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Bioactive Molecules from Extreme Environments

Edited by
Daniela Giordano

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Editor

Daniela Giordano

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Editor
Daniela Giordano
National Research Council
(CNR)
Italy

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

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Contents

About the Editor

Daniela Giordano, Ph.D., is a CNR (National Research Council) Scientist in Biochemistry. She began her work at CNR in 2011, working first at the Institute of Protein Biochemistry (IBP-CNR) and, since 2014, at the Institute of Biosciences and BioResources (IBBR-CNR) in Naples. She graduated in Pharmaceutical Chemistry at the University of Naples Federico II in 2002 and obtained a Ph.D. at the University of Sacro Cuore in Rome in 2007, spending a period of six months at Northeastern University in Boston. After receiving her Ph.D., she began her research experience with fellowships at the Institute of Protein Biochemistry (IBP-CNR) in Naples, focusing her research on the molecular basis of cold adaptation of oxygen-binding proteins in polar bacteria and fish. She spent many periods as a visiting scientist at the National Institute of Health and Medical Research (INSERM), Paris, the University of Antwerp, and the University of Buenos Aires, studying the recombinant expression of globins of Antarctic bacteria and fish and their kinetic and thermodynamic properties. In 2010, she participated in an Arctic cruise, the TUNU-IV expedition (TUNU is East Greenland in Inuit language), for the collection of Arctic samples. Her research interest is focused on Antarctic and Arctic marine organisms because they are amongst the most vulnerable species to climate change and are a valuable source of natural products that can function as start structures of new molecules for drug discovery. Indeed, she is now leading a new research line on the identification of bioactive compounds as promising industrial products (pharmacy, nutraceutics, cosmeceuticals) from marine polar organisms. The results of her research are summarized in over 60 publications on highly qualified international journals and book chapters.

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Commentary

Bioactive Molecules from Extreme Environments

Daniela Giordano [1,2]

[1] Institute of Biosciences and BioResources (IBBR), CNR, Via Pietro Castellino 111, 80131 Napoli, Italy; daniela.giordano@ibbr.cnr.it

[2] Department of Marine Biotechnology, Stazione Zoologica Anton Dohrn (SZN), Villa Comunale, 80121 Napoli, Italy

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Abstract: Marine organisms inhabiting extreme habitats are a promising reservoir of bioactive compounds for drug discovery. Extreme environments, i.e., polar and hot regions, deep sea, hydrothermal vents, marine areas of high pressure or high salinity, experience conditions close to the limit of life. In these marine ecosystems, "hot spots" of biodiversity, organisms have adopted a huge variety of strategies to cope with such harsh conditions, such as the production of bioactive molecules potentially valuable for biotechnological applications and for pharmaceutical, nutraceutical and cosmeceutical sectors. Many enzymes isolated from extreme environments may be of great interest in the detergent, textile, paper and food industries. Marine natural products produced by organisms evolved under hostile conditions exhibit a wide structural diversity and biological activities. In fact, they exert antimicrobial, anticancer, antioxidant and anti-inflammatory activities. The aim of this Special Issue "Bioactive Molecules from Extreme Environments" was to provide the most recent findings on bioactive molecules as well as enzymes isolated from extreme environments, to be used in biotechnological discovery pipelines and pharmaceutical applications, in an effort to encourage further research in these extreme habitats.

Keywords: Arctic/Antarctic; deep-sea; deep hypersaline anoxic basin; cold-adapted bacteria; halophilic microorganisms; marine natural product; enzyme; carotenoid; silver nanoparticle; marine bioprospecting

1. Foreword

Marine organisms produce a huge variety of natural products that confer important advantages, either as antibiotics or by allowing communication with other organisms and with the environment. These molecules have long been exploited in medical fields as antioxidants, antimicrobial and anticancer agents, and in biotechnological applications, attracting an increasing commercial interest from the biotech sector [1]. In 2016, 1277 new marine compounds were reported exerting interesting biological activities, and many of these are of bacterial origin [2]. The global market for marine-derived drugs is expected to be USD 2745.80 million by 2025 [3]. However, to date, few molecules isolated from marine organisms have been approved [4] and many marine ecosystems remain unexplored.

Due to the limited accessibility and remoteness, extreme marine environments are still largely underexploited in comparison with terrestrial ecosystems. Recent advances in the sampling and accessibility of extreme areas, together with the development of omics technologies, have opened new avenues for drug discovery. These remote habitats are promising reservoirs of biotechnological and biomedical applicability, and represent a good opportunity for compound discovery and bioprospecting. Marine organisms inhabiting extreme environments have adopted unique survival strategies for growing and reproducing under hostile conditions, biosynthesizing an array of biomolecules potentially valuable for many applications in the biotechnological sector, in the pharmaceutical and cosmeceutical industries and in the bioremediation.

Extreme environments are exposed to one or more environmental parameters, i.e., temperature, salinity, osmolarity, UV radiation, pressure or pH, showing values close to the limit of life. Polar and hot regions, deep sea, hydrothermal vents, marine areas of high pressure or high salinity, and acidic and alkaline regions are examples of extreme environments. In the Arctic and Antarctic regions, the sea ice can reach up to ~13% of the Earth's total surface. The Arctic Ocean is mainly characterized by light seasonality and cold temperatures with winter extremes, whereas Antarctica is considered as the driest, windiest and coldest place on Earth, completely isolated, geographically and thermally, from the other continents. Polar marine life has adapted to thrive in the ocean's most inhospitable conditions, where extremes of pressure and temperature and the absence of light have selected species with a unique range of bioactive compounds, either enzymes [5] or secondary metabolites [6].

The deep sea, below a depth of 1000 m, is recognized as an extreme environment. It is characterized by the absence of sunlight and the presence of low temperatures and high hydrostatic pressures. This environment becomes even more extreme in particular conditions, with extremely high temperatures of >400 °C in deep-sea hydrothermal vents, and with extremely high salinities and high pressures in deep hypersaline anoxic basins (DHABs). These habitats have been discovered on the sea floor in different oceanic regions, such as the Red Sea, the eastern Mediterranean Sea and the Gulf of Mexico. New DHABs, the Thetis, Kyros and Haephestus basins in the Mediterranean Sea, have been recently discovered. DHABs are microbial "hotspot", with dense microbial populations of unique bacterial lineages more metabolically active than those of the adjacent layers [7]. Deep-sea extremophiles, able to proliferate under these challenging parameters (pressure and temperature, pH, salinity and redox potential), have adopted a variety of strategies to cope with these extreme environments, such as the production of extremozymes, with thermal or cold adaptability, salt tolerance and/or pressure tolerance, and secondary metabolites with biomedical applications.

The majority of marine bioactive compounds comes from microorganisms as a prolific resource for novel chemistry and the sustainable production of bioactive compounds, bypassing the problem of the recollection of samples from the field, which is required for macroorganisms. Recently, the increasing number of whole microbial genomes and the development of genome mining approaches have accelerated the discovery of novel bioactive molecules, overcoming the requirement for the isolation or cultivation of microorganisms [8].

2. The Special Issue

The Special Issue "Bioactive Molecules from Extreme Environments" was aimed at collecting papers regarding bioactive molecules and enzymes isolated from organisms inhabiting extreme environments being used in biotechnological discovery pipelines and pharmaceutical applications. Special attention was paid to the species biodiversity of extreme habitats as a promising reservoir of untapped compounds with biotechnological potential.

In total, eleven articles were accepted and included in the Special Issue. Most of the articles in this Special Issue were focused on marine microorganisms, bacteria and fungi, with their chemical diversity and active metabolites able to cope with harsh habitats.

Five articles in this Special Issue were focused on enzymes from microorganisms in extreme environments. Many enzymes have been evaluated for industrial applications, for the production of pharmaceuticals, foods, beverages, paper, as well as in textile and leather processing and waste-water treatment. However, only a few of these are able to meet the industrial demands that include tolerance of harsh conditions of temperature, pH, salinity and pressure, maintaining high conversion rate and reproducibility. The market for enzymes is expected to reach USD 7.0 billion by 2023, from USD 5.5 billion in 2018 [9]. Enzymes currently on the market derive from mesophilic organisms. However, the enzymes isolated from extreme environments are endowed with unique catalytic properties, and might be of great interest in different sectors of biotechnology.

In Bruno et al. 2019 [5], the authors described microbial enzymes with a potential biotechnological interest isolated in either Arctic or Antarctic environments. Polar marine environments are a promising

research area for the discovery of enzymes with a potential industrial application. Cold-adapted enzymes are characterized by unique catalytic properties in comparison to their mesophilic homologues, i.e., higher catalytic efficiency, improved flexibility and lower thermal stability. These features make them particularly interesting for a potential commercial use in the food industry, agricultural production, synthetic biology, and biomedicine. Oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases, and their proposed biotechnological applications, were reviewed in this paper. Hydrolases are the most abundant class of cold-adapted enzymes with a potential industrial application in the detergent, textile, paper, and food industries. For example, lactases or β-galactosidases, due to their capacity to reduce lactose intolerance, have attracted the interest of many research group and industries, and some of these isolated from the Antarctic marine *P. haloplanktis* LMG P-19143 were patented [10] and produced in large quantities by NutrilabNV (Bekkevoort, Belgium). Other enzymes have found application in molecular biology; a cold-adapted uracil-DNA glycosylase isolated from a psychrophilic Antarctic marine bacterium was released by New England Biolabs [11], a cold-active nuclease, isolated from an Antarctic marine psychrophile, *Shewanella* sp. strain Ac10, was developed by Takara-Clontech [12], and a recombinant alkaline phosphatase isolated from the Antarctic bacterial strain TAB5 was developed by New England Biolabs [13].

In Liu et al. 2019 [14], samples of seal and penguin feces, soil and marine sediment, collected from Fildes Peninsula in Antarctica were used as sources for the bioprospecting of chitinase-producing microorganisms. After optimizing the medium components and culture conditions, a cold-adapted strain belonging to *Pseudomonas* was identified as the best producer of chitinase, exhibiting more than 50% of its catalytic activity even at 0 °C. The crude chitinase showed the significant inhibition of fungi *Verticillium dahlia* CICC 2534 and *Fusarium oxysporum* f. sp. *cucumerinum* CICC 2532, which can cause cotton wilt and cucumber blight, respectively, suggesting that the cold strain may be a competitive candidate for biological control in agriculture, especially at low temperatures.

Jin et al. 2019 [15] discussed the potential industrial applications of extremozymes, as thermophilic, psychrophilic, halophilic and piezophilic enzymes. Deep-sea thermophiles, able to grow at high temperatures of 41–120 °C, produce thermostable proteolytic enzymes, attractive for use in the detergent, food and feed industries. For example, an α-amylase from the thermophile *Thermococcus* sp., isolated from a deep-sea hydrothermal vent, is on the market, and is named Fuelzyme®, released by Verenium Corporation (San Diego, CA, USA) [16]. Deep-sea psychrophiles, exhibiting an active metabolism even at −25 °C, are a promising source of industrial cold-active enzymes with applicability in the textile, detergent, beverage, food and biofuel industries. Deep-sea halophilic enzymes have particularly great potential in the production of biodiesel, polyunsaturated fatty acids and food, or in the treatment of waste water containing high salt concentrations and starch residues. Deep-sea piezophilic enzymes, living up to 70–110 MPa, have shown high efficiency in applications for food production, where high pressures are used for the processing and sterilization of food materials.

Aguila-Torres and co-authors 2020 [17] reported a taxonomic identification and biochemical characterization of cypermethrin-degrading and biosurfactant-producing bacterial strains from samples collected in cypermethrin-contaminated marine sediment in Chilean northern Patagonia. This environment is considered extreme because of a wide range of temperatures, ranging from 4 to 20 °C, salinity, and a low nutrient availability. In addition, there has been an extensive use of cypermethrin as an antiparasitic pesticide in the salmon farming industry in northern Patagonia. Cypermethrin, used in agriculture and aquaculture, is considered a possible human carcinogen, able also to affect marine ecosystems. In northern Patagonia, a high concentration of cypermethrin has been reported in marine sediment. Among isolated strains, four strains exhibited the highest growth rate on cypermethrin, and high levels of biosurfactant production. An analysis of the genome sequence of these strains demonstrated the presence of genes encoding esterase, pyrethroid hydrolase and laccase, associated with the different biodegradation pathways of cypermethrin.

Bioremediation has become an important tool for removing pollutants from contaminated environments, taking advantage of the presence of microorganisms and their ability to clean

contaminated environments. Hydrocarbon-degrading bacteria are able to metabolize contaminants, developing specific pathways for sustaining their energetic and carbon requirements in the presence of them.

Varrella and colleagues 2020 [7] in their review described the environmental characteristics of DHABs, highlighting the unique bacterial lineages found in these extreme habitats. DHAB-derived microorganisms represent promising candidates for the bioremediation of oil hydrocarbons thanks to the presence of enzymes involved in pathways associated with hydrocarbon degradation. Proteobacteria represented by sulfate-reducing Deltaproteobacteria, as well as sulfur-oxidizing Gamma- and Epsilonproteobacteria, Actinobacteria, Deferribacteres and Euryarchaeota, were found across DHABs worldwide. In addition, viruses, found to be well-preserved in DHAB sediments, were able to control prokaryotic dynamics in these ecosystems. The variety of prokaryotes inhabiting DHABs represents an important source of polyextreme enzymes, such as esterase, lipase, α-amilase, pullulanase and xylanase, with applications in pharmaceutical, food, and beverage industrial processes, and many bioactive compounds with antiviral, antimicrobial and antitumor activities.

The marine chemical diversity varies from simple peptides and linear fatty acids to complex compounds such as terpenes, alkaloids and polyketides, etc., incorporating elements used in the marine environment as chemical defenses against predators. Natural products with different structures, able to perform a wide range of biological activities, make marine biomolecules valuable alternatives to many pathogenic bacteria and fungi, especially in the era of antimicrobial resistance. The most commonly reported activity is toward pathogenic agents, including viruses. Discovering novel and efficient antimicrobial molecules is becoming essential for natural-product chemistry, because of antibiotic-resistant microorganisms or even multidrug-resistance, and the development of new emerging infections. One example is represented by a potent antiviral agent isolated from the ascidian *Aplidium albicans*, which is under clinical trials in patients affected by Corona Virus SARS-CoV-2 [18]. Many papers in this issue are related to antimicrobial activity as well as anticancer activity. Cancer is the leading cause of death globally, and finding new molecules with anticancer activities remains a major challenge in the pursuit for a cure.

Zain ul Arifeen et al. 2019 [19] wrote a review in which they described the structure, biological activity, and distribution of secondary metabolites produced by deep-sea fungi in the last five years. Fungi living in deep-sea environments produce unique secondary metabolites for defense and communication. Despite being the producer of many important bioactive molecules, deep-sea fungi have not been explored thoroughly due to methodological and technical limitations. However, their abundance and presence in all possible extreme ecosystems make them an ideal source of new bioactive molecules with many applications. Polyketide- and nitrogen-containing compounds, polypeptides, ester and phenolic derivatives, piperazine derivatives and terpenoid compounds were the bioactive molecules isolated from deep-sea fungi and showing antibiotic, antimicrobial, antiviral activities as well as cytotoxicity against cancer cells. Most of these compounds were isolated from two fungal genera, i.e., *Penicillium* and *Aspergillus*. Among these natural products, terpenoid derivatives were the most abundant compounds with the strongest antibiotic and cytotoxic activities with respect to other classes of molecules. In particular, breviones, isolated from the deepest sediment-derived fungus *Penicillium* sp. (5115 m depth), showing the strongest cytotoxic activity against cancer cells, have the potential to be good candidates for anticancer drugs.

