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Bioprospection of marine microorganisms: biotechnological applications and methods

HEBE M. DIONISI*, MARIANA LOZADA, NELDA L. OLIVERA

Centro Nacional Patagónico (CONICET-CENPAT), Boulevard Brown 2915, Puerto Madryn (9120), Chubut, Argentina
**Correspondence. E-mail. hdionisi@cenpat.edu.ar*

ABSTRACT

Environmental microorganisms constitute an almost inexhaustible reserve of genetic and functional diversity, accumulated during millions of years of adaptive evolution to various selective pressures. In particular, the extent of microbial biodiversity in marine habitats seems to grow larger as new techniques emerge to measure it. This has resulted in novel and more complex approaches for the screening of molecules and activities of biotechnological interest in these environments. In this review, we explore the different partially overlapping biotechnological fields that make use of microorganisms and we describe the different marine habitats that are particularly attractive for bioprospection. In addition, we review the methodological approaches currently used for microbial bioprospection, from the traditional cultivation techniques to state of the art metagenomic approaches, with emphasis in the marine environment.

Key words: bioprospection, marine microorganisms, biotechnological applications, Argentina

RESUMEN

Bioprospección de microorganismos marinos: aplicaciones biotecnológicas y métodos. Los microorganismos ambientales constituyen una reserva prácticamente inagotable de diversidad genética, acumulada durante millones de años de evolución adaptativa a varias presiones selectivas. En particular, la magnitud de la biodiversidad microbiana en hábitats marinos parece crecer al emerger nuevas técnicas para medirla. Como resultado, se han comenzado a utilizar enfoques novedosos y más complejos para la búsqueda de moléculas y actividades de interés biotecnológico en estos ambientes. En este artículo de revisión, nosotros exploramos los diferentes campos de la biotecnología que utilizan microorganismos, los cuales se superponen parcialmente, y describimos los diferentes hábitats marinos que resultan particularmente atractivos para la bioprospección. Además, revisamos los enfoques metodológicos actualmente utilizados para la bioprospección microbiana, desde las técnicas de cultivo tradicionales hasta modernos enfoques metagenómicos, con énfasis en el medio ambiente marino.

Palabras clave: bioprospección, microorganismos marinos, aplicaciones biotecnológicas, Argentina

INTRODUCTION

Microbes, the most diverse and abundant group of organisms on Earth, constitute 60% of the total biomass and carry out more photosynthesis than the green plants (13). They are responsible for vital biogeochemical cycling without which life in our planet would not be possible. Microorganisms regulate the composition of the atmosphere, influence climate, recycle nutrients, and degrade pollutants (30). In addition, they provide us with many economically valuable products and processes (80). Despite their vital role in Earth sustainability and their current and potential biotechnological applications, much of the diversity and metabolic capabilities of the microorganisms still remains untapped (76). The greater part of microbial biodiversity is still represented by yet-to-be cultured microorganisms, which hinders our understanding of microbial ecosystem functioning (68). This is not an easy task as we know that microbial communities are extremely complex (with 100s or 1000s of distinct taxa identified in very small samples) and constantly changing in response to their environment (89). However, developing such an understanding is essential to meet many of the major challenges facing our civilization today: a sustainable supply of food and energy, human health, as well as facing climate change and environmental degradation (59, 89). Fortunately, a powerful and constantly expanding toolbox is starting to facilitate fundamental and applied research on microorganisms and their communities (17).

In billions of years of evolution, microorganisms have accumulated remarkable physiological and functional heterogeneity, and currently constitute an

almost inexhaustible reserve of genetic diversity (90). The biotechnological potential of this immense natural diversity can be further improved by tools such as enzyme engineering, metabolic engineering and directed evolution (79). As a consequence, isolated microorganisms and microbial communities have become an important source of biological products and activities with applications across all major industries, in the form of engineered microbial assemblages, cells, macromolecules, metabolites or bioactive compounds (76). The following partially overlapping fields make use of microorganisms: industrial biotechnology, pharmaceutical biotechnology, agricultural biotechnology and environmental biotechnology [Table 1, (50)].

