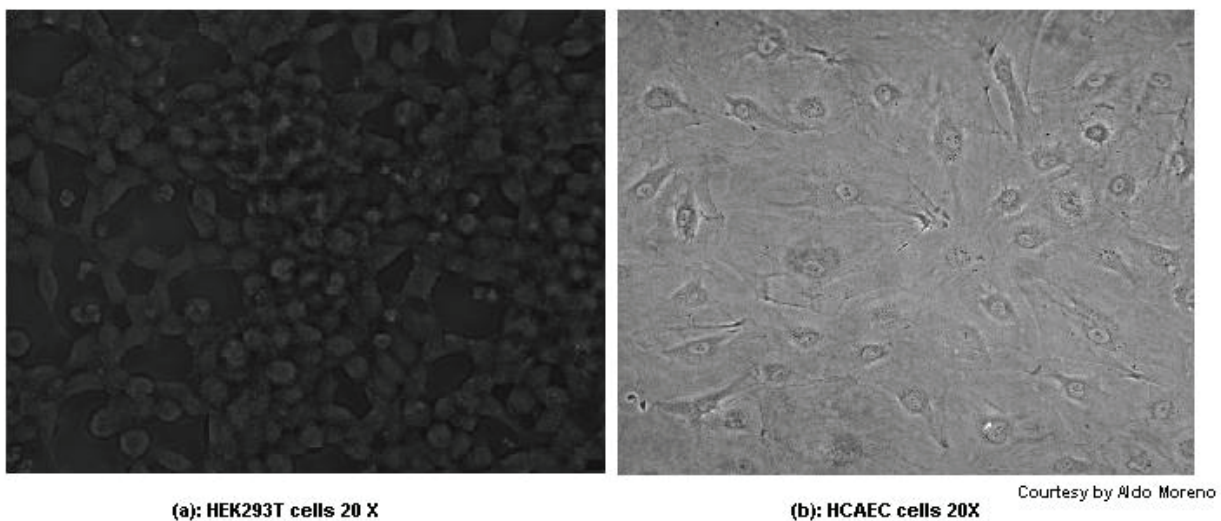


Fig. 1. Pictures of immortalized cells that resemble carcinogenic cells and cells of a normal tissue (20X). (a): immortalized cells of Human Embryonic Kidney Cells (HEK293T) visualized with 20X amplification. (b): Human Coronary Artery Endothelial Cells (HCAEC) visualized with 20X amplification. (A courtesy of Aldo M.Ulloa, UCSD, School of Medicine).

The incidence of cancer increases constantly constituting an enormous challenge for health institutions, in many of the cases, the medicines used in chemotherapy treatment provoke secondary toxicity or resistance (Isnard-Bagnis et al., 2005). For all of the above, there is an urgent need to discover new anticancer compounds from natural sources. Compounds like taxol, camptothecin, vincristine and vinblastine, are obtained from superior plants (Cragg & Newman, 1999), recently some medicines obtained from marine organisms have showed promising results when administered at different cancer stages.

The metabolic and physiological capacity that allows marine organism to survive in extreme conditions provides an enormous potential for the production of unique compounds that are not present in the terrestrial organisms. That is way marine organisms are an attractive source of compounds with pharmaceutical activity, (Faulkner, 2002). Seaweeds are recognized as one of the richest sources of new bioactive compounds of which reviews have been published recently on the biological activity of their derivative compounds (Blunt et al., 2006).

## 3. Resistance to antibiotics

The resistance to antibiotics is a phenomenon by which a microorganism stops being affected by an antimicrobial compound to which previously it was sensitive. It is a

consequence of the capacity of certain microorganisms (bacteria, virus and parasites) to neutralize the effect of the medicine. The resistance can come from the mutation of the microorganism or from the acquisition of resistance genes. The infections caused by resistant microorganisms do not answer to the ordinary treatment, which result in a long illness and the risk of death (WHO, 2011).

Approximately 440 000 new cases of multiresistant tuberculosis produce at least 150 000 deaths every year. In South East Asia infections of *Plasmodium falciparum* that are late in disappearing after the beginning of the treatment with artemisinins are arising, which indicates resistance of the parasite to this specific medicine. Resistance has been found also to the antiretroviral medicines that are used in the treatment of the HIV's infection (WHO, 2011).

A high percentage of the infections contracted in the hospitals are caused by very resistant bacteria, like Methicillin Resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecium* and several microorganisms Gram negative resistant to Vancomycin (WHO, 2011). New mechanisms can appear that can cancel completely the ability of antimicrobial drugs to act against bacteria. This could represent the last defense against multiresistant microorganism's strains. For example, a new  $\beta$ -lactamase, enzyme of the group of the carbapenemases that nowadays are named like NDM-1, gives resistance to the majority of the  $\beta$ -lactams medicines. The enzyme is linked to genes that are easily transferred between the common bacteria, and the infections caused by NDM-1's producer bacteria have no treatment or, if they have it, the therapeutic options are few (WHO, 2011).

#### **4. Factors that enhance the resistance appearance to the antimicrobial effects**

According to the World Health Organization (WHO) several factors exist that enhance the resistance to antibiotics. One problem is the lack of commitment of the government towards solving the problem, the bad definition of the responsibilities of the interested parts and the scarce participation of the consumers those results in the lack of coherent and coordinated methods to anticipate and to contain the resistance to the antimicrobial compounds. The improper and irrational use of antimicrobial drugs promotes conditions for the appearance of resistant microorganisms, which at once propagate. This happens, for example, when the patients do not take the complete treatment of a prescribed antibiotic or when the above mentioned medicine is of bad quality.

The nonexistence or weakness of the systems of alertness determines the lack of information that can guide politicians to make recommendations and to closely continue to monitor the resistance to the existing antimicrobial compounds. Also, the scarcity of diagnosis means more medicines and vaccines for the prevention and treatment of illness, also, the shortcomings on the subject of research and development, debilitates the aptitude to combat the problem (WHO, 2011).