Corral and co-authors 2019 [20] described the antimicrobial and anticancer molecules produced by microorganisms, illustrating their action mechanisms *in vitro*. Halophilic microorganisms, such as archaea, bacteria and fungi, widely distributed around the world, inhabit hypersaline ecosystems characterized by a salinity higher than seawater, i.e., 3.5% NaCl. They are a source of bioactive molecules with applications in biomedicine. The continuous increase in antibiotic resistance establishes an urgent need for the exploiting of natural and sustainable resources to find novel antimicrobial molecules. Bacteria of the genus *Nocardiopsis* and *Streptomyces*, belonging to the phylum Actinobacteria, were found to be the main producers of antimicrobial compounds, whereas among fungi the genus

Aspergillus was the most prolific. Likewise, *Nocardiopsis*, *Streptomyces*, *Bacillus*, *Halomonas* and *Aspergillus* were the most frequent producers of antitumoral molecules. Some of these compounds are promising candidates for preclinical trials.

Choi et al. 2019 [21] reported the characterization of deinoxanthin from a novel reddish *Deinococcus* sp. AJ005 isolated from seawater near King George Island in Antarctica, whose genome was recently completely sequenced. *Deinococcus* strains, Gram-positive bacteria, live in different habitats such as air, soils, and seas, and also at high altitudes and in Antarctic environments. They cope with these extreme habitats thanks to a variety of metabolic pathways, including the biosynthesis of antioxidants such as deinoxanthin. This carotenoid is particularly interesting for its use as an antioxidant, a cosmetic ingredient, and a food or feed additive, it being an efficient scavenger of reactive oxygen species and an anticancer agent. On the basis of genome annotation analysis, the authors proposed the deinoxanthin biosynthetic pathway, investigating the effects of culture conditions on the deinoxanthin biosynthesis in this strain.

John et al. 2020 [22] used a new *Pseudomonas* strain associated with the Antarctic marine ciliate *Euplotes focardii* to obtain silver nanoparticles (AgNPs) after incubation with 1 mM of $AgNO_3$ within 24 h. Nanoparticles (NPs) have become particularly interesting in biomedical sciences, drug-gene delivery, space industries, cosmetics and chemical industries. AgNPs show general antibacterial and bactericidal features, thus becoming promising tools in biomedical applications. Since the physiochemical methods used for AgNP synthesis are not convenient given their high energy consumption and the use of toxic reagents, there has been a growing need to develop a simple and low-cost approach to AgNP synthesis without toxic chemicals. An alternative to the chemical synthesis method is to use microbes to obtain nanoparticles. This easy and efficient biological method to synthesize AgNPs may be used against drug-resistant pathogenic bacteria, contributing to solving the problem of antibiotic resistance. The authors characterized the size and morphology of AgNPs and demonstrated that *Pseudomonas* AgNPs showed a higher antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* with respect to the chemically synthesized NPs. The results of this paper are related to the patent number 102019000014121 deposited in 06/08/2019.

Only two articles in this Special Issue are focused on macroorganisms, specifically the deep-sea Antarctic sponge *Latrunculia biformis*, collected from the Antarctic Weddell Sea shelf at a depth of 291 m, and the Antarctic krill *Euphausia superba* that represents the most abundant biomass in cold environments at the base of the Antarctic food chain.

In Li et al. 2019 [23], the authors isolated diverse discorhabdin alkaloids from the Antarctic deep-sea sponge *L. biformis*. Three known discorhabdins, (−)-discorhabdin L, (+)-discorhabdin A and (+)-discorhabdin Q, and three new discorhabdin analogs (−)-2-bromo-discorhabdin D, (−)-1-acetyl-discorhabdin L and (+)-1-octacosatrienoyl-discorhabdin L, were identified and characterized by bioactivity and molecular networking-based metabolomics and the chemical structures elucidated by extensive spectroscopy analyses. (−)-discorhabdin L, (−)-1-acetyl-discorhabdin L and (+)-1-octacosatrienoyl-discorhabdin L showed promising anticancer activities, demonstrated by the molecular modeling of the potential binding of discorhabdins to the anticancer targets involved in their anticancer activity. (−)-1-acetyl-discorhabdin L and (+)-1-octacosatrienoyl-discorhabdin L are the first discorhabdin analogs with an ester function at C-1 with (+)-1-octacosatrienoyl-discorhabdin L, which is the first discorhabdin bearing a long-chain fatty acid at this position.

The experimental article by Huang et al. 2020 [24] reported the first genome survey of the Antarctic krill *E. superba*, a very important marine organism in the Antarctic food chain. The high-throughput comparative identification of putative antimicrobial peptides (AMPs) and antihypertensive peptides (AHTPs) from whole-body transcriptomes of the Antarctic krill and its mesophilic counterpart, the whiteleg shrimp *Penaeus vannamei*, revealed that AMPs/AMP precursors and AHTPs were generally conserved, with interesting variations between the two crustacean species due to cold adaptation. This paper is a preliminary exploration of bioactive peptides in a polar key species of the trophic chain for the development of novel marine drugs.

3. Perspectives and Conclusions

The papers included in this Special Issue provide an overview of the growing interest in species biodiversity, highlighting the importance of marine extreme environments as sources of a unique marine chemical diversity of molecules. It is worth noting that six articles in this Issue are focused on molecules and enzymes isolated from Antarctica. This means that there is an increasing interest in this habitat because it is perceived as an important source of drug discovery. In fact, the unique environment and ecological pressures of marine polar regions might be the major drivers of a selection of unique biological communities able to biosynthesize new compounds with diverse biological activities. It is expected that, in the near future, more marine molecules from polar regions as well as from other extreme habitats will find their way into biomedical and biotechnological applications.

In conclusion, the Guest Editor thanks all the authors that contributed with their interesting articles to this Special Issue, all the reviewers for evaluating the submitted manuscripts, and the Editorial board of Marine Drugs, the Editor-in-Chief of the Journal Orazio Taglialatela-Scafati for their support, especially in this difficult period of the 2020 pandemic SARS CoV-2.

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Review

Enzymes from Marine Polar Regions and Their Biotechnological Applications

Stefano Bruno [1], Daniela Coppola [2,3], Guido di Prisco [2], Daniela Giordano [2,3] and Cinzia Verde [2,3,*]

[1] Department of Food and Drug, University of Parma, Parco Area delle Scienze 23A, 43124 Parma, Italy; stefano.bruno@unipr.it

[2] Institute of Biosciences and BioResources (IBBR), CNR, Via Pietro Castellino 111, 80131 Napoli, Italy; daniela.coppola@ibbr.cnr.it (D.C.); guido.diprisco@ibbr.cnr.it (G.d.P.); daniela.giordano@ibbr.cnr.it (D.G.)

[3] Department of Marine Biotechnology, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy

* Correspondence: c.verde@ibp.cnr.it or cinzia.verde@ibbr.cnr.it

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Abstract: The microorganisms that evolved at low temperatures express cold-adapted enzymes endowed with unique catalytic properties in comparison to their mesophilic homologues, i.e., higher catalytic efficiency, improved flexibility, and lower thermal stability. Cold environments are therefore an attractive research area for the discovery of enzymes to be used for investigational and industrial applications in which such properties are desirable. In this work, we will review the literature on cold-adapted enzymes specifically focusing on those discovered in the bioprospecting of polar marine environments, so far largely neglected because of their limited accessibility. We will discuss their existing or proposed biotechnological applications within the framework of the more general applications of cold-adapted enzymes.

Keywords: Arctic/Antarctic environment; biocatalysis; cold-adaptation; marine biotechnology

1. Introduction

The economic progress in the "Era of Biotech" demands new biocatalysts for a wide range of applications, from therapy to industrial manufacturing. Particularly, biocatalysis is an attractive alternative to chemical synthesis, as biocatalysts are biodegradable and non-toxic, they originate from renewable sources, they have high selectivity and they provide products in high yields [1]. Up to 40% of the industrially relevant chemical reactions that require organic solvents harmful to the environment could be substituted by enzymatic catalysis by 2030 [2]. Many industrial processes already benefit from the use of enzymes (e.g., proteases, amylases, cellulases, carboxymethylcellulases, xylanases) in the production of pharmaceuticals, foods, beverages, confectionery, paper, as well as in textile and leather processing and waste-water treatment. Most of these enzymes are microbial in origin because they are relatively more thermostable than their counterparts from plants and animals [3]. Their market is expected to reach $7.0 billion by 2023 from $5.5 billion in 2018, with an annual growth rate of 4.9% for the period 2018–2023 [4]. In this market, industrial detergents will reach $10.8 billion by 2022, with an annual growth rate of 4.2% for the period 2017–2022 [4], whereas the global market for enzymes in the food and beverages industries is expected to grow from $1.8 billion in 2017 to nearly $2.2 billion by 2022, with an annual growth rate of 4.6% from 2017 to 2022 [4].

The increasing demand for new or better biocatalysts with different substrate selectivity, chiral selectivity, stability and activity at various pH and temperatures is likely to be met through the investigation of microorganisms, the largest reservoir of biological activities in Nature. Marine microorganisms are particularly promising in this respect and it is predicted [5] that the global market for marine biotechnology, estimated at $4.1 billion in 2015, may reach $4.8 billion by 2020 and $6.4 billion

by 2025, with the identification of new marine-derived enzymes [6]. The bioprospecting of marine environments, i.e., the systematic search for new bioactive compounds or biomolecules, has been traditionally focused on temperate/tropical latitudes [7], and polar marine environments have been mostly neglected, mainly due to their limited accessibility. These habitats include not only seawater but also sediments and sea ice, where internal fluids remain liquid in winter. Although both polar regions contain freezing seas, their microorganisms have different evolutionary histories due to their different geography and land-sea distribution [8]. The northern polar region is characterized by extensive, shallow shelves of landmasses that surround a partially land-locked ocean [9], whereas the Antarctic region consists in a dynamic open ocean that surrounds the continent [10,11]. The opening of the Drake Passage between Tierra del Fuego and the Antarctic Peninsula 23.5–32.5 million years ago was the key event for the development of the Antarctic Circumpolar Current (ACC), partially responsible for cooling of the Antarctic waters. The Antarctic Polar Front, the northern boundary of the ACC, promoted the isolation of the Antarctic marine fauna [10,11]. In both polar regions, the constantly low temperatures have driven the evolution of cold-adapted microorganisms, which are classified as psychrophilic and psychrotolerant according to the physiological features of their growth [12]. In this review, the general term "cold-adapted" will be used to indicate "psychrophile" organisms indigenous to cold environments [13].

In view of recent breakthroughs in sampling methodologies, sequencing, and bioinformatics, polar marine environments have recently come into the scientific spotlight—also because of growing concerns about their role in global climate change dynamics. An increasing number of works demonstrated their biological diversity [14–19], which includes bacteria, archaea, yeasts, fungi and algae [20,21]. Among polar microorganisms, those defined as 'cold-adapted' are those that thrive in permanently cold environments, even at subzero temperatures in super-cooled liquid water, and that evolved the physiological and biochemical capability to survive and reproduce under these extreme conditions [22]. With a better knowledge of marine microorganisms of polar regions, previously inaccessible bio-products, both in terms of new bioactive metabolites and proteins/enzymes with potential commercial applications, have been described [23,24]. Their biotechnological use can take many forms, from agricultural production to industrial processes, food chemistry [25], synthetic biology, and biomedical uses [26,27]. It is expected that, in the near future, more cold-adapted bacteria and their enzymes will find their way to biotechnological applications.

In this review, we will describe recent findings in enzymes isolated in either Arctic or Antarctic regions, some of which are of potential biotechnological interest. Unlike previous reviews, we particularly focused on enzymes isolated from marine polar environments. Cold-adapted enzymes from non-marine environments (i.e., from soil or lakes) or from non-polar sources (i.e., deep sea) will be reported to suggest potential future applications for enzymes isolated from marine polar microorganisms or to highlight structure-function relationships common to all cold-adapted enzymes, regardless of their origin.

2. Methods for Enzyme Discovery and Engineering

The identification of enzymes from cultured or uncultured marine microorganisms have been carried out through several '-omics' techniques. Additionally, natural enzymes have been optimized through enzyme engineering to improve their biotechnological properties (Figure 1).

Omics techniques. The overall knowledge on polar microorganisms has recently increased through the application of "omic" approaches (environmental shotgun sequencing, metatranscriptomics, proteomics) that have revealed the peculiar properties of their communities [28].

High-throughput metagenomic screening approaches, using both sequence-based and function-driven screenings, are contributing to the identification of a large number of enzymes, with most industrial enzymes having being identified by traditional functional screening of (meta)genomic libraries [29–31]. Recently, rapid technological developments in bioinformatics have revolutionized the exploration of the microbial diversity for the discovery of enzymes with commercial impact [32].

The sequence-based analysis of their (meta)genomic DNA by high-throughput sequencing methods followed by in silico analysis was used to predict enzyme function [33] allowing an increase in the rate of discovery. Sequence-based metagenomic approaches represents a powerful tool by avoiding the intensive, expensive, and time-consuming lab work associated to classic screening by reducing the number of targets to be functionally tested. Nevertheless, the latter approach is only limited to the discovery of genes with high similarities to already deposited sequences, making the discovery of new enzymatic functions difficult [34].

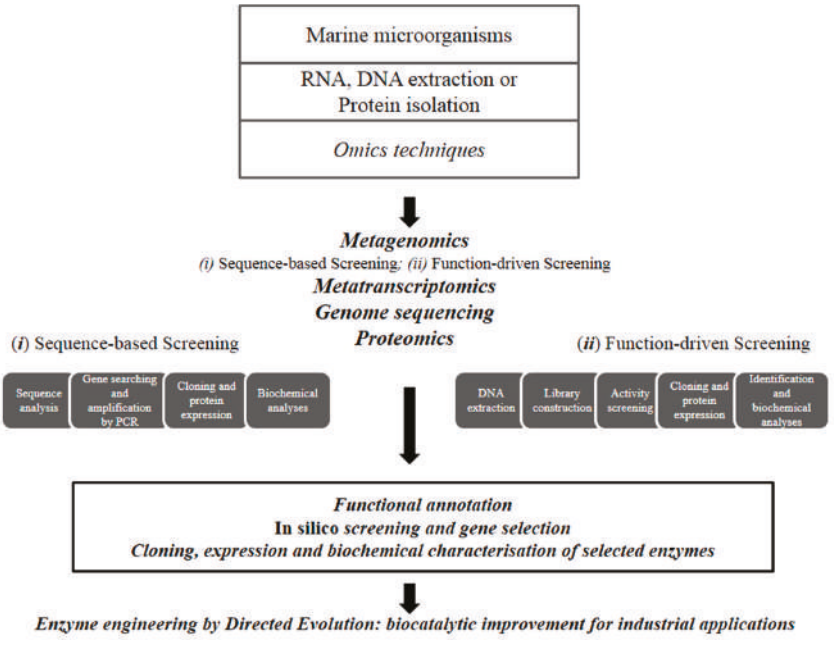

Figure 1. Workflow for the discovery of novel industrial enzymes by *omics* technologies.