Industrial biotechnology

Also known as white biotechnology, this field is based on the use of living cells and/or their enzymes for the sustainable production of chemicals, materials and fuels from renewable sources (79). This fast emerging area focuses on the development of clean bioprocesses with a reduction in greenhouse gas emissions, as well as energy and water usage (24). In the production of chemicals, biocatalytic reactions can replace a multi-step synthesis with a single step involving low energy and less material input, producing higher quality products at a lower cost (93). More importantly, they sometimes enable the synthesis of products that may not be possible to synthesize otherwise (26). Current challenges are the development of novel and improved biocatalysts for the production of chemicals, as well as a cost-effective production of these enzymes (26). It is expected that 20% of the global chemicals (fine chemicals, specialties, polymers, etc.) and 60 % of fine chemicals will be produced using biotechnology by 2020, which represents 800 billion US$ (50). Other area in which white biotechnology has shown considerable promise is the production of biodegradable polymers such as poly(hydroxyalkanoates), as the demand for these emerging biopolymers has been growing 20-30 % per year (24). Low productivity and high production costs currently represent obstacles for a wide commercial use of these polymers. However, their versatility has made them good candidates as high value low volume products, particularly for medical/biomedical use such as tissue engineering, cardiovascular products, drug delivery or wound management (36).

Pharmaceutical biotechnology

Developing new drugs, improving and replacing those that have become less effective and creating safer treatments and more efficient diagnostic tools for an ever-wider array of important diseases is essential to improve our quality of life (8). Pharmaceutical biotechnology (or red biotechnology) is used in the development and production of therapeutics, *in vivo* diagnostics and vaccines for both humans and animals. Other interests of this area include *in vitro* genetic diagnostics and the development of functional foods or nutraceuticals [Table 1, (87)]. The role of microorganisms in the pharmaceutical industry has been pivotal in the 80 years since the discovery of penicillin by Alexander Fleming. For example, actinomycetes have supplied >50 % of all antibiotics in use today, as well as important antitumoral, immunosuppressive and anthelmintic agents, and fungi have been producers of important drugs, such as penicillins, along with the most commercially successful drug class in history, antilipidemic statins (8).

Although the advent of molecular biology and genetic engineering yielded a profound transformation of the pharmaceutical industry, it is currently facing an unprecedented challenge. Since 1995, the number of new drugs launched into the market has declined by 50 %, despite the rapid technical progress during this period, doubled R&D investments and the increasing importance of biotech companies as a source

Table 1: Biotechnology fields and their products

Field	Color code	Key products
Industrial biotechnology	white	Fine chemicals, amino acids, vitamins, organic acids, detergents, biocatalysts and bioconversion agents polymers
Pharmaceutical biotechnology	red	Anti-tumor drugs, nutraceuticals, antibacterial compounds, immunosuppressants
Agricultural biotechnology	green	Biopesticides, antiparasite agents, food-processing agents, plant growth promoters
Environmental biotechnology	grey	Bioremediation, production of bioenergy

of innovative potential therapies (100). Furthermore, only one out of 5,000 compounds that are discovered ever reaches the customer and hardly a third of marketed drugs earn their own costs (95). In order to enhance the efficiency of drug discovery, there is a need not only for investments and emergent techniques and approaches [such as metagenomics or synthetic biology, (54)] but also new concepts. Biological and ecological models provide important tools for a rational search for active natural compounds (12), and evolutionary concepts could be of great help to streamline the drug-discovery pipeline, facilitating the discovery of targets and drug candidates (100).

Agricultural biotechnology

One of the major challenges for the twenty-first century is an environmentally sound and sustainable crop production, and agricultural (green) biotechnology applies microorganisms with this end [Table 1, (6)]. Plant-associated microorganisms have an important effect on plant health and growth by enhancing stress tolerance, providing disease resistance, aiding nutrient availability and uptake, and promoting biodiversity (52). Currently, products containing inoculants for biocontrol and plant growth promotion that take advantage of this beneficial association are available in the market. Strains of *Bacillus thuringiensis*, *Bacillus subtilis*, and *Pseudomonas fluorescens* are the most commonly used biopesticides, and the market for these products is growing 9-10 % a year, strongly tied to the growth in organic production (6). Microbial inoculants with plant growth-promoting rhizobacteria are also commercially available (52). Furthermore, there is potential to develop other products based on beneficial plant-microbe interactions, such as agents that reduce environmental stress or stimulate flavor compounds (2). However, our understanding of plant-microbe interactions is still incomplete, and more research is needed to avoid inconsistencies in the performance of these inoculants (52).