Right now, the WHO is focusing their efforts towards the regulation of the normatively by means of alertness, technical assistance, generation of knowledge and alliances, prevention and control of certain illnesses like tuberculosis, malaria, HIV, proper illnesses of the infancy, sexually transmitted diseases and hospitable infections; the quality, the supply and

the rational use of essential medicines; the safety of the patients; and the guarantee of certified laboratories.

On the other hand, the struggle against the resistance to the antimicrobial compounds was the issue of the World Day of the Health of 2011 (April 7). On this occasion, the WHO called to contain the spread of the resistance to the antimicrobial compounds by means of a set of politics that were recommended so that the governments can start to solve the problem (WHO, 2011).

## 5. Distribution and economic importance of marine algae

Marine algae are not only used in the discovery of new drugs, they are also used extensively as food on the Asian east coast (Japan, China, Korea, Taiwan and Vietnam), Indonesia, Peru, Canada, Scandinavia, Ireland, Wales, the Philippines and Scotland, among other places. From the economic point of view the marine algae represent an important resource of food and industrial input. The Caribbean Sea coast of Colombia contains innumerable species that have an economic value and are used as human food, medicinal products, fertilizers, fuel, and play an important role in the extraction of phycocolloids and hydrocolloids (Teas, 2007). All these products have a big industrial application. In spite of the speculation on the seaweed potential as a direct source of proteins and pharmaceutical products, the demand for phycocolloids will be the factor that will influence the future development of the marine algae world resources.

Many species have been exploited, but others like the genus *Sargassum* and *Codium* have been considered to be invasive for their capacity of adaptation and their high growth rate. Due to the fact that it has been established that marine algae are a potential source for new drugs their study should become a priority. The chemical screening of all the seaweeds and their related organisms is necessary in order to establish which species can be exploited without consequences and those that must be protected.

## 6. Marine algae as producers of secondary metabolites

In 2010, Mexican investigators found that the marine algae *Codium fragile*, *Sargassum muticum*, *Endarachne binghamiae*, *Centroceras clavulatum* and *Laurencia pacifica* possess compounds that inhibit the growth of Gram negative bacteria *Proteus mirabilis* (Villarreal-Gómez et al., 2010), which provokes 90 % of the infections caused by *Proteus*. The bacteria causes the production of big levels of urease that hydrolyze the urea to ammonia increasing the pH and therefore the formation of glazing of struvite, carbonate of calcium and/or apatite, causing the formation of kidney stones.

In the South-west coast of India, a group of scientists studied 13 groups of marine algae to evaluate the cytotoxic, larvicide, nematicide and ichthyotoxic activities on *Artemia salina* larvae. This Indian region is the only marine habitat with great marine algae diversity. 13 algae extracts between them *Dictyota dichotoma* and *Hypnea pannosa* showed lethal effect against the root nematode *Meloidogyne javanica*. *D. dichotoma* and *Valoniopsis pachynema* showed an ichthyotoxic activity. *A. orientalis*, *Padina tetrastromatica* and *Centroceras clavulatum* showed activity against the urban mosquito larvae *Culex quinquefasciatus* (Manilal et al., 2009). Another study done in the same country, found marine algae that belonged to

the family *Chlorophyceae* (*Caulerpa racemosa* and *Ulva lactuca*) and *Rhodophyceae* (*Gracillaria folifera* and *Hypneme muciformis*) that showed antibacterial activity against the Gram negative bacteria *E. faecalis*, *K. pneumoniae* and *E. aerogens*, as well as in the Gram positive bacteria *S. aureus* (Kandhasamy & Arunachalam, 2008). In a work done in the Iberian Peninsula with 82 marine algae (18 *Chlorophyceae*, 25 *Phaeophyceae* and 39 *Rhodophyceae*) the antibacterial and antifungal activity was analyzed to evaluate their application as natural preservatives for the cosmetic industry. The raw extracts of every taxon, prepared from fresh material as well as from lyophilized one, proved to have the opposite effect on three Gram positive bacteria, two Gram negative bacteria and yeast, by means of the agar diffusion technique. Sixty seven % of the studied seaweeds did not show antimicrobial activity against opposite the tested microorganisms. The biggest percentage of active taxon were presented in the group of the *Phaeophyceae* (84 %) followed by the *Rhodophyceae* (67 %) and finally the *Chlorophyceae* (44 %). The red seaweed presented the highest activity, with the widest spectrum. Inside this group, the most active species were *Bonnemaisonia asparagoides*, *Bonnemaisonia hamifera*, *Asparagopsis armata* and *Falkenbergia rufolanosa* (*Bonnemaisoniales*). As for the microorganisms, *Bacillus cereus* was the most sensitive and *Pseudomona aeruginosa* the most resistant. Three taxonomic groups showed seasonal change in the production of antimicrobial substances, being autumn the station with major percentage of active taxon for the *Phaeophyceae* and *Rhodophyceae*, while for the *Chlorophyceae* it was summer (Salvador et al., 2007).

Now a day there is a database that contains all the known natural compounds derived from marine algae, creating a crucial tool in the scientific community dedicated to the search of new useful compounds for medicinal purposes. This database provides the user with more than 3,600 released articles that describe 3,300 secondary metabolites originated from seaweeds, and it is still considered to be insufficient even though is growing every day. According to the database, *Phaeophytas* and *Rhodophytas* present significantly more quantity of bioactive compounds than *Chlorophytas*. The red seaweed *Laurencia* (*Ceramiales*, *Rhodomelaceae*) is one of the most prolific algae in the production of secondary metabolites derived from the sea. Sesquiterpenes, diterpenes, triterpenes and acetogenins (characterized by the presence of halogens atoms in their chemical structures) have been found present on this seaweed (John Davis & Vasanthi, 2011).