Some recent EU FP7-funded projects (e.g., MACUMBA and PHARMASEA) made valuable efforts in exploiting the potential of microorganisms (mostly bacteria and microalgae) for pharmaceutical, cosmeceutical, and nutraceutical applications [35]. In order to explore marine biodiversity in several environments, including extreme habitats, a pan-European consortium was instituted in 2015 [36] for the creation of one of the largest collections of marine enzymes, with a focus on those of interest for the industrial market [32]. At present, this collection contains about 1000 enzymes, of which around 94% are available in ready-to-use expression systems and 32% have been completely characterized [2,32,37]. However, the publication of gene/genome sequences is faster than the identification of new protein functions, the most important challenge in modern biology due to the lack of experimental evidence to support functional annotation of a large fraction of genes and proteins [38,39]. Indeed, there is a gap between the numbers of biological activities predicted in silico and the number of new enzymes described experimentally, with the proportion of the latter ones approaching 0% [38,39], despite successful approaches of enzyme screen programmes through metagenomic approaches [40].

In addition to genomic screenings, recent technological developments in the complementary fields of transcriptomics have developed tools suitable for further discoveries. Indeed, advances within the field of RNA sequencing [41] have allowed analysis of whole (meta)transcriptomes and made them available for the generation of high-quality protein sequence databases, facilitating enzyme identification. The functional annotation of selected genomes and transcriptomes is relevant

to infer associated biological functions and application of proteomics studies in the discovery of bioactive proteins.

As for proteomics approaches, the most commonly used method for enzyme discovery is bottom-up shotgun proteomics [42], in which proteins are enzymatically digested and the obtained peptides are analysed either by liquid chromatography coupled to electrospray ionization (ESI–LC–MS/MS) or by matrix-assisted laser desorption/ionization followed by time of flight mass spectrometry (MALDI–TOF–MS). A further direct method for enzyme discovery is activity-based proteomics, which takes advantage of enzyme class-specific substrates for the identification of groups of enzymes [43,44].

Enzyme Engineering

The ultimate value of biotechnology is the production and delivery of molecules or processes of interest for many applications; the emerging requirement for biotechnological products is to be energy saving, thus reducing environmental impacts [45]. Therefore, enzymes from cold-adapted organisms are important because their properties meet the ongoing efforts and needs to decrease energy consumption [46].

The ability of enzymes to catalyze a diverse set of reactions with exquisite specificity makes these proteins essential for reactions useful to humankind. Due their structural and functional complexity, the use of natural enzymes in biotechnology has been limited over the past decades. In fact, natural enzymes generally require modification for industrial use [47], given the improvements in catalytic efficiency, stereo-selectivity, and stability that can be reached through combinatorial engineering strategies [48]. Recent advances in science and technology have led to activity improvements for native and non-native substrates, synthesis of new chemistries and functional characterization of promising novel enzymes.

In the past, enzyme-based industrial processes were designed around the limitations of natural enzymes discovered in nature; today, enzymes can be engineered to fit the process specifications through enzyme engineering [1]. Enzyme engineering can be pursued by either rational site-directed mutagenesis based on currently available sequence and 3D structures deposited in the RCSB Protein Data Bank, or by directed evolution. Directed evolution is a key technology at the base of the development of new industrial biocatalysts [49–52] by random amino acid residues changes in the enzyme, largely carried out using PCR-based methods, followed by selection or screening of resulting libraries for variants with improved enzymatic properties. Changes in enzyme properties usually require simultaneous multiple amino-acid substitutions, creating exponentially more variants for testing. However, modern high-throughput screening methods, such as fluorescence-activated cell sorting [53–55] allow the screening of tens of millions of variants in a short time. Moreover, the best approach to create multiple mutations is to limit the choices by statistical or bioinformatic methods, such as the statistical correlation approach based on the ProSAR (protein structure-activity relationship) algorithm [56], to identify if a particular substitution is beneficial or not.

Furthermore, the development of synthetic biology methodology, through which large DNA sequences can be synthesized de novo, is increasing the ability for designing and engineering enzymes with new functions. Moreover, the possibility to synthesize whole-gene synthesis can also be used to build high-quality DNA libraries [1].

3. Cold-Adapted Enzymes and Their Biotechnological Applications

The bioprospecting of microorganisms living in extreme environmental conditions has already led to the discovery of several enzymes endowed with catalytic properties potentially useful for biotechnological applications [57], including some isolated from marine polar regions [23]. Moreover, the systematic investigation of the relationship between the structure and function of these enzymes—in comparison with their mesophilic homologs—has allowed recognising specific structural features associated with cold adaptation, which can be applied to the design of non-natural enzymes endowed with high activity at low/room temperatures. New catalytic activities are expected

to arise from the Enzyme Function Initiative (EFI), which was recently established to address the challenge of assigning reliable functions to enzymes discovered in bacterial genome projects [58]. The main aim of the initiative is the computation-based prediction of substrate specificity for functional assignment of unknown enzymes.

3.1. Applications of Cold-Adapted Enzymes

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The ability of enzymes to catalyze a diverse set of reactions with exquisite specificity makes these proteins essential for biochemical studies, as well as promising catalysts for reactions useful to humankind. Due to their structural and functional complexity, the use of natural enzymes in biotechnology has been limited over the past decades. In fact, natural enzymes generally require modification for industrial use, given the improvements in catalytic efficiency, stereo-selectivity, and stability that can be reached through combinatorial engineering strategies. Recent advances in science and technology have led to activity improvements for native and non-native substrates, synthesis of new chemistries and functional characterization of promising novel enzymes.

Protein engineering technologies make it possible to develop enzymes that are highly active on non-natural targets, in the presence of organic solvents, and even enzymes for chemical transformations not found in nature. Application of these advanced engineering technologies to the creation of biocatalysts typically starts with an integrated approach with iterative cycles of DNA mutation and selection to create—by directed evolution—enzymes with novel functions for biotechnological approaches [59]. Due to our enhanced understanding of how these enzymes work in extreme environments, we can look forward to increasingly use computational tools, such as Artificial Intelligence, and to design enzymes with new structures and functions [60].

Cold-adapted enzymes have been suggested as biotechnological tools for several reasons:

(1) They are cost-effective, e.g., lower amounts are required, due to higher catalytic efficiency at low temperature;

(2) They can catalyze reactions at temperatures where competitive, undesirable chemical reactions are slowed down. This property is particularly relevant in the food industry, where deterioration and loss of thermolabile nutrients can occur at room temperature;

(3) They catalyze the desired reactions at temperatures where bacterial contamination is reduced. There is a number of advantages in working at lower temperature (around 10–15 °C) than those currently used for large-scale industrial production;

(4) Most cold-adapted enzymes can be inactivated by moderate heat due to their thermolability, avoiding chemical-based inactivation. A striking application of this property has been described for the design of live vaccines. Mesophilic pathogens were engineered for production of thermolabile homologs of essential enzymes, making them temperature-sensitive (TS). The engineered strains are inactivated at mammalian body temperatures, thus losing pathogenicity, but retaining their entire antigenic repertoire. Duplantis and colleagues [61] were able to entirely shift the lifestyle of *Francisella* (*F. novicida*), responsible for tularaemia disease in mice, by substituting its genes encoding essential enzymes with those identified in an Arctic bacterium. The authors applied the same approach to *Salmonella enterica* and the Gram-positive *Mycobacterium* [62]. The TS *S. enterica* strains were shown to be safe in research or diagnostic laboratories but were still capable of stimulating a protective immune response [63]. The TS strain of *M. tuberculosis* could be a safe research or diagnostic strain that is incapable of causing serious disease in humans while being identical to wild-type *M. tuberculosis* except for the TS phenotype [62].

The isolation of cold-adapted enzymes from natural sources is mostly unfeasible and their recombinant expression through standard protocols is hindered by the relatively high growth temperature of the commonly used expression hosts, including *E. coli*. However, several recombinant expression systems have been devized for the production of cold-adapted enzymes [64], mostly aimed at the stabilization and solubilization of heat-sensitive proteins. Development of low-temperature expression system has been attempted in the psychrophilic *Arthrobacter* sp. isolated from a Greenland glacier [65]. An alternative strategy is to use the engineered Arctic Express Cells which co-express cold-adapted homologues of the *E. coli* GroELS chaperonines, Cpn60 and Cpn10, from *O. antarctica* [66]. This system allows protein processing at very low temperatures (4–12 °C), potentially increasing the yield of active, soluble recombinant protein.

3.2. Structural Features of Cold-Adapted Enzymes

A basic requirement of evolutionary cold adaptation is that the enzyme should afford metabolic rates comparable to those of mesophilic organisms at its working temperature. Indeed, the temperature-dependence of the catalyzed reaction constants is described by the Arrhenius Equation (1)

$$k = Ae^{-E/(RT)} \quad (1)$$

where k is the reaction rate, E is the activation energy of the reaction, R is the gas constant, T is temperature and A is a collision frequency factor. It shows that a decrease of 10 °C brings about a decrease of 2–3 fold in the reaction rate. Therefore, a mesophilic enzyme with an optimal working temperature around 37 °C is expected to work 16–80 times slower when brought to 0 °C [67]. Increases in enzyme expression in psychrophilic microorganisms to compensate for the slower reactivity have been observed only in a small number of cases. Rather, the most common evolutionary adaptation consists in the evolution of enzyme homologs endowed with increased catalytic efficiency, the k_{cat}/K_M ratio, either by an increase of k_{cat}—particularly for enzymes working at saturating concentrations of substrate—or by a decrease in K_M—particularly for enzymes working at sub-saturating concentrations of substrate. k_{cat} is almost invariably increased in cold-adapted enzymes, up to 10-fold. The trade-off between k_{cat} and K_M in cold-adapted enzymes has been comprehensively reviewed [68].

From a structural point of view, it is widely accepted that these kinetic parameters are associated with the increased flexibility of these enzymes [69,70]. This dynamics adaptation allows for the conformational rearrangements required for catalysis at low temperatures, making it possible for cold-adapted enzymes to maintain the same conformational space accessible to their mesophilic and thermophilic homologs at higher temperatures. Indeed, for the latter enzymes, the kinetic energy associated with low temperatures would be insufficient to overcome the kinetic barriers required for the conformational rearrangements associated with catalysis. The turnover number, or k_{cat}, is a good index of enzyme flexibility, as it reflects the rate of transition between all conformational states involved in the catalytic cycle.

Multiple structural features are usually associated with increased flexibility, involving both the active site and the peripheral regions. Commonly observed structural features are: Decreased core hydrophobicity, fewer prolyl residues in loops, increased hydrophobicity of the surface, more glycyl residues, lower arginyl/lysyl ratio, weaker interactions between subunits and domains, longer loops, decreased secondary-structure content, more prolyl residues in α-helices, fewer and weaker metal-binding sites, fewer disulfide bridges, fewer electrostatic interactions, reduced oligomerization and increase in conformational entropy of the unfolded state [71,72]. Many of these structural adaptations are summarized in Table 1. The crystal structures of 11 proteins isolated from the Antarctic marine oil-degrading bacterium *Oleispira antarctica* (α/β hydrolase, phosphodiesterase, transaldolase, isochorismatase, amidohydrolase, fumarylacetoacetate isomerase/hydrolase, 2-keto-3-deoxy-6-phosphogluconate aldolase, phoshonoacetaldehyde hydrolase, inorganic pyrophosphatase, and protein with unknown function) revealed that the most dominant structural feature is an increase in surface

hydrophobicity and negative charge, with higher Glu+Asp/Arg+Lys ratio compared to their mesophilic counterparts [73].

Table 1. Structural adaptations in cold-adapted enzymes and their effects on protein structure.

Molecular Adaptation	Effect	Reference
Decreased number of hydrogen bonds and salt bridges	Increased flexibility	[69,72]
Reduced proline and arginine content	Increased molecular entropy	[23,74]
Increased surface charged residues	Increased conformational flexibility	[23]
Reduced frequency of surface, inter-domain and inter-subunit ionic linkages and ion-network	Increased conformational flexibility and reduced enthalphic contribution to stability	[75]
Reduced core hydrophobicity/increased surface hydrophobicity	Reduced hydrophobic effect/ entropic destabilization	[70]
Increased accessibility of active site	Increased flexibility for substrate and cofactor binding	[76]
Loop extensions	Reduced stability	[77]

Adapted from [22].

In other instances, however, a small number of specific residues was recognized as solely responsible for the cold adaptation of enzymes. In the β-galactosidase from the polyextremophilic Antarctic archaeon *Halorubrum lacusprofundi*, the mutation of only six amino-acid residues in comparison to its mesophilic homologs resulted in altered temperature activity profiles, suggesting that a small number of mutations can indeed account for cold adaptation [71]. In other proteins, a limited number of regions were associated to cold adaptation: the characterization of chimeric derivatives of monomeric isocitrate dehydrogenases of *Colwellia maris* and *Pseudomonas psychrophila*—cold adapted and mesophilic, respectively—led to the identification of two regions responsible for their different thermal properties [78]. The relative contribution of local and global protein flexibility in determining cold adaptation was assessed by performing molecular dynamics simulations on cytosolic malate dehydrogenases orthologous from marine mollusks adapted to different temperatures [79]. A convergent evolution trend was observed, with a significant negative correlation between the adaptation temperature of the organism and overall protein flexibility, with regions involved in ligand binding and catalysis showing the largest fluctuation differences [79].

Generally, the higher flexibility at low temperatures brings about low stability at higher temperatures. However, thermolability, although common [70], is not universal in cold-adapted enzymes. Three superoxide dismutases from the Antarctic psychrophilic ciliate *Euplotes focardii* were recently showed to exhibit a melting temperature of around 50–70 °C, suggesting a combination of cold adaptation and relative tolerance to high temperatures [80]. GroEL and thioredoxin from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 were shown to be only marginally less stable than their *Escherichia coli* homologs [81].

It should also be highlighted that cold adaptation does not only consist in altered kinetic parameters of individual enzymes. For instance, sequencing and functional analysis of the genome the Antarctic marine oil-degrading bacterium *O. antarctica* revealed an array of alkane monooxygenases, osmoprotectants, siderophores and micronutrient-scavenging pathways [73] associated to cold adaptation.

3.3. Examples of Biotechnological Applications of Polar Enzymes

According to the type of catalyzed reaction, enzymes are classified into six main classes, i.e., oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases, as reported in Table 2. The proposed biotechnological applications of cold-adapted enzymes belonging to several of these classes have been extensively reviewed [23,25,82–85].

Table 2. Polar-active enzymes isolated from Antarctic and Arctic marine polar environments.