Environmental biotechnology

The main goal of environmental (grey) biotechnology is the sustainability of key resources such as water, soil and energy, through the management of microbial communities (65). The most traditional applications of environmental biotechnology are: (a) removing contaminants from water, wastewater, sludge, sediment, or soil, (b) increasing the quality of drinking water, (c) capturing valuable products from renewable resources (*e.g.* biomass, nutrients, metals, water, energy), and (d) sensing contaminants or pathogens in the environment (65). Potential future applications of microbial resource management are removing CO_2 or methane from the atmosphere or maintaining soil microbial community integrity when applying chemicals such as pesticides (89).

A deep understanding of microbial communities, which are extremely complex assemblages of microorganisms, is fundamental for their management, and partnerships between different disciplines are essential to reach this goal (65). The scientific foundation of environmental biotechnology is microbial ecology, which aims to understand microbial communities and how they interact with their environment (64). With the ultimate goal of predicting the behavior of microbial populations and communities, this discipline is currently maturing beyond the descriptive stage that characterized the last few decades. The interaction with other key disciplines, such as macroecology, ecosystem science, systems biology, engineering, material science and mathematical modeling, is also essential for the manipulation and control of microbial communities (89). In particular, mathematical models are the ideal tools for integrating large numbers of microbial, chemical and physical phenomena taking place within the microbial communities of interest (66).

MARINE MICROORGANISMS AND THEIR BIOTECHNOLOGICAL APPLICATIONS

Marine (blue) biotechnology aims to the development of products and other benefits for mankind from marine biodiversity, through the application of biological knowledge and cutting-edge techniques (59). The marine environment is the largest habitat on Earth, representing more than 70 % of the surface of our planet. Oceans include the greatest extremes of temperature, light and pressure encountered by life (53). Within the oceans, habitats can range from tropical sunlit surface waters to ocean trenches with 110 MPa pressure at 11 km below sea level. Furthermore, temperatures in the ocean can be over 350 ^{o}C in pressurized fluids in hydrothermal vents and as low as -35 ^{o}C in channels within the sea ice. These diverse marine environments still remain largely unexplored, understudied and underexploited in comparison with terrestrial ecosystems and organisms (59). However, as the success rate in finding previously undescribed active chemicals in marine organisms

is 500 times higher than that for terrestrial species, the use of marine biological resources for biotechnological purposes is currently blooming (4). Unfortunately, the exposure of marine ecosystems to anthropogenic stressors including, among others, overextraction of many components of the food web, pollution and habitat destruction, can lead to the extinction of irreplaceable marine species, dramatic changes in microbial communities and ultimately ecosystem collapse (67).

Oceans contain a rich variety of organisms, including at least 34 of the 36 living phyla, some of which are only found in the oceans (4). In particular, microorganisms are ubiquitous and highly diverse in the marine environment. Through billions of years of evolution marine microorganisms from the three domains of life, namely Bacteria, Archaea and Eukarya, have developed unique metabolic and physiological capabilities to thrive in marine habitats, even the most extreme ones (70). Many marine microorganisms are able to produce novel metabolites with biotechnological potential which are not often present in microbes from terrestrial origin. It is important to notice, however, that the recovery of a microorganism from the ocean does not necessarily imply that it is truly 'marine', as some organisms may be wash-in components from the terrestrial environment (60). Indeed, halotolerant species are frequently isolated from marine sources, especially in coastal environments where terrestrial input is significant.

The oceans contain various different habitats that are suitable for bioprospection. Microorganisms with biotechnological potential are present in pelagic and benthic habitats, and also can have symbiotic or epibiotic lifestyles. Effective competition and defense strategies common in surface-associated microorganisms, such as the production of toxins, signalling molecules and other secondary metabolites, constitute an unparalleled reservoir from a biotechnological perspective (15). In fact, bacteria living in complex associations with marine invertebrates are often proposed to be the producers of metabolites previously assigned to their hosts (71). The most striking example is the case of the microbial communities inhabiting marine sponges, which are among the richest sources of interesting chemicals produced by marine organisms (40). Due to the difficulties associated with the chemical synthesis of natural products and the cultivation of marine invertebrates, the cultivation of the bacterial symbiont or the heterologous expression of specific pathways could allow obtaining a sustainable, essentially unlimited supply of substances for testing and subsequent drug production (40).