## 7. Bacteria associated with marine algae

The marine ambience is a complex ecosystem with an enormous plurality of forms of life that are associated between themselves, the most common associations found are between eukaryotic cells and microorganisms (Eganet al., 2008). The surface of all the marine eukaryotic organism are covered by microbes that live adherent to diverse communities often immersed in a matrix or forming a bilayer (Pérez-Matos et al., 2007). Also, the specificity of the guest organism also has been demonstrated in studies that show the presence of the only adherent stable communities to organisms of the same species through that they live on geographically distant regions (Webster & Bourne, 2007).

In the recent years, the bioactive properties of marine algae and marine microorganisms have been analyzed, and in both cases positive results have been obtained. Many of the marine algae species often come accompanied by several bacterial strains which have been

taken of the sea together with the algae cells, or have been the result of a contamination in the algae culture. These mixed populations that are present in the culture and in the sea, show that the bacteria use organic substances secreted by living or dead algae cell. It has been observed that many types of seaweeds present a major growth in the presence of bacteria than in their absence. Some seaweed species need vitamins for their growth and possibly the bacteria are partially responsible for the production of these substances; some of them produce antibiotics (Jasti et al., 2005; Penesyan et al., 2010).

The number of natural products, discovered from several organism that include plants, animals and microorganisms, overcomes millions of compounds. Forty to sixty percent derives from terrestrial plants, from which twenty to twenty five % possesses bioactive properties such as antibacterial, anticancer, antifungal, antiviral and anti-inflammatory activity (Berdy, 2005).

Bacteria exist only in some seaweed species, as it is the case of *Leucobacter sp.*, collected in the Todos los Santos bay, BC. Mexico; which only was present in one out of six seaweeds analyzed (*Egregia menziessi*), (Villarreal-Gómez et al., 2010). This member of the *Actinobacteria* family, has also been associated to the nematode *Caenorhabditis elegans* (Muir & Tan, 2008). *Micrococcus* is another actinobacteria strain that has been associated only to *Egregia menziessi*. This strain is usually found in soil and water. It is catabolically versatile, with the skill of using unusual substrates like pyridine, herbicides, polychloric biphenyl's and oil. It can also biodegrade many environmental pollutants (Zhuang et al., 2003). The bacterial strain *Kocuria palustris* (Sm32), it is exclusively present in the brown seaweed *Sargassum muticum* that is considered to be an invasive species in many countries. This strain has industrial applications in the degradation of organic matter (Kovacs et al., 1999). The *Alcaligenes* found exclusively in the seaweed *Endarachne binghamiae*, are used for the industrial production of not standard amino acids (Madigan et al., 2005). Finally, the bacterial strain member of the genus *Alteromona*, was associated only with the seaweed *Laurencia pacifica*, generally isolated in sea water; this *Proteobacteria* has industrial use, since they produce polysaccharides of high molecular weight. Several of the bacterial strains phylogenetically related, have industrial application; therefore, it is necessary to study the chemical interactions seaweed - bacteria for a better understanding of the process of production of the different secondary metabolites, which produce these species.

## 8. Marine bacteria as producers of secondary metabolites

The marine microscopic communities are responsible of the change in the distribution of certain chemical elements in the sea. The autonomous aptitude of the marine organisms to produce substances biologically active that possibly accumulate, modify, kidnap and use toxins of other organisms, is a test of it (Mebs, 2000). For example, the lomaiviticins *a* and *b*, substances with antitumor potential were isolated for the first time from squids, and they contain the bacteria *Micromonospora lomaivitiensis*. In later experiments, this bacterium was isolated and cultivated in fermentation reactors, to finally determine that the bacteria were the real producers of lomaiviticin (He, 2001). Marine bacteria have often been considered to produce antibacterial and anticancer substances, allowing the ecological stability of the multiple marine ecosystems, the interrelations between epiphytic microorganism's ambiances, inhibiting the rival organisms and pathogenic microbes. The sharing or

competition mechanisms that are known between these microorganisms are diverse, including antibiotic production, bacteriocines, siderophores, lysosomes, proteases and even the pH alteration through the production of organic acids (Avendaño-Herrera et al., 2005).

In recent studies done in Todos Santos Bay, B.C. to bacteria associated to the seaweed surface it was found that bacteria of the family *Firmicutes*, *Proteobacteria* and *Actinobacteria* produce compounds capable of inhibiting the growth of HCT-116 colorectal cancer cells (Villarreal-Gómez et al., 2010). Also, it was found that the bacteria *Microbulbifer thermotolerans*, and *Pseudoalteromonas sp*, are capable of producing biofilms and produce chemical compounds that protect them from the other protozoans. An example of these compounds is violacein, an alkaloid that it is synthesized predominantly in biofilm, it has been found that in nanomolar concentrations violacein inhibits protozoan cells and induces programmed cellular death in eukaryotic cells. This bacterial producing biofilm secretes specific chemical substances for defense purposes and contribute to the persistence of these bacterial strains in different environments and provide an ecological and evolutionary context for the discovery of bacterial metabolites against eukaryotic cells (Matz et al., 2008).

*Bacillus sp* species have been found to possess chemical compounds with anticancer activity. Although this type of bacteria can grow in almost any substrate, it is possible to suggest that this species seems to have acquired the skill to synthesize compounds capable of inhibiting HCT-116 colorectal cancer cells (Villarreal-Gómez et al., 2010).