Marine Polar-Active Enzymes	Reaction	Organism Source	Origin of Sample	Applications/Potential Uses	References
HYDROLASES: EC 3 (Type of reaction: Hydrolytic cleavage AB + H_2O → AOH + BH)					
β-galactosidase	Hydrolysis of lactose into its constituent monosaccharides	*Pseudoalteromonas* sp. 22b	Alimentary tract of Antarctic krill *Thyssanoessa macrura*	Candidates for lactose removal from dairy products at low temperatures	[86,87]
β-galactosidase		*Pseudoalteromonas haloplanktis* TAE 79	Antarctic seawater		[88]
β-galactosidase		*Pseudoalteromonas haloplanktis* LMG P-19143	Antarctic seawater		[89]
β-galactosidase		*Guehomyces pullulans*	Antarctic sea sediment		[90]
β-galactosidase		*Enterobacter ludwigii*	Sediment samples of Kongsfgord, Arctic		[91]
β-galactosidase		*Alkalilactibacillus ikkense*	Ikka columns in South-West Greenland		[92]
α-Amylase	Cleavage of α-1,4-glycosidic linkages in starch molecules to generate smaller polymers of glucose units	*Pseudoalteromonas* sp. M175	Antarctic sea-ice	Detergent additive for its stain removal efficiency	[93]
α-Amylase§		*Glaciozyma antarctica* PI12	Antarctic sea-ice	Additives in processed food, in detergents for cold washing, in waste-water treatment, in bioremediation in cold climates and in molecular biology applications	[94]
α-Amylase		Bacterial strains	Sediment samples from Midtre Lovènbreen Arctic glacier		[95]
α-Amylase		*Alteromonas* sp. TAC 240B	Antarctic seawater		[96]
α-Amylase		*Pseudoalteromonas haloplanktis* *	Antarctic seawater		[97,98]
Xylanase	Hydrolysis of the main chain of xylan to oligosaccharides, which in turn are degraded to xylose	*Cladosporium* sp.	Antarctic marine sponges	Additives in textile and food industries, and bioremediation	[99]
Xylanase		*Flavobacterium frigidarium* sp.	Antarctic shallow-water marine sediment		[100]

Table 2. *Cont.*

Marine Polar-Active Enzymes	Reaction	Organism Source	Origin of Sample	Applications/Potential Uses	References
HYDROLASES: EC 3 (Type of reaction: Hydrolytic cleavage AB + H_2O → AOH + BH)					
Serine protease (Subtilisin)	Cleavage of peptide bonds	*Bacillus* TA39	Antarctic seawater	Additives in low-temperature food processing, food and textile industries, leather processing, detergent industry	[101,102]
Serine protease (Subtilisin)		*Bacillus* TA41	Antarctic seawater		[101,103]
Serine protease		*Colwellia* sp. NJ341	Antarctic sea-ice		[104]
Serine alkaline protease		*Shewanella* sp. Ac10u	Antarctic seawater		[105]
Acid protease		*Rhodotorula mucilaginosa* L7	Antarctic marine alga		[106]
Subtilisin-like serine protease		*Pseudoalteromonas* sp., *Marinobacter* sp., *Psychrobacter* sp., *Polaribacter* sp.	Antarctic seawater and thorax, abdomen and head of krill (*Euphausia superba* Dana)		[107]
Protease		*Pseudoalteromonas* sp. NJ276	Antarctic sea-ice		[108]
Subtilisin-like Serine proteinase		*Leucosporidium antarcticum* 171	Antarctic sub-glacial waters		[109]
Aminopeptidase		*Pseudoalteromonas haloplanktis* TAC125	Antarctic seawater		[110]
Aminopeptidase		*Colwellia psychrerythraea* 34H	Greenland continental shelf sediment samples		[111,112]
Serine peptidase		*Lysobacter* sp. A03	Penguin feathers in Antarctica		[113]
Serine peptidase		*Serratia* sp.	Coastal seawater in Northern Norway		[114]
Metalloprotease		*Pseudoalteromonas* sp. SM495	Arctic sea-ice (Canadian Basin)		[115]
Metalloprotease		*Sphingomonas paucimobilis*	Stomach of Antarctic krill, *Euphausia superba* Dana		[116]
Metalloprotease		*Psychrobacter proteolyticus* sp.	Stomach of Antarctic krill *Euphausia superba* Dana		[117]
Endopeptidase		Microbial source	Arctic marine microbial source	Candidate for molecular biology application: digestion of chromatin (ArcticZymes)	[118]

Table 2. *Cont.*

Marine Polar-Active Enzymes	Reaction	Organism Source	Origin of Sample	Applications/Potential Uses	References
HYDROLASES: EC 3 (Type of reaction: Hydrolytic cleavage AB + H_2O → AOH + BH)					
Lipase	Hydrolysis of long-chain triacylglycerol substances with the formation of an alcohol and a carboxylic acid	*Bacillus pumilus* ArcL5	Arctic seawater (Chukchi Sea)	Detergent additives used at low temperatures and biocatalysts for the biotransformation of heat-labile compounds	[119]
Lipase		*Pseudoalteromonas haloplanktis* TAC125	Antarctic seawater		[120]
Lipase		*Colwellia psychrerythraea* 34H	Arctic seawater		[121]
Lipase		*Polaromonas vacuolata*	Antarctic seawater		[122]
Lipase		*Psychrobacter* sp.	Antarctic seawater		[123,124]
Lipase		*Shewanella frigidimarina*	Antarctic seawater		[125]
Lipase		Bacterial strains	Arctic sediment samples from the snout of Midtre Lovènbreen glacier up to the convergence point with the sea		[95]
Lipase		*Psychrobacter* sp. TA144 **	Antarctic seawater		[126]
Lipase		*Psychrobacter* sp. 7195	Antarctic deep-sea sediment (Prydz Bay)		[127]
Lipase		*Moritella* sp. 2-5-10-1	Antarctic deep-sea water		[128]
Lipase		*Pseudoalteromonas* sp., *Psychrobacter* sp., *Vibrio* sp.	Antarctic seawater samples (Ross Sea)		[129]
Phytase	Hydrolysis of phytate to phosphorylated myo-inositol derivatives	*Rhodotorula mucilaginosa* JMUY14	Antarctic deep-sea sediment	Candidate for feed applications, especially in aquaculture	[130]
Esterase	Hydrolysis of simple esters, usually only triglycerides composed of fatty acids shorter than C 8	*Pseudoalteromonas arctica*	Arctic sea-ice from Spitzbergen, Norway	Additives in laundry detergents and biocatalysts for the biotransformation of labile compounds at low temperatures	[131]
Esterase		*Thalassospira* sp.	Arctic sea fan (*Paramuricea placomus*), Vestfjorden area (Northern Norway)		[132]
Esterase		*Oleispira antarctica*	Antarctic coastal waters		[73,133]
Esterase		*Pseudoalteromas haloplanktis* TAC125	Antarctic seawater		[134,135]
Esterase		*Pseudoalteromas* sp. 643A	Alimentary tract of Antarctic krill *Euphasia superba* Dana		[136]
Esterase		Marine Arctic metagenomics libraries	Arctic seawater and sediment from Barents Sea and Svalbard	Candidate for organic synthesis reactions and cheese ripening processes	[137,138]

Table 2. *Cont.*

Marine Polar-Active Enzymes	Reaction	Organism Source	Origin of Sample	Applications/Potential Uses	References
HYDROLASES: EC 3 (Type of reaction: Hydrolytic cleavage AB + H_2O → AOH + BH)					
Epoxide hydrolase	Hydrolysis of an epoxide to its corresponding vicinal diol with the addition of a water molecule to the oxirane ring	*Sphingophyxis alaskensis*	Arctic seawater	Candidate for the production of enantiopure epoxides in the pharmaceutical industry	[139]
S-formylglutathione hydrolase	Hydrolysis of S-formylglutathione to formic acid and glutathione	*Pseudoalteromonas haloplanktis* TAC125	Antarctic seawater	Candidates for chemical synthesis and industrial pharmaceutics	[140]
S-formylglutathione hydrolase		*Shewanella frigidimarina*	Antarctic marine environment		[141]
Polygalacturonase (pectin depolymerase)	Cleavage of glycosidic bonds between galacturonic acid residues	*Pseudoalteromonas haloplanktis*	Antarctic seawater	Additive in food industries, such as clarification of juice, in the process of vinification, yield and color enhancement and in the mashing of fruits	[142]
Pullulanase	Hydrolysis of α-1,6-glycosidic bonds in pullulan to produce maltotriose	*Shewanella arctica*	Seawater samples in Spitsbergen, Norway	Additive in food and biofuel industries	[143]
Invertase	Hydrolysis of the terminal non-reducing β-fructofuranoside residue in sucrose, raffinose and related β-D-fructofuranosides	*Leucosporidium antarcticum*	Antarctic seawater	Not defined (ND)	[144]
α-glucosidase	Hydrolysis of the non-reducing terminal α-glucopyranoside residues from various α-glucosides and related compounds	*Leucosporidium antarcticum*	Antarctic seawater	Additive in detergent and food industries	[144]
Cellulase	Hydrolysis of the β-1,4-D-glycosidic linkages in cellulose	*Pseudoalteromonas haloplanktis*	Antarctic seawater	Additive in detergent industry	[145]
Chitobiase	Hydrolysis of chitobiose to N-acetylglucosamine	*Arthrobacter* sp. TAD20	Antarctic sea sediments	ND	[146]

Table 2. *Cont.*

Marine Polar-Active Enzymes	Reaction	Organism Source	Origin of Sample	Applications/Potential Uses	References
HYDROLASES: EC 3 (Type of reaction: Hydrolytic cleavage AB + H_2O → AOH + BH)					
Alkaline phosphatase	Hydrolysis and transphosphorylation of a wide variety of phosphate monoesters	TAB5 strain	Antarctica^	Candidate for molecular biology application: dephosphorylation of DNA (New England Biolabs)	[147–149]
Alkaline phosphatase		*Shewanella* sp.	Intestine of Antarctic shellfish	Candidate for molecular biology application	[150]
Pyrophosphatase	Catalysis of the conversion of one ion of pyrophosphate to two phosphate ions	*Oleispira antarctica*	Antarctic deep sea	ND	[73]
Glycerophosphodiesterase	Catalysis of the hydrolysis of a glycerophosphodiester	*Oleispira antarctica*	Antarctic deep sea	ND	[73]
Endonuclease (Cryonase)	Cleavage of the phosphodiester bond the middle of a polynucleotide chain	*Shewanella* sp. Ac10	Antarctic seawater	Candidate for molecular biology application: digestion of all types of DNA and RNA at cold temperatures (Takara-Clontech)	[151]
Exonuclease	Cleavage of the phosphodiester bond at either the 3′ or the 5′ end	Arctic marine bacterium	Arctic marine microbial source	Candidate for molecular biology application: 3′-5′ exonuclease specific for single stranded DNA (ArcticZymes)	[152]
Ribonuclease	Hydrolysis of the phosphodiester bonds among the nucleic acid residues of RNA	*Psychrobacter* sp. ANT206	Antarctic sea-ice	Candidate for molecular biology applications	[153]
Uracil-DNA glycosylase	Hydrolysis of the N-glycosidic bond from deoxyuridine to release uracil	Antarctic marine bacterium	Antarctic marine microbial source	Candidate for molecular biology application: release of free uracil from uracil-containing single-stranded or double-stranded DNA (New England Biolabs)	[154]

Table 2. *Cont.*

Marine Polar-Active Enzymes	Reaction	Organism Source	Origin of Sample	Applications/Potential Uses	References
OXIDOREDUCTASES: EC 1 (Type of reaction: Transfer of hydrogen or oxygen or electrons between molecules AH + B → A + BH; A + O → AO; A^-+B→A+B^-)					
Phenylalanine hydroxylase	Catalysis of the hydroxylation of L-Phe to form tyrosine	*Colwellia psychrerythraea* 34H	Arctic marine sediments	ND	[76]
Alcohol dehydrogenase	Catalysis of the interconversion of alcohols to their corresponding carbonyl compounds	*Moraxella* sp. TAE123	Antarctic seawater	Candidate for asymmetric synthesis	[155]
Alanine dehydrogenase	Catalysis of reversible deamination of L-alanine to pyruvate	*Shewanella* sp. Ac10u, *Carnobacterium* sp. St2	Antarctic seawater	Candidate for enantioselective production of optically active amino acids	[156]
Leucine dehydrogenase	Catalysis of reversible L-leucine and other branched chain L-amino acids deamination reaction to the corresponding α-keto acid	*Pseudoalteromonas* sp. ANT178	Antarctic sea-ice	Candidate for medical and pharmaceutical industry applications	[157]
Malate dehydrogenase	Catalysis of reversible oxidation of malate to oxalacetate	*Flavobacterium frigidimaris* KUC-1	Antarctic seawater	Candidate for detection and production of malate under cold conditions	[158]
Isocitrate dehydrogenase	Catalysis of decarboxylation of isocitrate to α-ketoglutarate and CO_2	*Desulfotalea psychrophila*	Arctic marine sediments	ND	[159]
L-threonine dehydrogenase	Catalysis of dehydrogenation at the β-carbon (C3) position of L-threonine	*Flavobacterium frigidimaris* KUC-1 ***	Antarctic seawater	ND	[160]
Superoxide dismutase	Catalysis of the dismutation of superoxide anion radicals into molecular oxygen and hydrogen peroxide	*Pseudoalteromonas haloplanktis*	Antarctic seawater	Candidates for applications in agriculture, cosmetics, food, healthcare products and medicines	[161]
Superoxide dismutase		*Marinomonas* sp. NJ522	Antarctic sea-ice		[162]
Superoxide dismutase		*Pseudoalteromonas* sp. ANT506	Antarctic sea-ice		[163]
Superoxide dismutase		*Psychromonas arctica*	Arctic sea-ice and sea-water samples		[164]
Superoxide dismutase		*Rhodotorula mucilaginosa* AN5	Antarctic sea-ice		[165]

Table 2. *Cont.*

Marine Polar-Active Enzymes	Reaction	Organism Source	Origin of Sample	Applications/Potential Uses	References
OXIDOREDUCTASES: EC 1 (Type of reaction: Transfer of hydrogen or oxygen or electrons between molecules AH + B → A + BH; A + O → AO; A^-+B→A+B^-)					
Catalase	Catalysis of degradation of hydrogen peroxide into water and molecular oxygen	*Bacillus* sp. N2a	Antarctic seawater	Candidate for textile and cosmetic industries	[166,167]
Glutathione reductase	Catalysis of the reduction of oxidized glutathione to produce reduced glutathione	*Colwellia psychrerythraea*	Antarctic seawater	Candidate as an antioxidant enzyme in heterologous systems	[168]
Glutathione peroxidase	Catalysis of the reduction of hydrogen peroxide and other organic peroxides	*Pseudoalteromonas* sp. ANT506	Antarctic sea-ice	ND	[169]
Thioredoxin reductase	Catalysis of the reduction of thioredoxin	*Pseudoalteromonas haloplanktis* TAC125	Antarctic seawater	ND	[170]
Glutaredoxin	Catalysis of the reduction of protein disulfides in glutathione-dependent reactions	*Pseudoalteromonas* sp. AN178	Antarctic sea-ice	ND	[171]
Peroxiredoxin	Catalysis of the reduction of hydrogen peroxide, peroxynitrite and a wide range of organic hydroperoxides	*Psychrobacter* sp. ANT206	Antarctic sea-ice	Candidate for food and pharmaceutical industries	[172]
Dihydroorotate oxidase	Catalysis of the stereospecific oxidation of (S)-dihydroorotate to orotate	*Oleispira antarctica*	Antarctic deep sea	ND	[73]

Table 2. *Cont.*

Marine Polar-Active Enzymes	Reaction	Organism Source	Origin of Sample	Applications/Potential Uses	References
TRANSFERASES: EC 2 (Type of reaction: Transfer of groups of atoms AB + C → A + BC)					
Aspartate aminotransferase	Catalysis of transamination reaction of L-aspartate and α-ketoglutarate into the corresponding oxaloacetate and L-glutamate	*Pseudoalteromonas haloplanktis* TAC125 ****	Antarctic seawater	ND	[173]
Glutathione S-transferase	Catalysis of conjugation of reduced glutathione with various electrophilic compounds and ROS	*Pseudoalteromonas* sp. ANT506	Antarctic sea-ice	ND	[174]
Hydroxymethyl-transferase	Catalysis of reversible conversion of L-serine and tetrahydropteroylglutamate to glycine and 5,10-methylenetetrahydropteroylglutamate. Cleavage of many 3-hydroxyamino acids and decarboxylation of aminomalonate	*Psychromonas ingrahamii*	Arctic polar sea-ice	Candidate as a pharmaceutical, agrochemicals and food additive	[175]
LIGASES: EC 6 (Type of reaction: Covalent joining of two molecules coupled with the hydrolysis of an energy rich bond in ATP or similar triphosphates A + B+ ATP → AB + ADP + Pi)					
Glutathione synthetase	Catalysis of formation of glutathione from L-γ-glutamylcysteine and glycine	*Pseudoalteromonas haloplanktis*	Antarctic seawater	ND	[176]
DNA ligase	Catalysis of the formation of a phosphodiester bond between adjacent 5′-phosphoryl and 3′-hydroxyl groups in double stranded DNA	*P. haloplanktis* TAE 72	Antarctic seawater	Candidate for applications in molecular biology	[177]