In recent years, marine microorganisms living under extreme conditions have also been the focus of bioprospecting efforts as novel sources of biomolecules with biotechnological applications (18). Deep-sea habitats (*e.g.* hydrothermal vents, sinking particles, animal guts, biological surfaces or deeply buried subseafloor sediments) have a variety of unique ecological niches that have in common the presence of extremely elevated hydrostatic pressure (14). Furthermore, typically piezophilic organisms can be either psychrophilic or thermophilic in nature, due to the cold temperatures of the deep ocean or due to their proximity to hydrothermal vents (81). There is a wide range of biotechnological applications for piezophilic microorganisms, such as deep-sea waste disposal, the production of novel natural products or the development of biocatalysts for high-pressure bioreactors (81).

Other microbial communities exposed to extreme environmental conditions are those inhabiting intertidal marine ecosystems. Microorganisms from intertidal zones must be able to tolerate rapid and repeated fluctuations in environmental conditions including temperature, light and salinity, and are exposed to wave action, ultraviolet radiation, as well as periods of drought (48). Intertidal microbial communities grow preferentially as biofilms on natural and artificial surfaces, and within these protective microenvironments, they are subjected to intense biological and chemical interactions leading to the production of various interesting secondary metabolites (15). Intertidal and supratidal microbial communities are also a promising source of natural sunscreens. In response to intense solar radiation, cyanobacteria and other microorganisms have evolved a variety of defense mechanisms including the biosynthesis of UV-absorbing/screening compounds that offer the potential for development of novel UV blockers for human use (12).

Although the oceans are a hotbed of microbial diversity with immense biotechnological potential, most biotechnological products on the market today derived form terrestrial species (3). A long-held belief of many scientists that seawater contained few microbes and that marine microorganisms were hard to isolate and maintain in the laboratory environment were the main reasons for overlooking marine microorganisms in bioprospecting efforts (60). However, more recently novel isolation techniques have been developed, and a wealth of additional information has been

uncovered by culture-independent methods that showed the incredible diversity of marine microbes with vast swathes of uncharacterized metabolism (23). Actually, the extent of marine microbial biodiversity, and consequently natural products potential, seems to be limitless and growing larger as new techniques emerge to measure it (27). High-quality research in the field of marine biotechnology is one of the key-factors for successful innovation in exploiting the vast diversity of marine life. On the next section, traditional as well as state of the art techniques used to study marine microorganisms with biotechnological applications will be reviewed.

METHODOLOGICAL APPROACHES

Traditional culture revisited

Since the fermentation studies of Louis Pasteur in the 1870s, cultivation has been the usual approach to screen for and develop new products and capabilities of microbial origin. Microbiology evolved into a highly culture-directed science, for which isolation and identification of strains constituted the obligate preliminary step of any basic or applied research work. Nowadays, culture collections provide pure and identified strains for reproducible experimentation, as well as serve as repository of newly described microorganisms testable for future biotechnological applications (86). The ability to culture strains, and to characterize their physiology is still regarded as an important advantage for bioprospection (33). However, there is a limit to the extent of the real biodiversity that is currently accessible in this way, as only a small proportion of environmental microorganisms are isolated in culture in any given media (62). Traditional rich media often select for fast-growing, opportunistic microorganisms (97), while microorganisms producing secondary metabolites are often slow-growing, "K-strategy" bacteria, which are rarely selected in traditional culture. As a consequence, environmental microbes with potential biotechnological interest can remain untapped due to the fact that the culture media used is not suitable for their growth. Today, one of the main challenges for the full exploitation of the biotechnological potential of marine microorganisms remains increasing the ratio of cultured *vs.* the "yet uncultured" majority of microbes (33).

Sometimes, traditional cultivation coupled with a certain ecological knowledge ("where to look for what") can be sufficient for the isolation of strains with worthwhile biotechnological capabilities. For example, it is known that surfaces of marine eukaryotes are covered by microorganisms distinct from the ones of the surrounding water. These microbial epibionts produce antimicrobial compounds that allow them to outcompete other surface colonizers (58). In a recent survey, 325 epiphytic bacterial isolates were obtained from the surfaces of marine algae, by means of traditional cultivation. Thirty-nine of them showed antimicrobial activity, highlighting the biotechnological potential of targeted isolation techniques (58). Awareness of the attachment properties of sediment bacteria has also been instrumental for enhanced recovery in culture. In this case, the use of a dispersion and differential centrifugation technique for the extraction of cells from marine sediment, together with media containing several different carbon sources, lead to the isolation of *Actinomycetes* genera only reported for terrestrial environments (47).