Selective response mechanisms exist against certain organisms, as shown in marine biofilms of *Bacteroidetes*, *Planctomycetes*, *a,c* – and *d* *Proteobacteria*, where the production of chemical substances as violacein has been observed. This compound works as a defense mechanism against certain specific predators like the protozoa consuming bacteria. This allows the successful persistence of the bacterial biofilm in several marine environments (Matz et al., 2008). Studies done in seaweed collected in the same coastal area, share similar defense mechanisms and inhibit the growth of Gram negative bacteria *Proteus mirabilis* and *Klebsiella pneumoniae* (Villarreal-Gómez et al., 2010), creating an interesting ecological and biotechnological role, and becoming a great subject for the search of marine natural products.

The ethanolic extracts of *Grateloupia doryphora*, *Ahnfeltiopsis duroillaei*, *Prionitis decipiens*, *Petalonia fascia* and feathery Bryopsis of the central coast of Peru, presented antibacterial effect against the clinical strains *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 and not clinical strain *Staphylococcus aureus* ATCC 6633. The ethanolic extract of *B. plumosa* presented the biggest antibacterial effect against two strains of *S. aureus*, being evident in their inhibition halos, while the extract of *P. fascia* showed major antibacterial activity, acting on 3 mentioned above strains (Magallanes et al., 2003)

Studies done on bacteria associated with the marine worms of the *Polychaetes* species show a strong antimicrobial activity that can be used as a potential resource for the development of new medicines (Sunjaiy-Shankar et al., 2010).

The following table shows some examples of bacterial strains with bioactivity and the sources where they were obtained. It is possible to appreciate the diseases that can be fought utilizing the secondary metabolites from different types of bacteria. This emphasizes the importance of microorganisms as an ideal source of bioactive compounds.



Bacteria	Gram (+ or -)	Activity	Target organism	Disease
<i>Pseudomonas bromoutilis</i>	-	Anticancer	<i>Staphylococcus aureus</i> , <i>Streptococcus pneumoniae</i> , <i>Streptococcus pyogenes</i>	Pneumonia, osteitis, arthritis, endocarditis, localized abscesses
<i>Chromobacteria marinum</i>	-	Antibacterial	<i>Escherichia coli</i> , <i>Pseudomona aureginosa</i> , <i>Staphylococcus aureus</i>	Pneumonia, osteitis, arthritis, endocarditis, localized abscesses
<i>Flavobacteria uliginosum</i>	-	Anticancer	Sarcoma-180 cells	Viral tumor
<i>Bacillus sp.</i>	+	Anticancer	HCT-116 cells	Colorectal Cancer
<i>Lactococcus lactis</i>	+	Anticancer	Human papilloma virus type 16 (HPV-16)	Cervical Cancer
<i>Staphylococcus aureoverticillatus</i>	+	Anticancer	Tumor cells	Tumors
<i>Marinobacter drocarbonoclasticus</i>	-	Antibacterial (siderofore)	<i>Mycobacteria tuberculosis</i> , <i>Bacillus anthracis</i>	Tuberculosis, carbuncle (anthrax like)

Table 1. Microorganism's producers of bioactive substances.

### 9. Interactions between bacterial species

Studying the diversity of bacterial species and knowing their inter and intraspecific interactions, makes the search for secondary metabolites in bacteria easier. For many years, researchers have managed to use different culturing methods that allow them to create

dense bacterial populations that yield a great amount of extracts that can be used to investigate their bioactive properties.

Not only the seaweed - bacteria interactions can influence the secretion of bioactive substances, but also the interactions that exist between bacterial species that inhabit the same ecosystem. There are different types of interactions between bacterial species and other organisms; these can be positive (metabiosis and symbiosis) or negative (parasitism, predation and competition). For their high population density, nitrogen content and their relative incapability to escape from predators, the bacteria have served as food for diverse groups of organisms (Schlegel & Jannasch, 2006).

As a rule, bacteria have been considered to be the prey of many organisms, including other bacteria species or other types of microbes. This is the case of the genes involved in producing Pilli type IV which were identified in the periplasmic strain B of *Bdellovibrio bacteriovorus* 109J and in the epibiotic strain *Bdellovibrio* sp. JSS, both Gram negative proteobacteria, which are forced predators of other Gram negative bacteria (extracellular). Using immune fluorescence microscopy the presence of the pilli was observed in the phase of cellular attack, confirming that the Pilli type IV plays a role in the invasion of other Gram negative bacteria on the part of *Bdellovibrio* (Qin et al., 2010).

Another type of interaction between the bacteria is the competition. Some bacteria are eliminated by different species when the environmental resources are limited; therefore they produce compounds that impress negatively in their competitors. Generally, these antimicrobial compounds that are produced in the environment are difficult to detect, for example the bacteria incapable of producing antibiotic compounds must reproduce very fast compared to those that do. It has been found that in actinomycetes, bacterial species that have the slowest reproduction rates are the greatest producers of antibiotic compounds (Mahmoud & Koval, 2010).

In some cases, the seaweeds dissolve organic substances that are used as nutrients by the bacteria; nevertheless some of the bacteria do not obtain nutrients this way or from any other animals. They adhere to them only for physical support and obtain their nutrients directly from the surrounding environment (Rheinheimer, 1992).

Bacteria can have interactions among themselves in order to find mutual benefits. Such is the case of *Escherichia coli* and *Proteus vulgaris*; these two species can coexist in a rich in lactose and urea medium. In this case, *Escherichia coli* degrade lactose while *Proteus vulgaris* degrades urea. The final products of these degradations can be used by another organism as a carbon and nitrogen source (Rheinheimer, 1992).

## 10. Techniques used for bacteria identification

To this date, several bacterial identification methodologies are known to have contributed a great deal of information for the study of their diversity. Traditionally, the studies to characterize the bacterial diversity in environments are based on the assumption that the cultivation techniques allow the recovery of most of the microorganisms in a sample [39]. Nevertheless, in the database analysis of DNA sequences, it has been observed that the bacteria obtained by standard microbiological techniques represent just a limited proportion of samples from natural sources (Escalante-Lozada et al., 2004).