Table 2. *Cont.*

Marine Polar-Active Enzymes	Reaction	Organism Source	Origin of Sample	Applications/Potential Uses	References
LYASES: EC 4 (Type of reaction: Cleavage of C-C, C-O, C-S, C-N or other bonds by other means than by hydrolysis or oxidation RCOCOOH → RCOH + CO_2)					
γ-carbonic anhydrase	Catalysis of CO_2 hydration to bicarbonate and protons	*Colwellia psychrerythraea*	Antarctic cold ice sediments	Candidates for biomedical applications	[178]
γ-carbonic anhydrase		*Pseudoalteromonas haloplanktis*	Antarctic seawater		[179,180]
Pectate lyase	Cleavage of the α-1,4 glycosidic bonds of polygalacturonic acid into simple sugars	*Pseudoalteromonas haloplanktis* ANT/505	Antarctic sea-ice	Candidate for detergent industry	[167,181]
Acid decarboxylase	Catalysis of decarboxylation of 3-octaprenyl-4-hydroxybenzoate to produce 2-polyprenylphenol	*Colwellia psychrerythraea* 34H	Arctic marine sediments	ND	[182,183]
ISOMERASES: EC 5 (Type of reaction: Transfer of group from one position to another within one molecule AB → BA)					
Sedoheptulose 7-phosphate isomerase	Catalysis of the conversion of sedoheptulose 7-phosphate to D-glycero-D-mannoheptose 7-phosphate	*Colwellia psychrerythraea 34H*	Arctic marine sediments	Candidate for biocatalysis under low water conditions	[184]
Triose phosphate isomerase§	Catalysis of the isomerization of dihydroxyacetone phosphate to D-glyceraldehyde 3-phosphate	*Pseudomonas* sp. *π*9	Antarctic sea-ice		[185]
Triose phosphate isomerase		*Moraxella* sp. TA137	Intestine of Antarctic fish		[186]

* previously known as *Alteromonas haloplanktis* [187], ** previously known as *Moraxella* TA144 [188], *** formerly known as *Cytophaga sp.* KUC-1 [189], **** formerly known as *Moraxella* TAC 125 [190]. ^it was isolated from Dumont d'Urville Antarctic Station but it was not possible to ascertain the marine origin. § only characterization of genes.

Currently, approximately 65% of more than 3000 enzymes with an industrial application are hydrolases, used in the detergent, textile, paper, and food industries [167,191]. Hydrolases, classified as Class 3 (EC 3) by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), catalyze the hydrolysis of chemical bonds. Herein, we focus on some recent examples of cold-adapted enzymes, particularly hydrolases, isolated from marine polar microorganisms. More examples of microbial enzymes isolated in Arctic or Antarctic marine polar regions are reported in Table 2. To our knowledge, this is the first table including only cold-active enzymes isolated from marine polar microorganisms. Indeed, several recent reviews do not distinguish between polar and non-polar enzymes or between marine and non-marine origin. The latter distinction is crucial to guide bioprospecting in search for novel activities.

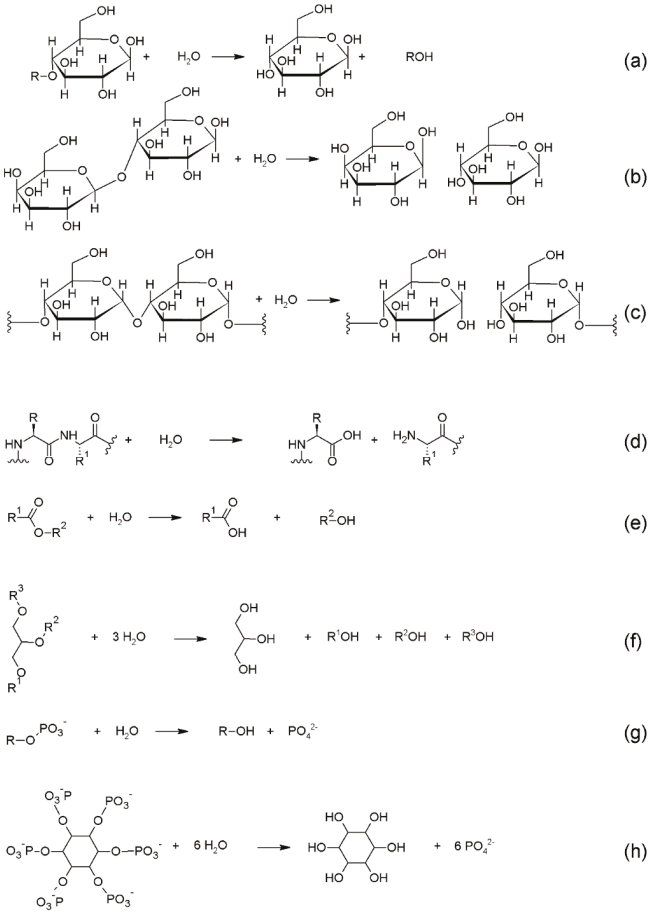
3.3.1. Glycoside Hydrolases

Glycoside hydrolases (GH; EC 3.2.1.), or glycosidases, hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety (Scheme 1a), overall processing a wide variety of complex substrates [192]. They are extremely common in nature, with more than 100 GH families currently recognized and grouped in the Carbohydrate-Active Enzymes database (CAZy), which provides a continuously updated list of families [193]. Their function ranges from the degradation of complex carbohydrates for their metabolic use (cellulases, amylases), to the defense against pathogens (lysozyme, chitosanase), to the synthesis of the oligosaccharide groups of glycoproteins. Their biotechnological applications are manifold, especially in the food processing industry, where enzyme activity at low temperatures is desirable to slow down spoilage and loss of nutrients. For this reason, cold-adapted hydrolases are usually in need [194].

Lactases or β-galactosidases (EC 3.2.1.23) (Scheme 1b) offer a clear example in this respect: with more than 70% of the world population suffering from lactose intolerance, the enzymatic removal of lactose from milk and dairy products using β-galactosidases is now common. The enzyme catalysing the hydrolysis of lactose into its constituent monosaccharides, glucose, and galactose, has drawn the interest of many research groups and enterprizes due to nutritional (lactose intolerance) and technological (crystallization), challenges associated with lactose [195]. The isolation of several cold-adapted β-galactosidases has been reported. Among them, some are from Antarctic organisms, e.g., *H. lacusprofundi*, isolated from the hypersaline Deep Lake [71,196], *Arthrobacter* species from soil samples [197,198], and marine *Pseudoalteromonas* species [86–88,199]. Other enzymes were isolated from marine organisms from the Arctic region, including those from *Enterobacter ludwigii* [91] and *Alkalilactibacillus ikkense* [92]. A cold-active β-galactosidase from the Antarctic marine bacterium *P. haloplanktis* TAE 79 was applied to lactose hydrolysis at low temperatures during milk storage [88] and was proposed for the production of tagatose, a natural monosaccharide with low caloric value and glycemic index [200]. A cold-active lactase isolated from the Antarctic marine *P. haloplanktis* LMG P-19143 was patented [89] and is also produced in large quantities by Nutrilab NV (Bekkevoort, Belgium). Yeasts are important sources for β-galactosidase production. *Guehomyces pullulans* 17-1, isolated from the Antarctic sea sediment, synthesize both extracellular and cell-bound β-galactosidases [90]. A 1,3-α-3,6-anhydro-L-galactosidase—an enzyme involved in the final steps of the agarolytic pathway and used in the industrial processing of agar—was identified in the agarolytic marine bacterium *Gayadomonas joobiniege* G7 and was shown to be active between 7.0 and 15 C [201].

Amylases (Scheme 1c) are also interesting enzymes for industrial processes, in detergent formulations and in food industry for beer and wine fermentation and for the preparation of bread and fruit juice. They hydrolyse starch to maltose, maltotriose, glucose monomers and limit dextrins. They can be classified in exoamylases (β-amylase EC 3.2.1.2; glucoamylase, EC 3.2.1.3; α-glucosidase, EC 3.2.1.20) endoamylases (α-amylase, EC 3.2.1.1) and debranching (pullulanases, EC 3.2.1.41; isoamylases, EC 3.2.1.68; dextrinases, EC 3.2.1.142) according to the specificity of the hydrolysis reaction they catalyze [194]. In the detergent industry, cold-active amylases not isolated from polar regions are already available on the market [167], i.e., Stainzyme® (active at temperatures between 30

and 70 °C) and Stainzyme® Plus (active below 20 °C) released by Novozymes and Preferenz™ S100 (active at 16 °C) released by DuPont Industrial Biosciences. In the textile industry, Genencor-DuPont developed Optisize® COOL, using a cold-active amylase for the desizing of woven fabrics at low temperatures. Other cold-active, detergent-stable α-amylases, not isolated from polar regions, were identified from *Bacillus cereus* GA6 [202] and from the marine bacterium *Zunongwangia profunda* [203]. The latter was isolated from surface seawater in the costal area of Fujian, China, but shows a cold-adapted and salt-tolerant α-amylase activity. An α-amylase produced by the psychrophilic yeast *Glaciozyma antarctica* PI12 was recently characterized [94].



Scheme 1. Principal enzymatic activities of cold-adapted enzymes investigated for biotechnological applications. (**a**) Generic reaction catalyzed by glycoside hydrolases, with a glucose derivative as substrate. (**b**) Reaction catalyzed by lactases, with conversion of lactose to galactose and glucose. (**c**) Reaction catalyzed by amylases on glucose polymers, with glucose or maltooligosaccharides as final products. (**d**) Generic reaction catalyzed by proteases, which catalyze the hydrolysis of peptide bonds of proteins or peptides. (**e**) Generic reaction catalyzed by esterases, which hydrolyse ester bonds. (**f**) Reaction catalyzed by lipases, with the hydrolysis of fatty acids from acyl glycerol. In this case, the full hydrolysis of a triglyceride to glycerol is represented. (**g**) Generic reaction catalyzed by phosphatases with the hydrolysis of phosphate monoesters. (**h**) Reaction catalyzed by phytases. In this case, the full hydrolysis to phosphate ions and inositol is represented.

Cold-adapted enzymes from marine polar regions could successfully be employed in these applications, and some of them are already under investigation or in use. A cold-adapted α-amylases from the Antarctic sea-ice bacterium *Pseudoalteromonas* sp. M175 exhibited resistance towards all the tested commercial detergents and was shown to improve their stain removal efficiency [93]. Heat-labile α-amylases displaying high activity at low temperature isolated from bacteria of Antarctic seawater were structurally studied [96,97].

Pullulanases (EC 3.2.1.41), debranching enzymes that hydrolyse α-1,6-and α-1,4-linkages in pullulan, starch, amylopectin and various related oligosaccharides are interesting enzymes in starch processing. There are different pullulanase groups according to their substrate specificities and reaction products. Pul13A is a type-I pullulanase isolated from Arctic seawater strain *Shewanella arctica* in Spitsbergen, Norway. It is able to hydrolase α-1,6-glycosidic bonds in pullulan to produce maltotriose at low temperature, displaying low thermostability at elevated temperatures. It represents a potential candidate for industrial applications such as starch degradation for ethanol-based biofuel production [143].

Although poorly studied, cold-active xylanases (EC 3.2.1.8) are also interesting in the food industry for bread making, as they convert the insoluble hemicellulose of dough into soluble sugars, thus yielding soft and elastic bread. Three cold-adapted xylanases from psychrophilic bacteria were shown to improve dough properties and bread volume with respect to mesophilic orthologues [204]. The xylanase pXyl from *P. haloplanktis*, isolated from soil samples [205], was very efficient in improving the dough properties and bread volume due to its high activity at low temperature [205]. The psychrophilic enzyme is now sold by Puratos (Grand-Bigard, Belgium). Antarctic marine fungi from marine sponges, which are the dominant macroinvertebrates in many benthic communities, are considered new and promising sources of cold-active xylanases. *Cladosporium* sp. isolated from marine sponges collected in King George Island, Antarctica, showed high xylanase activity at low temperature and very low thermal stability [99]. Also *Flavobacterium frigidarium* sp. nov., an aerobic psychrophilic and halotolerant bacterium isolated from marine sediment of shallow waters surrounding Adelaide Island in Antarctica, exhibited xylanolytic activity [100].

Cellulases, used in detergents for color and brightness care, have been classified according to their activity into endo- (EC 3.2.1.4) and exo-cellulases with exo-β-1,4-glucan cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). *P. haloplanktis* is able to convert the cellulose into an immediate nutritive compound for plants by hydrolysis. *P. haloplanktis* secretes a multi-modular endocellulase composed N-terminal catalytic module, a linker region and the cellulose-binding module. Structural adaptations to cellulose hydrolysis at low temperatures have been identified in the catalytic module and unusually long linker region by using both X-ray diffraction and small angle X-ray scattering methods [145].

Leucosporidium antarcticum strain 171, widespread in the cold marine waters below the Antarctic ice, was isolated from a seawater sample collected at a depth of 100 m in Admiralty Bay, King George Island. The yeast was found to produce cold-adapted invertases (β-D-fructofuranoside fructohydrolases, EC 3.2.1.26) and glucosidases (α-D-glucoside glucohydrolases, EC 3.2.1.20). Their synthesis may facilitate assimilation of β-fructofuranosides and α-glucopyranosides in cold environments where nutrient availability is fluctuating [144].

Chitin, one of the most abundant organic compounds in nature, is a structural polysaccharide composed of N-acetylglucosamine (GlcNAc) residues. Chitobiases (EC 3.2.1.29) found in bacteria, fungi, and eukaryotes, hydrolyse chitobiose to GlcNAc, which is further converted to glucosamine by a deacetylase. A cell-bound chitobiase was isolated from the marine psychrophile *Arthrobacter* sp. TAD20 collected along the Antarctic ice. The cold-adapted chitobiase overexpressed in *E. coli* displayed four functionally independent domains: (i) The catalytic domain, (ii) the galactose-binding domain, and (iii) the immunoglobulin-like domain followed by (iv) the cell-wall anchorage signal. The enzyme exhibited features that are typical of cold-adapted enzymes, with unusually low-K_m and high-k_{cat} values, with improved flexibility around the active site for efficient activity at low temperatures [146].

3.3.2. Proteases

Proteases (EC 3.4) catalyze the hydrolysis of peptide bonds (Scheme 1d). This catalytic activity has evolved multiple times, yielding enzymes with different catalytic mechanisms [206]. These enzymes are classified as exo-peptidases (EC 3.4.11-19), when they cleave the terminal amino acid, and as endo-peptidases (EC 3.4.21-99), when the peptide bonds they hydrolysed is internal. Proteases have several industrial applications, particularly in the food industry and as components of laundry detergents [207,208]. Both thermophilic [209] and cold-adapted homologs [210] are of biotechnological interest, depending on the intended working temperature of the process. To obtain cold-active proteases, two approaches have been pursed: mutagenesis of mesophilic enzymes or identification and expression of naturally occurring psychrophilic proteases. The former approach was applied to subtilisins from *Bacillus* species, mutated to increase their activity at low temperature. Particularly, the subtilisin savinase from *Bacillus* was mutated to produce a thermostable variant with increased activity at low temperature [211]. The latter approach has also been investigated.