Mimicking the source environment can add new value to the traditional cultivation approach. Changing a relatively simple condition such as temperature allows the growth of psychrophilic bacteria capable of producing cold-adapted enzymes, useful for shortening industrial processing times and saving energy costs (99). Accounting for habitat heterogeneity also improves the culturability of free-living bacteria inhabiting certain environments. The use of different electron acceptors greatly enhanced the recovery in culture of diverse phyla of bacteria from sediments (37). On the other hand, when aiming to recover epibionts, cultivation in surface attachment improves not only the recovery rate but also the production of secondary metabolites, because bioactive molecules are preferably produced in the biofilm state (69). The most extreme example in which ecological knowledge has been key for success is the cultivation of microbial symbionts, due to their highly specific requirements derived from their adaptation to the host. For the successful isolation of *Oscillatoria spongeliae*, a cyanobacterial endosymbiont which produces the antibiotic originally attributed to the sponge *Dysidea herbacea*, the designed medium resembled the sponge mesophyl; it was slightly hyperosmotic with respect to seawater (28).

When the microorganisms of interest require relatively simple growth conditions the development of pilot-scale cultivation adapted for a particular biotechnological need has been possible. Examples in the pharmaceutical industry are the production of the antitumoral Salinosporamide A by *Salinispora tropica* through fermentation in saline media for its use in clinical trials (82), and the production of antiparasitic

manzamine alkaloids by *Micromonospora* sp. isolated from sponges (3). Another interesting example is the cultivation of autotrophic microalgae as a source of oil for the biodiesel industry (45). This is recognized as the most efficient and environmentally-friendly way of producing lipids, since oil content can reach 80 % biomass and directly relies on sunlight (10). These microorganisms, grown in open or closed systems (called photobioreactors), can be marine or freshwater derived. The current challenge remains to achieve high cultivation densities and to modify current photobioreactors to reduce operating costs (41, 45).

Novel culturing techniques

Marine microbes are at the top list of "uncultivable by conventional methods", (33). Our lack of knowledge of the environmental and nutritional requirements of the targeted microorganisms, as well as the loss of biological cell-to-cell interactions due to the isolation, seem to be some of the possible explanations for this limitation (33). In addition, the immense complexity of the marine microbial world attempts against the recovery of a significant fraction of this diversity. Sophisticated high-throughput cultivation techniques have recently emerged as an alternative to overcome these limitations. With the aid of novel cultivation methods, the proportion of microorganisms from marine environments represented in culture has increased in the published records [from 10 % in 2000 to 25% in 2009; (42)].

A combination of high-throughput screening with the use of the natural source environment as culture media represented the first important progress in the large scale cultivation of marine bacteria. In a landmark study, high-throughput dilution-to-extinction culture was applied to marine bacterioplankton, by means of the dilution of seawater bacteria up to 1-10 cells per well in microtiter plates, using low-nutrient filtered marine water. Growth was subsequently detected by fluorescence microscopy in cell arrays (9). This method, which is both powerful and sensitive, allowed the cultivation of many previously uncultured members of the bacterioplankton community. For example, the first representative of the SAR11 clade, the most abundant marine bacterial group and only previously detected by culture-independent methods, was recovered (61). In later studies, this approach coupled to long-term incubation at low temperatures allowed the recovery of new variants of the members of these oligotrophic microorganisms (77). Similarly, Kaeberlein *et al.* (34) greatly improved the proportion of culturable bacteria from marine sediments by using a diffusion chamber. In this device, microbial cells are inoculated in an agar matrix which is separated from the source environment by membranes, isolating the cells but allowing nutrients and growth factors to pass through. The authors reported an average increase of the cells able to be recovered in culture, from 6 % to 22 % (34). Another version of this approach is the microbial trap, which selectively enriches for actinomycetes by allowing the filament colonization of the sterile agar through membranes with 0.2 μm pores (22).