It has been proposed that non-cultivable bacteria are microorganisms phylogenetically related to the cultivable ones, but in a physiological state that makes them recalcitrant. The

explanation is that some cultivable bacteria can turn in viable, but not cultivable when exposed to adverse environmental conditions and reverse to the cultivable state after the favorable conditions for their growth are restored (McDougald et al., 1998).

To study bacterial diversity, it is important to use molecular techniques that include the amplification of the 16S ribosomal gene using the polymerase chain reaction technique (PCR) in order to isolate and characterize their genetic material (Prieto-Davó et al., 2008).

## 11. Microbial diversity cultivation dependent techniques

To go as far as knowing all the chemical compounds produced by bacteria extracted from different environmental samples, their isolation and cultivation, is necessary to prepare organic extracts that could be evaluated chemically and biologically. The techniques used are generally known as microbial diversity cultivation dependent techniques or traditional methods of cultivation (Joint et al., 2010). These techniques are based on the need that the microorganisms have of taking from the environment a series of compounds that are used as energy source and synthesizing the cellular constituents necessary for their survival, like C, N, S, P, Ca, K, Na, Mg, Mo, Cu and Zn. Some microorganisms need to take the light energy or to oxidize chemical compounds; others need to obtain their carbon source from CO<sub>2</sub> or through carbonated organic compounds. Some microorganisms need a nitrogen source, for which they fix the atmospheric nitrogen, or take it from NH<sub>4</sub><sup>+</sup>. Others are capable of reducing NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup> to use it as an ammonium source, or, to use free amino acids. All these characteristics, as well as temperature, pH and salinity, have demonstrated to create a propitious ambient, for the isolation and purification of microorganisms originated from environmental samples (Rheinheimer, 1992).

Therefore, the collected environmental samples are exposed to different conditions and forms of cultivation that help to obtaining microorganisms capable of adapting to the established conditions. This allows to study the microorganism's diversity, as well as to cultivate them from different environmental samples.

## 12. Molecular techniques for bacteria identification

The molecular characterization of bacteria has had an enormous impact on the safety of microbiological industrial processes like the bio pharmacology and food industry as well as public health, since it establishes the source of contamination in a much more precise form and therefore to identify the strains involved in the process and to establish their trajectory.

The most used gene for bacterial molecular identification is the 16S ribosomal RNA gene, nevertheless, there have also been used other genes that can codify for 5S ribosomal proteins, and 23 S ribosomal RNA.

The ribosomes and ribonucleic acids (ribosomal RNA) are proteic complexes with the only function of synthesizing proteins. The ribosomes structure is preserved between the different life kingdoms (Plantae, fungi, animaleae, etc.). In the prokaryotes, the ribosomes are composed by two subunits: a big 50S, and small 30S subunits. The subunit 50S contains a 23S, one 5S rRNA and more than 30 different proteins. The subunit 30S contains one 16S rRNA and 20 additional proteins. Since the rRNA gene function is limited by the structure, certain regions in the rRNA genes that are in contact with other components in the ribosome must be preserved;

the sequences between the preserved regions have major mutation valuations. The preserved regions are useful to determine distant relations (genus), while the regions with higher mutation rate or variable regions are useful to distinguish organism closely related (species) (Eickbush & Eickbush, 2007). These characteristics of rRNA genes are excellent molecular chronometers for phylogenetic analysis and taxonomic classification of cellular organisms.

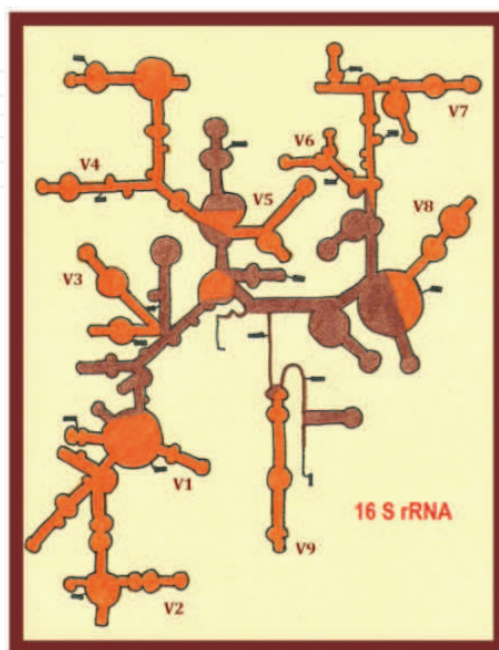


Fig. 2. Scheme of Bacterial 16S ribosomal RNA that shows the variable sequences (V1 to V9). The variable regions area used to characterized bacteria by species level, and the constant region is used to correlated the genus.

In general, the phylogenetic trees based on the 16S and 23S rRNA genes can be used in parallel (De Rijk et al., 1995), while the 5S rRNA gene it is considered not to contain sufficiently long sequences to do significant statistical comparisons. The detailed phylogenetic studies based on the genetic sequences 16S and 23S provide comparisons in three primary domains Bacteria, Archaea and Eukarya (Woese et al., 1990). Nevertheless, one of the disadvantages of the 23S rRNA gene on 16S rRNA in the phylogenetic studies is the rare use of the taxonomic classification for the absence of primers that amplify a considerable length sequence and the difficulty of genes sequences too long for the current technologies.

Previously the 16S rRNA and 23S rRNA genes have been compared and the results show that the 16S rRNA gene has more closely related typical sequences, with major length, insertions and/or deletions and possibly a better phylogenetic resolution for their high genetic change (Blunt et al., 2005). Nevertheless, recent studies indicated that the 23S rRNA gene also contains conserved regions for the design of primers capable of amplifying a wider length with one grade similar to the primers used in the of 16S rRNA genes (Hunt et al., 2006; Pei et al., 2009).