Commercially exploited cold-adapted proteases include a protease from *Bacillus amyloliquefaciens*—a non-polar soil bacterium—commercialized as Purafect Prime L™, which is active at 20 °C, and the protease Properase™ from *B. alcalophilus*, which combines activity at high pH and low temperature and Excellase™. They are commercialized by Genencor (Genencor International Inc., Palo Alto, CA, USA). A cold-adapted extracellular aspartic protease was isolated from the yeast *Sporobolomyces roseus* isolated from an underground water sample drawn from the disused silver and lead mine "Luiza" (Zabrze, Poland) and was proposed as biocatalyst in the food industry, particularly in cheese and soy-sauce production, meat tenderization, and as bread additive [212]. It was also proposed in the production of antioxidant peptides from dairy and animal proteins, as hydrolysis of beef casein by this enzyme yielded peptides endowed with antioxidant activity [212].

Bioprospecting of cold-adapted bacteria from the polar regions could further increase the variety of cold-active proteases for industrial use. Several recent examples of such enzymes have been reported. Particularly, Antarctic fungi capable of producing proteases have been recently reviewed [85]. Two cold-adapted subtilisins from Antarctic marine *Bacillus* TA39 [102] and TA41 [103] showed high activity at low temperatures but also limited stability. A mutant with unaltered activity at low temperatures but higher stability was obtained through directed evolution [101]. A subtilisin-like cold-adapted serine peptidase was isolated from the Antarctic bacterium *Lysobacter* sp. A03, and is both active at low temperatures and resistant to higher temperatures [113].

A protease from the Antarctic bacterium *Janthinobacterium lividum* obtained from the Polar BioCenter (Korea Polar Research Institute) [213], recombinantly expressed and purified, was reported. Among the fungal proteases identified from polar marine samples, the extracellular subtilase lap2, from the Antarctic yeast *Leucosporidium antarcticum* 171, exhibited a low optimal temperature (25 °C) poor thermal stability, and high catalytic efficiency in the temperature range 0–25 °C [109].

The extracellular protease released by *Rhodotorula mucilaginosa* L7 strain was isolated from an Antarctic marine alga and was shown to be stable in the presence of high concentrations of NaCl [106]. A new cold-adapted protease with a potential biotechnological application, largely represented amongst Antarctic bacterial genus (*Pseudoalteromonas* sp., *Marinobacter* sp., *Psychrobacter* sp., *Polaribacter* sp.), exhibits a higher catalytic efficiency at lower temperatures compared to its mesophilic counterpart [107]. The extracellular cold-active protease from the marine psychrophilic bacterium *Pseudoalteromonas* sp. NJ276, for its catalytic activity and broad substrate specificities, has a potential application in low-temperature food processing, in particular in the preservation of milk at low temperature [108]. A secreted a cold-active serine protease identified in *Colwellia* sp. NJ341, isolated from Antarctic sea-ice, showing a 30% of activity at 0 °C and a better thermostability than other cold-active proteases, represents a good candidate for industrial applications, particularly in processes where there may be a risk of microbial contamination or a temperature instability of reactants or products [104].

An aminopeptidase produced by the marine psychrophile *Colwellia psychrerythraea* strain 34H was structurally investigated, displaying structural features (fewer proline residues, fewer ion pairs,

and lower hydrophobic residue content) related to the cold adaptation and able to increase the flexibility for activity in the cold [111].

Besides their industrial applications, proteases have also long been used for therapeutic applications, particularly in the treatment of cardiovascular diseases, sepsis, digestive disorders, inflammation, cystic fibrosis, retinal disorders, and psoriasis [214]. The proteases so far approved by the US FDA derive from mesophilic organisms. However, the cold-adapted ones are endowed with a higher catalytic efficiency [215] and might, therefore, be of great interest. Indeed, they evolved to be efficient at low temperatures and, if thermostable enough, they would further increase their reaction rates at 37 °C, the intended temperatures for therapeutic applications. To overcome their thermal instability, mutational studies have been conducted [83].

As for general applications in molecular biology, proteinase, an endopeptidase from an Arctic marine microbial source, was developed by ArcticZymes [118] for the digestion of chromatin, thus releasing naked DNA. As it is thermolabile, it can be inactivated at temperatures compatible with RNA integrity and DNA as double strands.

3.3.3. Lipases and Esterases

Esterases (EC 3.1.1) hydrolyse ester bonds (Scheme 1e). Lipases in particular (EC 3.1.1.3) are glycerol-ester hydrolases that catalyze the hydrolysis of triglycerides to fatty acids and glycerol (Scheme 1f). They are widely used in organic synthesis and as laundry detergents [216]. Although thermophilic homologs might appear more promising for industrial applications for their higher stability under harsh conditions, cold-adapted homologs are also actively investigated for their high activity at low temperatures [217]. Moreover, lipases are promising tools for the preparation of chiral molecules in the pharmaceutical industry, where low working temperatures are desirable to reduce the rate of non-catalytic side reactions. Particularly, the predictable enantiopreference of lipases allows the determination of the absolute configuration of secondary alcohols using the lipase-catalyzed kinetic resolution and the use of cold-active lipases in organic solvents is excellent for the preparation of single-isomer chiral drugs, and the enantioselective or regioselective preparation of alcohol and amine intermediates in the synthesis of pharmaceuticals [218].

There are already examples of lipases identified in organisms isolated from the polar regions which are currently on the market. Lipozyme® CALB is a non-specific lipase from *Candida antarctica* which was successfully used in the resolution of racemic alcohols, amines, and acids, and in the preparation of optically active compounds from meso substrates, as well as for the regio-selective catalysis in the selective acylation of different carbohydrates [219]. Lipases from *C. antarctica* catalyze hydrolysis on several substrates and are thermostable, a surprising property considering their origin. CALB is currently commercialized by Novozyme (Bagsværd, Denmark). The lipase from *C. antarctica* is also available in an immobilized form and commercialized as Novozym 435 (Novozyme, Bagsværd, Denmark).

More cold-adapted lipases were recently discovered in Arctic and Antarctic microorganisms. A lipase from the psychrotolerant yeast *Rhodotorula* sp. Y-23 isolated in the Nella Lake, East Antarctica, exhibited the highest V_{max} at 15 °C and high compatibility with commercial detergents, which brought about an increase in activity, making it a potential candidate in detergent formulation active at low temperatures [220]. The Gram-positive psychrotrophic Arctic bacterium *Arthrobacter gangotriensis*, obtained from an soil sample, was recently shown to exhibit lipolytic activity associated with a lipase that can work in a wide pH range and that exhibits good stability [221]. The bacterium *Bacillus pumilus* ArcL5 with lipolytic activity was isolated from the Chukchi Sea within the Arctic Ocean; the lipase BpL5, was recombinantly expressed in *E. coli* and was shown to retain 85% of its activity at 5 °C. Two mutants active against tricaprylin were also characterized [119].

Other lipases were identified in Antarctic soils [222]. The lipase LipG7 from the Antarctic filamentous fungus *Geomyces* sp. P7 was unusually thermostable and was proposed as an enantioselective biocatalyst [223].

Other cold-active lipases were identified in the Antarctic marine *P. haloplanktis* TAC125 [120], in the Arctic marine bacterium *C. psychrerythraea* 34H [121], *Psychrobacter* sp. of Antarctic marine origin [123,124,126] and Antarctic deep-sea water strain *Moritella* sp.2-5-10-1 [127,128].

Esterases (EC 3.1.1.1), hydrolases that catalyze the cleavage of simple esters such as triglycerides with short chains of fatty acids (less than eight carbon atoms) (Scheme 1e) [194], are interesting enzymes in organic synthesis and cheese ripening processes [137]. Cold-adapted esterases were identified in Arctic marine bacteria *Pseudoalteromonas arctica*, [131] and *Thalassospira* sp. [132] and the Antarctic marine bacteria *O. antarctica* [133] and *P. haloplanktis* TAC125 [134,135]. Moreover, a cold-adapted esterase was identified from *Pseudoalteromonas* sp. strain 643A, isolated from the alimentary tract of Antarctic krill *Euphasia superba* Dana. It displayed 20–50% of maximum activity at 0–20 °C, and the optimal temperature was close to 35 °C. The optimal pH for enzyme activity was around 8.0; however, it was stable between pH 9 and 11.5 [136].

3.3.4. Phosphatases

Alkaline phosphatases (EC 3.1.3.1), which catalyze the hydrolysis of phosphate monoesters (Scheme 1g), have an important application in molecular biology for the dephosphorylation of DNA linearized at the 5′ end to avoid its re-circularization during cloning processes. A recombinant alkaline phosphatase isolated from the Antarctic bacterial strain TAB5 [147,148] was developed by New England Biolabs [149]. The crystal structure of a cold-active alkaline phosphatase from a psychrophile, *Shewanella* sp. isolated from the intestine of an Antarctic shellfish, revealed local flexibility, responsible for the high catalytic efficiency at low temperatures [150].

Phytic acid is the phosphate ester of inositol. The phosphate groups in this form are not available for absorption by animals, with the partial exception of ruminants, where the hydrolysis is carried out by the rumen microbiota. Phytases (EC 3.1.3.) have been used for decades to enrich animal food of absorbable phosphate groups, both for non-ruminant livestock and fish [224] (Scheme 1h). Thermophilic phytases have been suggested for industrial use [225], but cold-adapted homologs were also proposed, especially in aquaculture, where low temperatures might be limiting for the activity of the mesophilic orthologues. In this view, the purification and characterization of novel cold-adapted phytases from the Antarctic marine *R. mucilaginosa* strain JMUY14 [130] and *Pseudomonas sp.* strain JPK1 isolated from soil were reported [226].

3.3.5. Other Hydrolases

A cold-adapted uracil-DNA glycosylase (EC 3.2.2.27 and EC 3.2.2.28) of Antarctic marine source was released by New England Biolabs as thermolabile uracil-DNA glycosylase [154]. This hydrolase, isolated from a psychrophilic marine bacterium, catalyzes the release of free uracil from uracil-containing single-stranded or double-stranded DNA; it is sensitive to heat and can be inactivated at temperatures above 50 °C.

Cryonase, a cold-active nuclease, isolated from an Antarctic marine psychrophile, *Shewanella* sp. strain Ac10 and patented by Awazu and colleagues [227] was developed by Takara-Clontech [151]. It is a recombinant endonuclease that can digest all types of DNA and RNA (single-stranded, double-stranded, linear or circularized) at low temperatures, frequently used during DNA digestion of samples in the presence of heat-labile proteins. A 3′–5′ exonuclease specific for single-stranded DNA and derived from an Arctic marine bacterium was released by ArcticZymes [152].

Antimicrobial enzymes endowed with bactericidal or bacteriostatic properties are an emerging strategy to combat pathogens, particularly by degrading their DNA, polysaccharides, and proteins, or by interfering with biofilm formation, or by catalysing reactions which result in the production of antimicrobial compounds [228]. For example, N-acyl homoserine lactones are signaling molecules involved in bacterial quorum sensing and their hydrolysis can reduce growth and biofilm formation of bacterial pathogens. A cold-adapted N-acylhomoserine lactonase (EC 3.1.1.81) was recently identified in Antarctic *Planococcus* sp. isolated from a soil sample collected on Lagoon Island (Antarctica) and

was shown to attenuate the pathogenicity of *Pectobacterium carotovorum*, a plant pathogen that causes soft-rot disease [229]. As the enzyme was shown to be thermolabile at the human body temperature, it was suggested as a safe antimicrobial agent in the treatment of crops, as it would be inactivated upon ingestion.

3.3.6. Other Enzymes

Besides hydrolases, other enzymes have been isolated in marine microorganisms from the polar regions and, for some of them, a biotechnological application has been proposed.

Recently, few enzymes showing signatures of cold adaptation in their activity and structure have been isolated and fully characterized from the Arctic marine bacterium *C. psychrerythraea* strain 34H and also included a phenylalanine hydroxylase (PAH), an oxidoreductase, with high-catalytic efficiency at 10 °C, high thermostability and low affinity for substrate, probably due to enhanced flexibility of the active site [76]. PAH (EC 1.14.16.1) catalyzes the conversion of L-Phe to L-Tyr by para-hydroxylation of the aromatic side-chain and recently there has been an increased number of studies on the structure and function of PAH from bacteria and lower eukaryote organisms. Almost all characterized bacterial PAHs are monomeric and display a fold similar to the catalytic domain of mammalian PAHs.

Many oxidoreductases that catalyze redox reactions of industrial interest have been described in cold-adapted bacteria. The crystal structure of a Fe-superoxide dismutase, Fe-SOD (EC 1.15.1.1) isolated from the marine *P. haloplanktis*, revelead that cold-adapted enzyme displays high catalysis at low temperature increasing the flexibility of its active site without modifying the overall structure [161]. SODs have been used in the pharmaceutical and cosmetic industries, food, agricultural, and chemical industries [169]. Antioxidant defense is an important component of evolutionary adaptations in the cold to face increased levels of reactive oxygen species (ROS). The cold waters of the polar regions promote the formation of ROS and would be expected to lead to enhanced ROS damage of DNA and membrane lipid peroxidation in polar species [72]. *P. haloplanktis* TAC125 copes with increased O_2 solubility by deleting entire metabolic pathways that generate ROS as side products [230]. In contrast, the Arctic bacterium *C. psychrerythraea* has developed an enhanced antioxidant capacity, owing to the presence of three copies of catalase genes, as well as two superoxide-dismutase genes, one of which codes for a nickel-containing superoxide-dismutase, never reported before in proteobacteria [231].

A recombinant form of glutathione reductase (EC 1.8.1.7) from the Antarctic *C. psychrerythraea*, was recently characterized [168]. Since the cold-adapted enzyme displays activity also at moderate temperatures when overexpressed in *E. coli,* it may have some potential for industrial applications to protect cells and tissues from oxidative stress. Malato dehydrogenase (MDH) (EC 1.1.1.37) was purified from the Antarctic marine bacterium *Flavobacterium frigidimaris* KUC-1 and, among cold-adapted MDHs, was shown to be the most thermolabile and cold-active [158]. Thioredoxin and thioredoxin reductase (EC 1.8.1.9) from *P. haloplanktis* TAC125 were obtained as recombinant proteins. Both proteins exhibit activity at 10 °C [170]. The recombinat cold-adapted peroxiredoxin (EC 1.11.1.15) was biochemically characterized from the Antarctic marine psychrophilic bacterium *Psychrobacter* sp. ANT206, which had an optimum growth temperature of 10–12 °C. These enzymes may be relevant for many applications in food and medicine, mostly for their ability to protect super-coiled DNA from oxidative stress [172].

A cold-adapted leucine dehydrogenase (EC 1.4.1.9), a NAD^+-dependent oxidoreductase, with unique substrate specificity was cloned from the Antarctic marine psychrotrophic bacterium *Pseudoalteromonas* sp. ANT178 and was shown to retain 40% of its maximal activity at 0 °C. Being the key enzyme in the enzymatic conversion of L-leucine and other branched chain L-amino acids in the corresponding α-keto acid, it was suggested as biocatalyst in the pharmaceutical industry [157].