Microdroplet encapsulation in an agarose matrix, combined with growth detection by flow cytometry, led to the recovery of new clades from the marine environment (97, 98). The advantage of the approach is that the microdroplets are physically distinct and, because they are much larger than bacterial cells, they can be manipulated. It also provides the matrix for development of a microcolony because the agarose is porous and nutrients and signaling molecules can diffuse into the growing colony and waste metabolites can diffuse out (33, 97). Interestingly, this method seems to enrich in rare microorganisms, as several of the cultivated representatives could not be detected in the environmental gene library from the same sample (97).

The bottleneck of these approaches seems to be the adaptation of the strains recovered in culture to laboratory conditions ("domestication"). Many of the strains forming microcolonies in diffusion chambers could only undergo a limited number of divisions in Petri dishes (34). In further experiments, successive rounds of *in situ* cultivation in the chambers allowed for a larger recovery of cells (44). Also, most of the strains able to grow on Petri dishes in the first study were indeed mixed cultures, highlighting the importance of chemical signaling for microbial growth (34). The co-culturing with "helper strains", followed by the identification of an oligopeptide signal, allowed previously uncultured strains to be successfully isolated in the laboratory (44, 56). Currently, second-generation high-throughput automated methods are being developed from these environmental cultivation devices. One example is the development of the isolation chip (Ichip), a culture/isolation device composed of several hundreds of miniature diffusion chambers each inoculated with a single environmental cell (55). Another is the micro-Petri dish, a device supported by porous material and reaching a million growth compartments (31).

Genomics

Since the publication of the first genome in 1995 (19), the field of microbial genomics has continue to grow exponentially, in such a way that more sequence data is produced that the one that can be analyzed (38). The milestone of 1,000 available prokaryotic genomes has been reached in 2009 (39), and in only 19 months that number increased more than 65 % with more than 4,900 genomes reported as "in process" by the end of June of 2011 (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). The analysis of annotated genomes shows that the biosynthetic potential of microorganisms has been greatly underestimated. For example, for a given organism there are far more biosynthetic gene clusters in its sequenced genome than currently known metabolites (88). In addition, many of the genes which emerge from these studies have no homologues in the databases or have unknown functions, indicating that despite data accumulation, our knowledge of the microbial world is still scarce (88). In an effort to increase this knowledge for marine bacteria, in 2004 the Marine Microbial Genome Sequencing Project was launched. This large-scale international effort comprises the genome sequencing and automatic annotation of hundreds of ecologically relevant microorganisms isolated from diverse marine habitats (http://camera.calit2.net/microgenome/). Its importance lies not only on the knowledge gained about particular isolates, but in the power to provide scaffolds for interpretation of the ever-growing culture-independent sequence data.

A strategy to sequence and assemble at least part of the genome of an uncultured microorganism is single-cell genomics, where DNA from individual cells is obtained, followed by whole-genome amplification and sequencing (27). This methodology has recently been used to obtain approximately two-third of the genome of a sponge symbiont belonging to candidate phylum *Poribacteria*, revealing unprecedented insights into the metabolism and lifestyle of this possibly ancient bacterium (73). Woyke *et al.* (94) used a similar approach involving fluorescence-activated cell sorting and multiple displacement amplification to obtain genome sequences of two abundant uncultured marine flavobacteria.

Metagenomics

Metagenomics can be defined as the analysis of genomic DNA from a microbial community (23). It usually starts with the direct extraction of the DNA from an environmental sample (the metagenomic DNA), without a previous culturing step (Fig. 1). This DNA can be analyzed essentially in the same way than genomic DNA from a single microorganism, for