Molecular methods of characterization based on the amplification of fragments belonging to the 16S ribosomal RNA gene through automated techniques constitute a rapid, trustworthy

and simple method of molecular genotyped bacterial and fungoid strains. The aptitude to identify microorganisms at species level and at the same time to establish a comparative analysis of the different analyzed strains constitutes a time saving way to identify the food pollutant potential and therefore to eradicate the pollutant focus or to facilitate the recognition of the producing strains of some bioactive metabolite capable of inhibiting the growth of other bacterial strains or carcinogenic cells.

The 16 S ribosomal gene (16S rRNA) is constituted by a region preserved through evolution, the mutations in this gene can usually be tolerated, since these mutations would only affect ribosomal RNA, nevertheless, the number of mutations are not completely well-known, the regions that are affected by them are met like "hot commercials" which present a considerable number of mutations, these areas are not the same in all species. The 16S rRNA gene mutations can affect the susceptibility to antimicrobial agents which can be an indicator to distinguish the phenotypic resistance to these agents. Nevertheless these characteristics do not affect the gene use for taxonomical identification at the genus and species level. 16S rRNA possesses a length of 1, 550 bp and it is composed by variable regions and conserved regions, with enough interspecific polymorphisms to provide a valid statistical characterization. The primers that are usually designed for the amplification of this gene, are based on the complementary chain of the conserved regions in the beginning of the gene at about 540 bp or at the end of the sequence around 1,550 bp and the sequence of the variable region is used for taxonomic comparison intentions, making 1500bp the minimum length that should be used to compare DNA sequences. The 16S rRNA gene sequence has been determined for a big number of strains; these sequences are included in the biggest database of nucleotides known as GenBank. This database contains more than 20 million sequences of which more than 90, 000 correspond to the 16S rRNA gene. . In general, the comparison of the 16S rRNA gene sequences allows the differentiation between organisms at genus level in most bacteria phyla, also classifies the strains at multiple levels, including species and sub species (Clarridge III, 2004).

### **13. Extraction of bioactive compounds from bacteria associated with marine algae**

To do a search of bioactive compounds from marine algae the following protocol is proposed:

#### **a. Isolation of the bacterial strains from the seaweed surface**

After being collected, the seaweed is placed in an Erlenmeyer flask and is rinsed with distilled water; a small sample from the flask is taken with a sterile swab and is inoculated in a general media that allows the growth of most of the bacteria present in the seaweed surface. Then the media with inoculate is incubated for periods of 24 to 48 hours at 25 °C, until the developing colonies start to emerge. Later the bacteria will be purified up to the third generation to assure the integrity of pure colonies and finally it is important to take one of the colonies of every purified strain and cryopreserved it in 15 % glycerol at -70 °C.

#### **b. Macroscopic morphology and Gram Stain**

The pure colonies are examined macroscopically evaluating their characteristics such as size, form, elevation, margin, color, type of surface, thickness, consistency, smell and pigments

production. Consecutively microscopic classification of the bacterial strains, such as the Gram stain, is necessary (Gram, 1884). The Gram stain is a technique of bacterial characterization based on the chemical composition of the cellular wall of the bacteria. The Gram positive bacteria present a cytoplasmic membrane, have a thick peptidoglycan layer, contain teicoic acids and lipoteicoic acids that serve as chelating agents and certain type of adhesions; the bacteria representative of this group are *Firmicutes* and *Actinobacteria*, which includes many well-known genus like *Bacillus*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium*.