Aminotransferases (EC 2.6.1.), belonging to the class of transferases, have also been isolated from marine polar microorganisms, although no industrial application has been suggested so far. The aspartate aminotransferase, a ubiquitous transaminase enzyme that catalyzes the conversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate, was isolated from the marine psychrophilic bacterium *P. haloplanktis* TAC125 and characterized from a structural and functional point of view [173].

It was shown to be thermolable, being inactivated at 50 °C. Its optimal working temperature is 10 °C lower than its *E. coli* homolog.

Photolyases (EC 4.1.99.3) were identified in the genera *Pseudomonas*, *Hymenobacter* and *Sphingomonas*, isolated in fresh waters in Antarctica (King George Island, Fildes Peninsula). Present in all living forms, except placental mammals and some marsupials, they are resistant to UV-radiations, being able to reverse DNA lesions (cyclobutane pyrimidine dimers and pyrimidine photoproducts [232] and, as UV radiations are known to be linked to skin cancer, they may have some potential in the cosmetic and pharmaceutical industries.

Cold-active γ-carbonic anhydrases (EC 4.2.1.1), able to catalyze the physiologic reaction of CO_2 hydration to bicarbonate and protons, were isolated from the psychrophilic marine bacteria *C. psychrerythraea* [178] and *P. haloplanktis* [179] cloned and characterized.

A cold-active DNA ligase from the psychrophile *P. haloplanktis* TAE72, isolated from Antarctic seawater at the Dumont d'Urville Antarctic Station, displays activity at temperatures as low as 4 °C [177]. DNA ligases (EC 6.5.1.) are enzymes involved in DNA replication, DNA recombination and DNA repair. They are commonly used in molecular biology to catalyze the formation of a phosphodiester bond between adjacent 5′-phosphoryl and 3′-hydroxyl groups in double stranded DNA. Currently, DNA ligases on the market, such as the recombinant versions of bacteriophage-derived DNA ligases, T4 and T7 ligases, and *E. coli* DNA ligases, are enzymes active at temperatures above 15 °C, where residual nucleases may interfere with the ligation process [167]. The possibility to use psychrophilic DNA ligases active at low temperature may guarantee high specific activity at low temperatures, thus avoiding unwanted reactions, carrying out the reaction in shorter times with respect to mesophilic ligases.

The crystal structure of the sedoheptulose 7-phosphate isomerase from the marine psychrophilic organism *C. psychrerythraea* 34H was determined. The Arctic bacterium produces extracellular polysaccharide substances to cope with cold and the isomerase is essential for producing D-glycero-D-mannoheptose 7-phosphate, a key mediator in the lipopolysaccharide biosynthetic pathway [184]. Other polar enzymes belonging to the class of isomerase are triose phosphate isomerases (EC 5.3.1.1), enzymes involved in the glycolytic pathway, identified in the Antarctic marine bacteria *Pseudomonas* sp. π9 [185], isolated from sea-ice and *Moraxella* sp. TA137 [186], isolated from the intestine of fish caught in Terre d'Adelie in Antarctica.

4. Perspectives and Conclusions

Marine microorganisms, whose immense genetic and biochemical diversity is only beginning to be appreciated, may become a rich source of novel enzyme activities in the near future. The marine bioprospecting of polar regions has begun only relatively recently but has already yielded success stories. Taking into account the evidence that the total number of species, and thus most likely biochemical diversity in the oceans, is higher than on land, there is a good reason to believe that a larger number of marine natural products from low-temperature environments may reach different sectors of biotechnology in the near future. For those reasons, a large number of research and development programmes in oceans are in progress worldwide. Although the genomic, functional and physiological knowledge of individual organisms is necessary to understand how enzymes and products work, more efficient strategies are needed to overcome the limits of the cultivation of microbes, especially those living in extreme environments such as the polar ones. The specific properties of polar marine microorganisms that make them unique and biotechnologically interesting are also responsible for their resistance to handling and cultivation. The most significant challenges are (i) the current lack of ability to produce extreme compounds (including enzymes) on large scale; (ii) the need to generate stronger synergies among marine scientists and industries at earlier levels to compensate the chronic underfunding of basic research and before large-scale production; (iii) the need to develop or improve technology transfer pathways for data and to secure access to fair and equitable benefit sharing of marine genetic resources. Metagenomics, genome engineering and systems biology will be fundamental to efficiently produce larger quantities of known and novel bioresources.

In the race to find new products for biotechnology, although there are good reasons to be optimistic, diverse strategies need to be adequately supported and funded in the academic context. The future perspectives require active collaboration between academia and industry at the beginning to support the research thus reducing the time lapse from the discovery to the industrial applications.

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Review

Deep Hypersaline Anoxic Basins as Untapped Reservoir of Polyextremophilic Prokaryotes of Biotechnological Interest

Stefano Varrella [1], Michael Tangherlini [2] and Cinzia Corinaldesi [1,*]

[1] Department of Materials, Environmental Sciences and Urban Planning, Polytechnic University of Marche, 60131 Ancona, Italy; s.varrella@univpm.it
[2] Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy; michael.tangherlini@szn.it
* Correspondence: c.corinaldesi@univpm.it

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Abstract: Deep-sea hypersaline anoxic basins (DHABs) are considered to be among the most extreme ecosystems on our planet, allowing only the life of polyextremophilic organisms. DHABs' prokaryotes exhibit extraordinary metabolic capabilities, representing a hot topic for microbiologists and biotechnologists. These are a source of enzymes and new secondary metabolites with valuable applications in different biotechnological fields. Here, we review the current knowledge on prokaryotic diversity in DHABs, highlighting the biotechnological applications of identified taxa and isolated species. The discovery of new species and molecules from these ecosystems is expanding our understanding of life limits and is expected to have a strong impact on biotechnological applications.

Keywords: marine prokaryotes; microbial diversity; polyextremophiles; deep hypersaline anoxic basins; blue biotechnologies; extremozymes; polyextremophiles; limits of life

1. Introduction

Deep-sea ecosystems (waters and seabeds of the ocean beneath 200 m depth) are the largest, most remote, and least explored biomes of the biosphere, comprising more than two-thirds of the oceanic volume [1–3]. They are characterized by absence of light, an average depth of approximately 4200 m, temperatures below 4 °C, and a hydrostatic pressure of about 40 MPa; taken together, these factors encompass some of the harshest environments on our planet, representing a challenge for the existence of life [2]. Over the last few decades, many deep-sea surveys have resulted in the discovery of highly diversified and peculiar habitats [2,4–6], including hydrothermal vents, cold seeps, mud volcanoes, and deep hypersaline anoxic basins, where life conditions are even more extreme [7]. Among these, deep hypersaline anoxic basins (DHABs) are defined as polyextreme ecosystems [8,9].

DHABs were discovered at the end of the last century on the seafloor in different deep-sea areas (at depths ranging from 630 m to 3580 m) around the globe (Figure 1), including the Mediterranean Sea [10,11], the Red Sea [12–14] and the Gulf of Mexico [15]. Intriguingly, the discovery of new DHABs is still ongoing, such as with the recent discovery of the new Thetis, Kyros, and Haephestus basins in the Mediterranean Sea [16–18]. To date, with the recent finding of these new DHABs, 35 basins have been discovered around the world. The Bannock, Tyro, Urania, L'Atalante, and Discovery basins are the deepest known DHABs, being far below the photic zone (3200–3500 m deep), and are located along the Mediterranean Ridge in the Eastern Mediterranean Sea, an accretionary complex subjected to continental collision [19]. Two of the most studied DHABs in the Red Sea are the Shaban and the Kebrit deeps. The Shaban Deep comprises four depressions at a depth of 1325 m, whereas the Kebrit Deep is a rounded basin of approximately 1 km in diameter found at a depth of 1549 m [20].

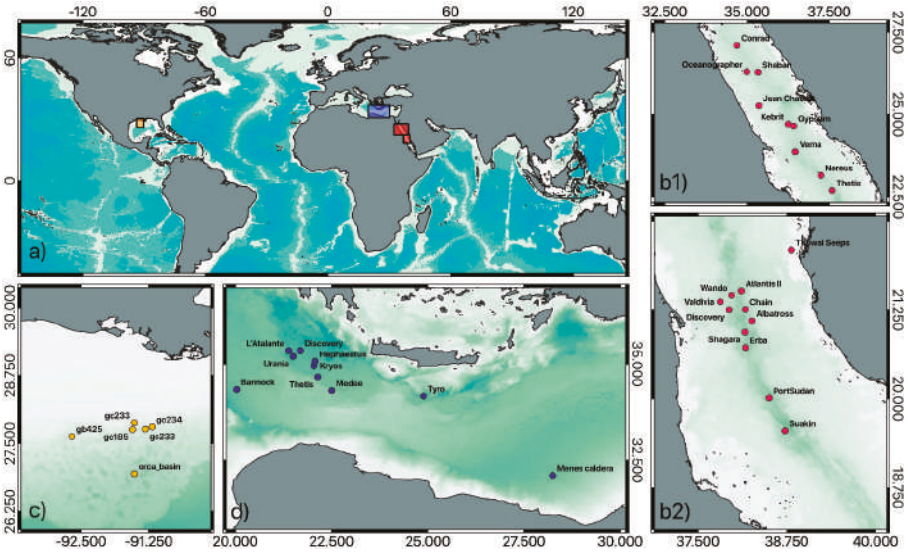

Figure 1. Global distribution of deep hypersaline anoxic basins (DHABs) (**a**). Locations and corresponding names of DHABs identified in the Red Sea (**b1**–**b2**), the Gulf of Mexico (**c**), and the Mediterranean Sea (**d**).

Different sampling strategies have been adopted to explore the general physical structure of DHABs. For instance, Mediterranean DHABs have been sampled through a rosette with Niskin bottles equipped with a conductivity, temperature, and depth (CTD) sensor and connected to a live camera to monitor the sampling operations [21–23]. Geochemical data of DHABs, such as those located in the Gulf of Mexico, have been collected through a brine-trapper, which was used to collect vertically water from different layers of the seawater–brine interface [24].

Despite the different geological features found in DHABs, most of them are derived from the re-dissolution of evaporitic minerals, like halite (NaCl-mineral) and kieserite ($MgSO_4$-mineral), after exposition to seawater due to tectonic activity [25,26]. This determines a salt-induced stratification of the water column (Figure 2), which drives the formation of a stable, dense, hypersaline brine lake with a variable thickness, ranging from one to tens of meters; this brine lake represents a polyextreme environment because its conditions hinder oxygen exchange, creating euxinic conditions, including high hydrostatic pressure, extremely low water activity and chaotropicity, and sharp oxy-, picno-, and chemoclines at the seawater–brine interface [21,23,27]. The salt concentration progressively increases over depth in the overlying halocline interface, reaching brines values up to 7–10 times higher than those existing in seawater [28].

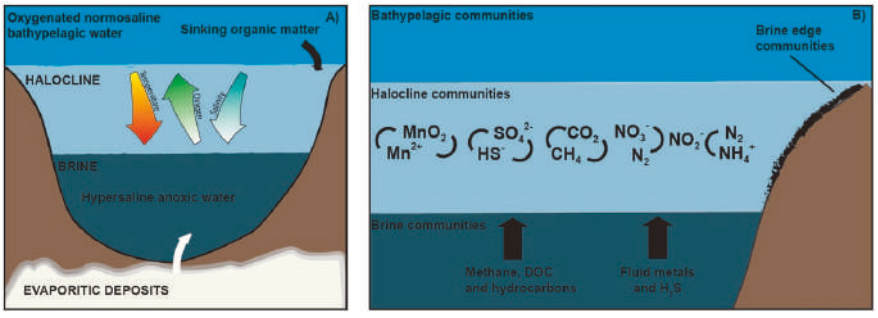

Figure 2. Simplified vertical section of a DHAB. The transition from the overlying seawater to the brine is commonly referred to as the halocline or brine–seawater interface, which is characterized by gradients of temperature, salinity, pH, and dissolved oxygen (**A**); the main biogeochemical processes taking place within the halocline are shown in (**B**). From left to right, the manganese cycle, the sulfate reduction and sulfide oxidation cycle, the methanogenesis and aerobic (anaerobic) methane oxidation cycle, and the anammox and denitrification cycle that occur in the halocline are shown [8,21,23,28–30]. DOC: Dissolved Organic Carbon.

Chemical and physical characteristics are specific to each DHAB and greatly vary depending on how the brine was formed along with the geographic localization (Table 1). The majority of the DHABs are thalassohaline (most of the dissolved ions are represented by those composing the overlaying seawater), whereas the Discovery, Kryos, and Hephaestus basins are athalassohaline and are characterized by high Mg^{2+} concentrations likely deriving from the dissolution of magnesium chloride salts (i.e., bischofite [18])

Table 1. Minimum and maximum values of the main physicochemical variables observed among DHABs.

Environmental Parameters	Ranges	DHABs	Location	References
Temperature	Min: 14 °C	La Medee	Mediterranean Sea	[31]
	Max: 68 °C	Atlantis II	Red Sea	[32]
Depth	Min 630 m	GC233	Gulf of Mexico	[33]
	Max: 3580 m	Discovery	Mediterranean Sea	[17]
Na^+	Min: 1751 mM	GC233	Gulf of Mexico	[24]
	Max 5300 mM	Tyro	Mediterranean Sea	[34]
Cl^-	Min: 2092 mM	GC233	Gulf of Mexico	[24]
	Max: 10,154.3 mM	Discovery	Mediterranean Sea	[17]
Mg^{2+}	Min: 8.7 mM	GB425	Gulf of Mexico	[24]
	Max: 5143 mM	Discovery	Mediterranean Sea	[17]
K^+	Min: 17.2 mM	Orca	Gulf of Mexico	[35]
	Max: 471 mM	La Medee	Mediterranean Sea	[31]
Ca^{2+}	Min: 1 mM	Discovery, Kyros	Mediterranean Sea	[17]
	Max: 150 mM	Atlantis II	Red Sea	[36]
SO_4^{2-}	Min: <1 mM	GB425; GC233	Gulf of Mexico	[24]
	Max: 333.1 mM	L'Atalante	Mediterranean Sea	[17]
Sulfide	Min: 0.002 mM	GC233	Gulf of Mexico	[24]
	Max: 16 mM	Urania	Mediterranean Sea	[16]

Overall, DHABs can be subdivided into four different systems: the seawater–brine interface, brines, the brine–sediment interface, and the sediments underlying the brines. Each of these features is characterized by specific conditions such as the steep halocline at the water–brine interface or the anoxic conditions of the sediments beneath the brines. In addition, the geochemical characteristics of each DHAB are mostly dependent on their geological evolution and origin. The high density of the brine prevents their mixing with the overlying oxygenated seawater, thus making the DHABs

completely anoxic [16]. Their different hydrochemistry and physical separation for thousands of years has made these systems greatly interesting for scientists due to their potential similarity with extraterrestrial environments [18,34,37]. Despite their extreme conditions, many studies have provided evidence of a highly active prokaryotic community and of the presence of living metazoans, greatly extending our knowledge regarding the limits of organisms' adaptions to life [20,27,38–48]. These organisms require specific adaptations for withstanding numerous physicochemical stresses [49].

The complex structure and conditions of the DHABs, such as the presence of the steep halo- and oxyclines, have been found to influence the distribution, structure, and richness of the microbial communities living in these environments [19]. Many studies have been focused on the halocline, which entraps nutrients, sinking organic materials, minerals, and microbial cells, and creates environmental gradients of great interest not only for identifying and isolating novel organisms but also for clarifying their metabolic strategies employed for adapting to extreme conditions [19]. The variable accumulation of metals and nutrients, especially in the halocline, supports the presence of different ecological niches exploited by highly diverse microorganisms with peculiar features [38]. However, to date, our knowledge of how these organisms are affected and contribute to the geochemical properties of the DHABs is still limited.