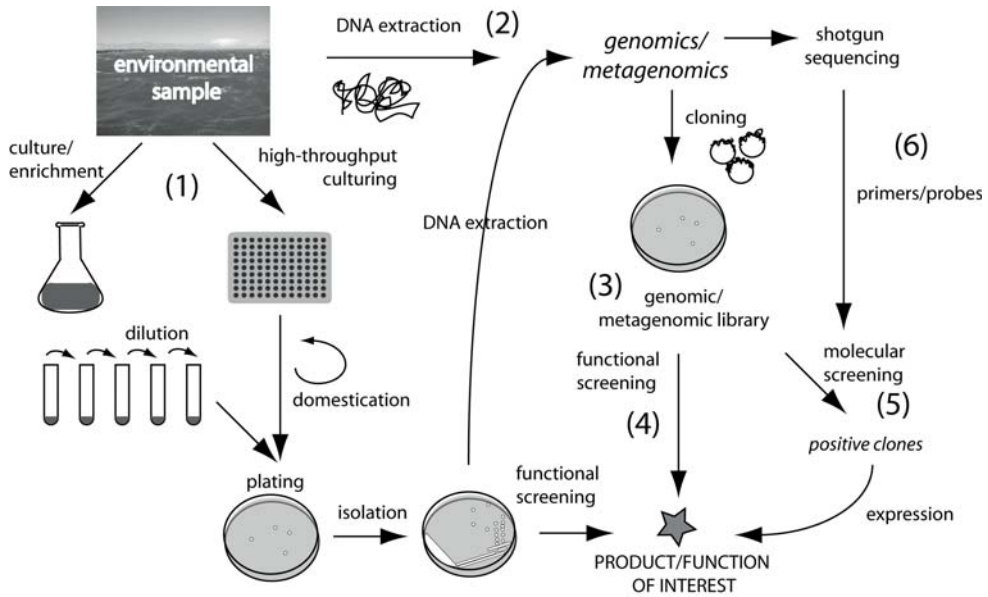

Figure 1. Main culture-dependent and independent methods used for bioprospection of microorganisms. From an environmental sample, microorganisms can either be cultured using traditional or high-throughput methods (1). Alternatively, DNA can be extracted directly from the sample (culture-independent methods, 2). DNA from pure cultures or the environmental sample can be cloned to generate genomic or metagenomic libraries, respectively (3). These libraries can be screened by functional (4) or molecular (5) methods to search for biomolecules of interest. Shotgun sequencing can aid in the design of molecular screening methods (6)

example by randomly sequencing the metagenomic DNA using next-generation sequencing technologies (49). With this approach, called random shotgun metagenomics, the community coverage will depend on the how diverse the community is and the chosen sequencing depth. Ultimately, it results in a profile of the genes contained in the metagenomic DNA and therefore in the community (23). Sequence assembly of the short individual reads obtained by these technologies still represents a challenge in complex communities, and this often limits the biotechnological value of the obtained information (51). If the assembly of the genes or gene clusters of interest is possible, they can be synthesized and cloned in order to produce the desired product. The term "synthetic metagenomics" has been recently proposed to define a new biocatalyst discovery approach that involves *in silico* identification of hypothetical target sequences followed by automated chemical DNA synthesis and heterologous expression (5). Synthetic metagenomics has recently been used to obtain *de novo* functional methyl halide transferase enzymes, useful for biofuel production, using information from the GenBank database (5). Advantages of this approach include the exploitation of the existing sequence databases and the possibility of codon optimization prior to their expression into the desired host (5).

The second metagenomic approach is to clone the isolated DNA using appropriate vectors to construct a metagenomic library, which is then screened for the desired capability (Fig. 1). Each clone will contain a fragment of the genome of a single microorganism of the community and, in theory, the whole genomic information of all members of the community can be captured if sufficient clones are obtained. Different types of vectors based on insert length can be used for this purpose: plasmids (which clone less than 10-15 kb), cosmids and fosmids (25-40 kb), or BACs (100-200 kb). The choice of vector depends on the environmental DNA quality, targeted genes and screening strategy. Small vectors such as plasmids will have high copy numbers and as a consequence can support stronger gene expression, while larger vectors have the advantage that they can hold complete pathways (74). For a complex community, the metagenomic library will need to contain a great number of clones (often hundreds of thousands) to be able to find clones containing the desired pathway or gene (84). Substrate enrichment and physical separation of metabolically active DNA *(e.g.* by stable isotope probing) previous to the cloning step can help increase hit rates (72).