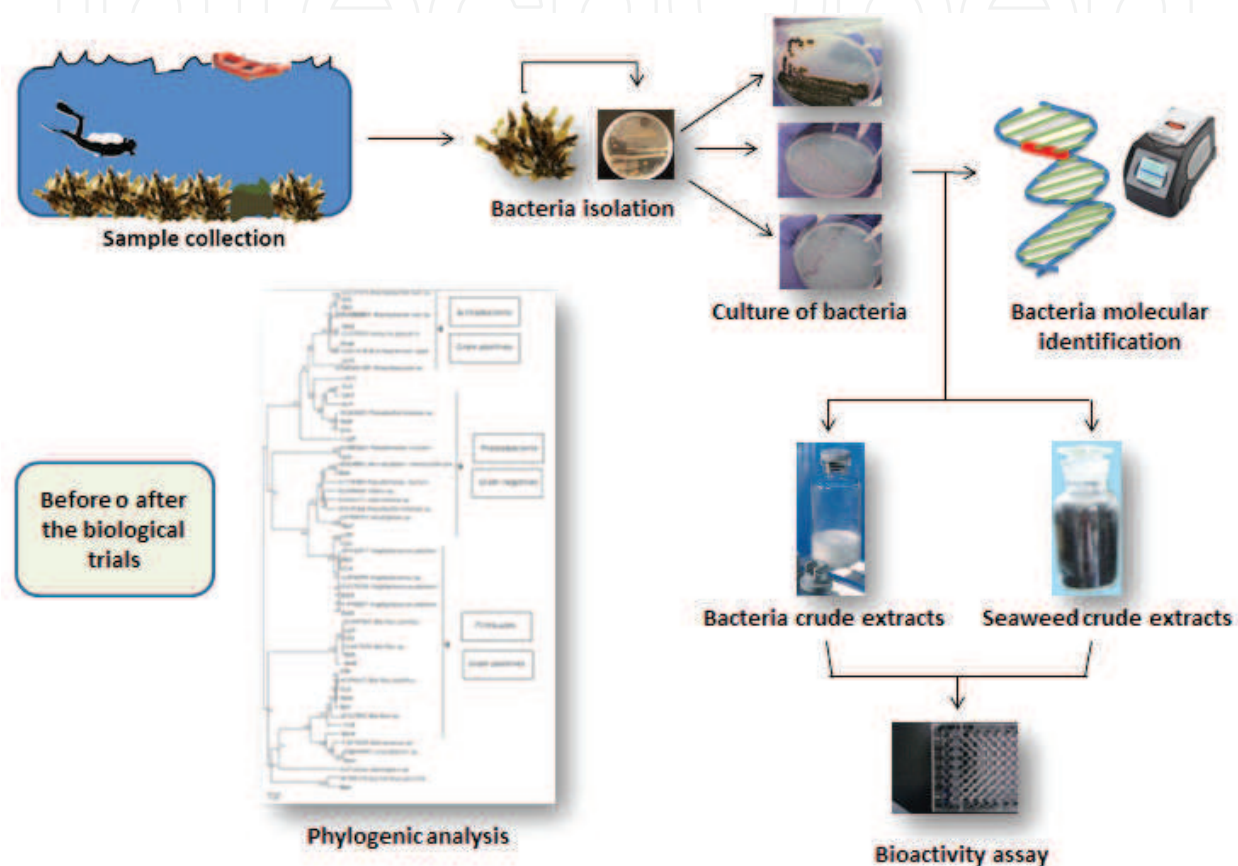


Fig. 3. Proposed methodology for the search of new bioactive compounds in bacteria associated with the surface of marine algae.

On the other hand, the Gram negative bacteria contain a thin cellular wall of peptidoglycan and an external membrane that covers their cellular wall. The external membrane contains diverse proteins, like porins or channels protein that allow the path of certain substances. Also they present a structure of lipopolysaccharides (LPS). The bacteria that predominate in this group are Proteobacteria, including to *Escherichia coli*, *Salmonella* and other enteric bacteria like *Pseudomonas*, *Moraxella*, *Helicobacter*, *Stenotrophomonas*, *Bdellovibrio*, acetic acid bacteria, Legionnaire's disease and the proteobacteria alpha like *Wolbachia* among others (Madigan et al., 2005).

Previous studies done with bacterioplankton demonstrated that most of the marine bacteria are Gram negative; but in recent studies with marine sediments evidence has shown that most of the bacteria that conform the marine sediments seem to be Gram positive (Gontang et al., 2007).

In studies made to the surface of the marine alga *Monostroma undulatum* (Gallardo et al., 2004) Gram negative bacteria belonging to the genus: *Vibrio* (20 %), inactive *E. coli* (18 %), *Flavobacteria* (11 %), *Flexibacter* (9 %), *Moraxella* (9 %), *Pseudomona* (9 %), *Aeromonas* (2 %), *Acinetobacter* (2 %), *Cotophaga* (2 %), *Photobacteria* (2 %) and *Alteromonas* (2 %); predominated and only one Gram positive was found, *Staphylococcus*.

### c. Phylogenetic analysis

Since bacterial identification was mentioned previously and bacterial characterization with molecular techniques is crucial for the achievement of phylogenetic studies that allow to have an account of the cultivable bacterial species that are present in a similar community, there are several investigators who agree with the idea, that bacteria with closely related DNA sequences produce very similar compounds. If this is true, just by knowing the bacterial ecology of a certain area it will be possible to predict the types of compounds that could be isolated from the bacteria present in that particular area.

The DNA sequences can be analyzed using the BLAST database (Basic Local Alignment Search Tool) that is a GeneBank database integral function (Altschul et al., 1999). To align the existing sequences between themselves it is possible to use the Clustal X (Staley & Ta, 1985) and Bioedit programs (Hall & Brown, 2001) for manual alignments. For the phylogenetic tree construction it is possible to use the following indications: Bootstrap test of phylogeny (1000 repetitions), p-distance joining neighbor, using the MEGA4 program (Tamura et al., 2007) using segment sequences of up to 1500 bp.

### d. Biological assays and mean lethal dose DL<sub>50</sub>

A bioassay can be defined as any test that involves living organisms. With them, it is possible to evaluate the effects of any substances or material in terms of the biological answer they produce. The main target in this type of analysis is to evaluate the level of stimulus that is necessary to obtain a response in a group of individuals of a population. The level of stimulus that causes response in 50 % of the individuals under study is an important parameter of characterization denoted like average lethal dose (DL<sub>50</sub>). The amount of time during which the stimulus is exhibited, must be specified, for example, 24 hours DL<sub>50</sub>, this in order to compare and to estimate the relative potency of the stimulus. For the DL<sub>50</sub> determination of the first step is securing the % of survival cell for every analyte and every target, the mortalities is corrected by Abbott's formula (Abbott, 1925).

$$M = \frac{me - mb}{1 - mb}$$

Where:

*M* = Mortality.

*me* = optical density in the extract.

*mb* = optical density in the target.

With the mortalities corrected, the obtained information is introduced in the software "BioStat 2008" (<http://www.analystsoft.com>) using Probit analysis to obtain the DL<sub>50</sub> values (STATPLUS, 2008).

## 14. Conclusion

Natural products are a very important resource for the elaboration of medicines. Although a big number of plants, microbes and marine resources have been evaluated in the search of new bioactive compounds, it turns out to be insufficient and it is necessary and important to continue with the search of new secondary metabolites, especially those that are endophytes microorganisms of seaweed. The bad use given to antibiotics has resulted in the development of bacteria strains that are resistant to many of the known drugs. This situation has led to a forced search for new antibiotic compounds, being the seabed a propitious site for exploration and future drug development. Also, the treatment with chemotherapy for the diverse causes of different types of cancer that at present today, appears effective, so the investigation becomes necessary in the chemistry of the natural products. The methods of bacterial culture and identification have become very promising especially, those done through molecular techniques, by which is possible to identify a strain up to species and sometimes at subspecies level. The diverse relationships that exist between microorganisms and their guests provoke that bacterial compounds can eventually be used as a source of new drugs for human well-being.