The screening of the metagenomic library relies on two complementary approaches: function-based and sequence-based. In function-based screenings, the desired function is detected or selected (74). It has the obvious advantage that the identified clone is already functional, and that it bears the potential to identify novel types of genes or pathways. However, gene expression in a heterologous host can be problematic, as the host genetic machinery often cannot recognize signals in the foreign DNA (91). Bioinformatic simulations have estimated that less than 50 % of enzymatic activities may be actually recovered in random cloning in *Escherichia coli* (21). A possible solution is to engineer the host cell machinery in order to enhance recognition of foreign genes. For example, an *E. coli* strain with modified ribosomal proteins was able to recognize genes from high G+C content bacteria twelve times more efficiently than the unmodified host (7). Heterologous hosts other than *E. coli* are also increasingly used for specific purposes. Examples include *Streptomyces, Pseudomonas, Rhizobium* and *Sinorhizobium* strains (35, 84). The development of expression vectors also functional in these bacteria (shuttle vectors) is a fundamental requirement for this purpose. Cosmid, fosmid and BAC vectors with extended host ranges have already been developed (1, 57).

Up to date, three different types of function-driven approaches are recognized: phenotypical detection of the desired activity, heterologous complementation of host strains or mutants and induced gene expression (74). In the first case, the clones of the metagenomic library are subjected to activity assays in order to detect the specific metabolic function. However, sometimes there are no assays available for the desired functions, or the signals are very faint to be readily detected (42). Heterologous complementation relies on the requirement of the target gene presence for growth [*e.g.* antibiotic resistance (63) or DNA polymerases (75)]. This approach represents a fast and effective way to analyze a large number of clones, with almost no false positives. Substrate induced gene expression (SIGEX) and its variants utilize specific expression vectors which are capable of producing fluorescence coupled to the activity of interest (83, 85, 92). SIGEX was developed to recover catabolic genes, based on the knowledge that catabolic gene expression is induced mainly by specific substrates, and is often controlled by regulatory elements close to the gene. A promoter-trap *gfp*-expression vector was used in combination with fluorescence-activated cell sorting for high-throughput selection of clones in liquid cultu-

res, for isolating aromatic-hydrocarbon catabolic operon fragments from a groundwater metagenomic library (83). A disadvantage of this approach is that constitutive expression systems or those with transcriptional regulators that are distantly situated cannot be detected.

In marine biotechnology, the successful expression of enzymes from psychrophilic microorganisms is of particular interest. Due to the misfolding that occurs in thermolabile proteins at higher temperatures, low activities are commonly observed at conventional screening temperatures. Decreasing cultivation temperatures of the *E. coli* host can recover folding and activity, but there is a concomitant reduction in growth and heterologous protein synthesis rates (16). This problem has been overcome using an *E. coli* strain carrying chaperone genes from *Oleispira antarctica,* which was able to functionally express a temperature sensitive esterase (18). This type of promising system could be adapted for the construction and functional screening of metagenomic libraries from cold environments (35).

In contrast to function-based screening strategies, the application of sequence-driven approaches involves the use of specific primers or probes to screen the library, designed based on conserved regions in the genes of interest. As they can only be designed based on previous knowledge, variants of known proteins tend to be retrieved by this approach (78). Nevertheless, this strategy has succeeded in the identification of novel genes, for example broad-range alkane monooxygenases from deep sea sediments (96). Alternatively, sequences of the genes identified by a functional approach can also be used as a source of *de novo*, unbiased genetic information to maximize the discovery process (17). Random next-generation sequencing of the metagenomic DNA from the source environment could also be applied to this end.

Biotechnology-driven metagenomic studies of marine environments have mainly focused on industrial enzymes (35, 42, 99) or pathways of pharmaceutical interest (35). Lipases (25, 29, 32, 43), esterases (11, 20) and monooxygenases (96) are the enzymes most often targeted in metagenomic libraries, usually by functional screening. Marine sediments are the environments often used for this type of studies, as they are now recognized as deposits of an enormous and mostly unexplored microbial diversity. They have been found to be even more phylogenetically diverse than any other environment type including soil (46). It is therefore foreseeable that this promising environment continues to be explored in the search of new biotechnological applications.

Microbial communities, in particular those inhabiting marine environments, represent an invaluable source of genetic information that can help solve pressing issues for our society. The bioprospection of these communities, which involves searching for products and activities of interest, is currently experiencing a revolution due to the development of an expanding methodological toolkit that allows the mining of biodiversity with a depth never possible before. The first part of this review highlighted the most promising research areas in this field, and describes traditional as well as advanced methodologies that can be used for the bioprospection of marine microorganisms. In the second part of this revision, we will discuss the potential of Argentinean marine environments for the bioprospection of microorganisms, as well as the challenges and opportunities for Argentinean researchers in this field.

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