### a. Future work in Drug Discovery

The strategies for drug discovery has been evolving constantly, today researchers do not conform with the finding of new and potent metabolites, now and for future days it has become important to do phylogenetic studies, structure elucidation of chemical compounds, bioinformatics approaches, genomics, proteomics, reverse pharmacology and so on., One fundamental field that has to be developed is the improvement of more efficient culture media, because we need to culture all the strains of bacteria to be able to evaluate and separate its compounds, in the meanwhile, we can identify the genes of cultivable and non-cultivable bacteria by molecular techniques to compare and try to demonstrate that the strains of bacteria with very similar DNA sequences have equally similar metabolites and culture requirements, if this is true, before we start screening for drug discovery purposes, researchers must do phylogenetic studies of bacterial population of a certain determinate area and decide which strains cultivate and which not, these strategies will make the drug discovery process less expensive and faster.

Methodological improvements studies based on the characterization of the extracellular polymeric substance produced by marine microorganisms and a better understanding of host-microbe interactions, should be us to provide further insight into the adaptive strategies against microbial pathogens and establish the extent to which secondary metabolites regulate microbial interactions.

The new soft ionization methods: Matrix-Assisted Laser Desorption Ionization (MALDI) and Electrospray Ionization (EI) are the recent approach used in a variety of new and innovative Mass Spectrometric (MS) applications. With them, is easier to analyze surfaces, they are tolerant to impurities and do not require extensive sample preparations. A sensitive and precise Mass Spectrometric approach like Desorption Electrospray Ionization (DESI) should be used to measure the physical location and quantities of natural products on biological tissue surfaces, cells or even complex mixed-species assemblages. These MS



imaging techniques known as “molecular eyes” are very precise and represent the last technological advance used to locate natural products in biological tissues (Esquenazi et al., 2009) allowing the study of the interface between the confluence of natural products chemistry, biology and ecology.

Bioinformatics is the part of molecular biology that involves working with biological data, typically using computers, with the goal of enabling and accelerating biological research. Bioinformatics comprises a wide range of activities: data capture, automated recording of experimental results; data storage and access, using a multitude of databases and query tools; data analysis; and visualization of raw data and analytical results (Pollock & Safer, 2001).

Today, many recently developed or discovered drugs with antibacterial and anticancer activity fail in clinical trials because of inefficiency for the anticipated indication or unexpected toxicity (Kola & Landis, 2004). Apparently, it remains hard to establish a clear link between antagonism or organism of a specific target and its influence in human illness and its target associated toxicity.

A significant cause for these high attrition rates is the often misjudged complexity of protein function in higher order organisms, in which, abundant protein-protein interactions, feedback loops and redundancies play a role. The collection of recognized pathways that can be found in public databases and commercial tools do not effectively address these issues because they are mainly a reflection of experimental data that are obtained from isolated cell lines and tissues. They address typically, the signaling events that lead to binding of transcription factors to the DNA, but do not detail the pleiotropic effects that arise downstream from the induced transcriptional program, which are most important in provoking the systems response to the signaling events and may determine, the capacity and toxicity of a drug (Pollock & Safer, 2001).

Most comparative genomics tools are intended at studying conservation of single genes or gene families, whereas computationally tools address orthologous biology, i.e. conservation of the entire pathways in which the target is involved, are unusual. This truly obstructs the output and success of translational investigation from pre-clinical to clinical studies (Pollock & Safer, 2001).

The developing of bioinformatics tools that addresses the above problems will allow for quicker and better experiments aimed at evaluating multiple targets and drugs for further clinical development. This will be a first step to reduce the high attrition rates associated with drug development (Pollock & Safer, 2001).

Clinical events or phenomena not reported previously following the administration of a known or new drug can offer valuable perceptions for drug development. Natural products have provided many such unexpected bedside interpretations. Researches in genomics, proteomics and metabolomics have stimulated the discovery of many new molecules, which are yet to be tracked for their drug-like activities. A new discipline called Reverse Pharmacology (RP) has been designed to decrease costs, time and toxicity.

The scope of reverse pharmacology is to understand the mechanisms of action at multiple levels of biology and to optimize safety, efficacy and acceptability of the leads in natural

products based on relevant science in this approach, as the candidate travels a reverse path from 'clinics to laboratory' rather than classical "laboratory to clinics". Actual humans are used as the ultimate model and in-depth investigation of the effects of drugs and the nature of disease progression is becoming ever more feasible because of advances in clinical biomarkers and systems biology. This articulates both structure of the system and components to play indispensable role forming symbiotic state of the whole system (Patwardhan et al., 2008).

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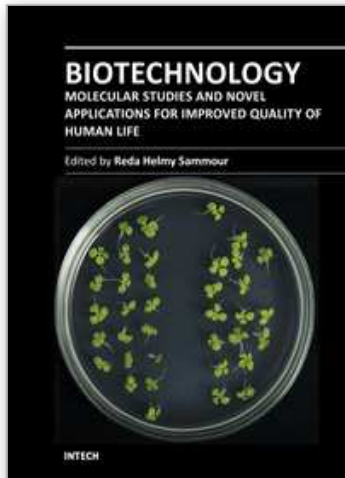
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## **Biotechnology - Molecular Studies and Novel Applications for Improved Quality of Human Life**

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This book deals with the importance of application of molecular biology as an approach of biotechnology for improvement of the quality of human life. One of the interesting topics in this field, is the identification of the organisms that produce bioactive secondary metabolites. It also discusses how to structure a plan for use and preservation of those species that represent a potential source for new drug development, especially those obtained from bacteria. The book also introduces some novel applications of biotechnology, such as therapeutic applications of electroporation, improving quality and microbial safety of fresh-cut vegetables, producing synthetic PEG hydro gels to be used as an extra cellular matrix mimics for tissue engineering applications, and other interesting applications.

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