The presence of life in these extreme environments has raised important questions about the molecular mechanisms that extremophiles have developed to overcome harsh conditions. Many studies have highlighted several peculiar adaptive strategies of halophilic microorganisms for maintaining stability and functionality of all their cellular components under such conditions [50,51]. Hence, microorganisms inhabiting extreme saline habitats not only have been considered useful subjects for ecological and evolutionary studies [50] but also hold an outstanding ability to produce bioactive molecules and enzymes, which can also be exploited for industrial and biotechnological purposes as well as for human wellness [52,53]. Considering the promising biotechnological potential of bacteria and archaea from DHABs due to their capability to live under extreme conditions, the present review provides an outline of the prokaryotic biodiversity in DHABs, highlighting their potential in producing enzymes and bioactive molecules for industrial, pharmaceutical, and environmental applications.

2. Prokaryotic Assemblages of DHABs

The specific characteristics and geochemical conditions of each DHAB have driven the development of different and highly-stratified communities. Brines and the seawater–brine interfaces, indeed, represent the most widely-studied domains within DHABs from both a taxonomic and ecological/functional point of view [21,23]. The halocline is a microbial "hotspot", harboring dense microbial populations that appear to be more metabolically active than those of the adjacent layers, with the presence of unique bacterial lineages having been found [21,22,44,54,55]. Several microbial lineages have been identified within DHAB brines. In particular, many members of the new Mediterranean Sea Brine Lake lineages (MSBL1–6) have been found extensively across hypersaline basins [56] from the Mediterranean to the Red Sea (despite their name), and include Archaea (e.g., MBSL1, which are sugar-fermenting organisms capable of autotrophic growth [23,56,57] and other major divisions of bacteria (MSBL2–6 [21,38]). Interestingly, the bacterial MSBL2 lineage has shown high similarity to the SB1 division found in the Shaban Deep brine pool, located in the Red Sea, which represents a novel halophilic lineage within bacteria, with no close cultivated relatives observed so far [28,38]. Similarly to the MBSL lineages, bacteria belonging to candidate division KB1 have been identified for the first time within the Kebrit Deep basin (Red Sea [20]) and subsequently have also been found in other DHABs of the Red Sea [58], as well as in other basins (e.g., the halocline of Mediterranean Sea brine pools and pools from the Gulf of Mexico [17,21,22,31,59]). Bacteria from this division can import and/or produce glycine betaine in response to osmotic stress [59]. The KB1 glycine betaine transport systems seem to aid not only in maintaining osmotic balance but also have a role in methane production [59]. Delta- and *Epsilonproteobacteria* are also widely distributed across DHABs. 16S rRNA gene libraries from the Bannock, Hephaestus, and L'Atalante basins (Mediterranean Sea) have provided

evidence of the presence of sulfate-reducing *Deltaproteobacteria* (in particular belonging to the ANME-1 clade, responsible for the anaerobic oxidation of methane [16]) and sulfur-oxidizing Gamma- and *Epsilonproteobacteria* [21,22]. In the GC233 basin within the Gulf of Mexico, combining geochemical data and molecular analyses, different *Deltaproteobacteria* sulfate-reducers (related to *Desulfosarcinales*, *Desulfobacterium*, *Desulfobulbus*, and *Desulfocapsa*) and sulfide-oxidizing *Epsilonproteobacteria* have been found, leading to the hypothesizing of the presence of a sulfur-cycling microbial community [15,60].

Archaea associated with the ammonia-oxidizing *Thaumarchaeota* Marine Group I have also been found across several DHABs worldwide [18,21,22]. In particular, they have appeared to be the most representative prokaryotic members in different Red Sea DHABs, though with different contributions: in the Atlantis II and Discovery, 99% of archaeal operational taxonomic units (OTUs) were found to belong to the phylum *Thaumarchaeota*, whereas in the Erba basin the percentage was about 64% [30]. Members of this phylum are capable of fixing CO_2 and oxidizing methane, contributing to dark primary production [61]. Overall, the dominant thaumarchaeal lineage is closer to the genus Nitrosopumilus [62]. The adaptation of this genotype to the hostile brine–sediment interface environment can be possible not only by increasing intra-cellular salt concentrations [63] but also for the presence of "acidic tuned" membrane proteins which show optimal activity and stability at high salinity [64]. Furthermore, genomic analyses have revealed the presence of specific pathways for taking up a mixture of osmolytes and other genes encoding for the biosynthesis of ectoine/hydroxyectoine, which are not present in mesopelagic clades [30]. However, different DHAB geochemistry may shape other thaumarchaeal lineages. Genomic analyses have revealed a newly isolated methanogenic archeon from the sulfide-rich halocline of Kebrit, which holds adaptive traits (e.g., osmoprotection and oxidative stress response) for counteracting the harsh local conditions [65].

Apparently, as high salinity is one of the main features of DHABs, halophilic organisms have been found across all the basins, and most of the isolated halophilic strains also display interesting metabolic features. In particular, 33 halotolerant bacterial strains have been isolated from the halocline of the Urania, Bannock, Discovery, and L'Atalante basins [66]. For instance, *Halanaeroarchaeum sulfurireducens* M27-SA2 is a sulfur-reducing and acetate-oxidizing haloarcheon isolated from the Medee basin [67]. Moreover, several novel strains have been isolated from Red Sea DHABs, such as *Halorhabdus tiamatea* (a non-pigmented, fermenting member of the *Halobacteriaceae* [68]) and *Haloplasma contractile* (a highly unusual contractile bacterium belonging to the Haloplasmatales order, which can grow under 0.2–3.1 M NaCl conditions [69]. Two other strains of a novel species, *Marinobacter salsuginis* SD-14BT and SD-14C, have also been isolated from the halocline of the Shaban Deep [70].

In general, prokaryotic diversity and activity appear to be less marked in sediments under brines than in deep-sea control sediments [55]. This is likely due to the cumulative physico-chemical stressors that greatly limit the survival of microorganisms which could be better adapted to the extreme DHAB chemocline [27,55,71]. Proteobacteria, Actinobacteria, Deferribacteres, and Euryarchaeota have been found in sediments underlying either Discovery Deep or Atlantis II [72]. In the sediments of L'Atalante, OTUs belonging to the *Pseudoalteromonas*, *Halomonas*, and *Pseudomonas* genera have been observed to be the most represented within the abundant Gammaproteobacteria class, suggesting a mixed assemblage of halophilic and halotolerant microorganisms [55]. In addition, metatranscriptomic analyses have revealed that, in the sediments underlying the Urania basin, most transcripts are affiliated with rRNAs of the genera *Pseudomonas*, *Rhodobacter*, and *Clostridium*, and with sequences associated with mitomycin antibiotics typically produced by *Streptomyces* [71]. Prokaryotes inhabiting DHAB sediments are killed by viruses, which may represent the main mechanism of top-down control of prokaryotic dynamics in these ecosystems [42]. Since viruses are found to be well-preserved in DHAB sediments, they can shape prokaryotic assemblages [41]. Based on this information, it is possible to hypothesize that prokaryotes of DHABs can produce specific molecules against viral infections.

3. Biotechnological Potential of Prokaryotes Inhabiting DHABs

Generally, marine microorganisms represent an untapped source for the discovery and development of new biomolecules due to their rich biodiversity and genetic capacity to produce unique metabolites [73–75]. In this regard, it is well documented that many taxonomically novel marine species are promising sources of new bioactive compounds with noteworthy pharmaceutical activities, which can become sources of novel therapeutic agents [52,76]. In particular, marine extreme environments, like deep-sea and polar ecosystems or DHABs, have been revealed to be a rich source of secondary metabolites with novel structures and outstanding biological activities [28,52,77].

Due to the limited accessibility and remoteness of such extreme ecosystems and the need for sophisticated instruments for exploring and investigating them, they are still largely understudied and underexploited in comparison with terrestrial ecosystems.

Over the last few years, the advancement of technologies for deep-sea exploration [78] and "-omics" (e.g., environmental shotgun sequencing and metatranscriptomics) for the analysis of environmental strains of prokaryotes has revolutionized bioprospecting in extreme environments, thus increasing our knowledge of the genetic potential of microbial communities for the discovery of enzymes with a commercial value [79,80]. In addition, functional screening of extremophile metagenomes could represent a valuable approach to identify novel antibacterial and anticancer agents. In this regard, bioinformatic tools like the metabolite analysis shell (antiSMASH) have recently been used to detect from metagenomic samples collected from the Atlantis II, Discovery, and Kebrit DHABs promising specialized metabolism gene clusters (SMGCs) coding for products with reported antibacterial and anticancer effects, namely terpenes, peptides, polyketides, and phosphonates [81]. Two clones belonging to these libraries which exhibited antibacterial effects were screened by high-throughput sequencing (NGS) and bioinformatic analyses along with cytotoxicity assay (MTT) testing of the whole cell lysates against different cancer cell lines (MCF-7, U2OS, and 1BR-hTERT) [82]. Although culture-independent approaches have radically changed microbial bioprospecting in extreme environments, the development of biotechnological applications must be accompanied by the corresponding study of pure cultures. In this regard, despite the great biodiversity-highlighted trough metagenomics in DHABs, so far less than 100 bacterial strains (Figure 3, Table S1) have been isolated and cultured for testing their extracts in few biotechnological applications [66,83–85]. Bioinformatic analyses on the phylogeny of the 16s rDNA sequences of those cultured strains (carried out by aligning them on the SILVA database v132 on the ACT server [86]) showed that most of these prokaryotes are affiliated with Gammaproteobacteria and Bacilli; in addition, several sequences within the same database were found to be phylogenetically related to the cultured strains (Table S2), further suggesting that more prokaryotic strains with adaptations to polyextreme ecosystems with biotechnological potential might be found within the same clades of already-cultured strains. Novel sampling and cultivation methods should be developed as alternatives to overcome culture limitations, especially in extreme environments [87].

Since the beginning of the new millennium, a number of studies have indicated the beneficial roles of extremophilic marine prokaryotes, which are a relevant but still underexplored source of bioactive molecules of commercial significance [74,88,89]. Extremophiles undoubtedly show unique capabilities and adaptations which allow them to thrive in systems characterized by harsh environmental conditions [90]. In fact, polyextremophilic microorganisms utilize alternative metabolic pathways and adaptive mechanisms which have important applications in industrial and environmental fields [50]. Since these microorganisms live in a biologically competitive environment for space and nutrients, they have developed mechanisms of defence against competitors and predators for their own survival, synthesising secondary metabolites of great value in pharmaceutical and biotechnological applications [91,92]. The advances in genome sequencing of extremophilic microorganisms have allowed us to provide a comprehensive understanding of their applications [93–95]. Moreover, microbes with large genomes, usually inhabiting complex harsh environments, can produce a vast array of secondary metabolites [96,97].

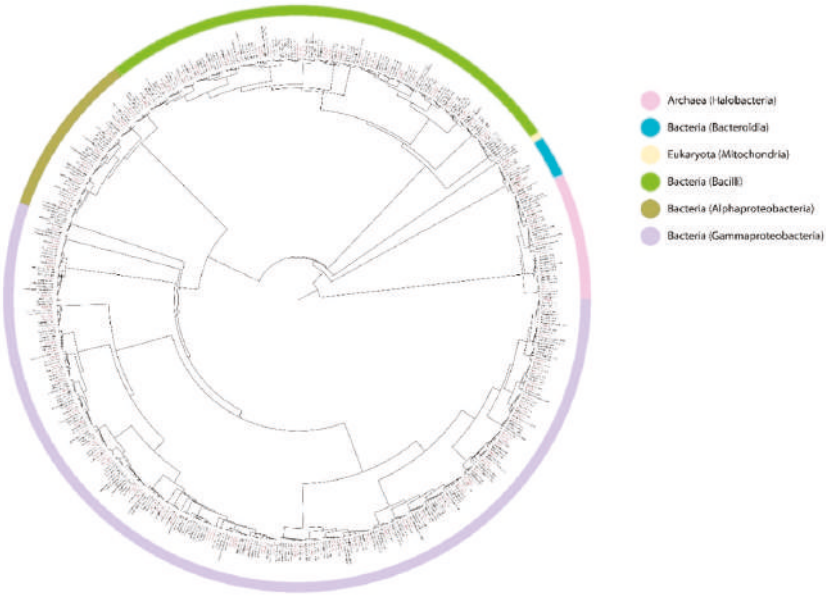

Figure 3. Phylogenetic tree of bacterial and archaeal strains isolated from DHABs. The tree was built using 16S rRNA gene sequences from [66,83–85] and phylogenetically close 16s rRNA sequences from the SILVA database v132.

3.1. DHABs as a Hidden Treasure for Biodiscovery of Pharmaceuticals

Over the last 50 years, the development of new multidrug-resistant pathogens, along with the consequent increase in infectious diseases, has become an important issue for human wellness [98]. Furthermore, anticancer chemotherapeutic resistance is recently becoming a biomedical challenge, arising either intrinsically or extrinsically, after therapy [99]. Thus, the need for the discovery and development of novel antimicrobial and chemotherapeutic drugs with new modes of action is nowadays becoming of fundamental importance [100,101]. Since most of the antibiotics currently available on the market have been extracted from terrestrial organisms or derived semisynthetically from fermentation products, the isolation of microorganisms from marine habitats represent an interesting possibility which can lead to the discovery of novel structures with antibiotic activity [102,103].

As such, the prokaryotic genera identified in DHABs isolated from different marine environmental sources, including extreme environments, represent an authentic treasure of many bioactive compounds useful for biomedical applications (Table 2).

Table 2. Bioactive molecules for pharmaceutical use produced by prokaryotes directly isolated from DHABs and promising bioactive molecules produced by prokaryotic taxa which have been identified in DHABs and isolated from other marine systems.

Marine Prokaryotes	Product	Bioactivity	Environmental Sources	Ref.
Alteromonas macleodii	Dithiolopyrrolone	Antibiotic and antitumor	Erba and Nereus DHABs	[84,104]
Alteromonas sp. B-10-31	Marinostatins B-1, C1, and C2	Serine protease inhibitor	Coastal seawater	[105]
Bacillus sp.	Macrolactins A–F	Cytotoxic, antimicrobial, antiviral	Deep sea	[106–109]
Bacillus halodurans	Enfuvirtide	Antiviral	Nereus DHAB	[84,110]
Bacillus MK-PNG-276A	Loloatins A–D	Antimicrobial	Great barrier reef	[111]
Bacillus sp.	Bogorol A	Antimicrobial	Seawater	[112]
Bacillus sp. CND-914	Halobacillin	Antitumor	Deep-sea sediments	[113]
Bacillus sp. MIX-62	Mixirins A–C	Antitumor		[114]
Bacteroidetes rapidithrix HC35	Ariakemicins A and B	Antimicrobial, cytotoxic	Sea mud	[115]
Erythrobacter sp.	Erythrazoles A and B Erythrolic acids A–E	Cytotoxic	Mangrove sediments	[116,117]
Halobacteroides lacunaris TB21	R-LPS	Immunomodulator	Thetis DHAB	[118]
Halomonas LOB-5	Loihichelins A–F	n.a.	Deep sea hydrothermal vents	[119]
Halomonas meridiana	n.a.	Antitumor	Nereus DHAB	[84]
Halomonas sp. GWS-BW-H8hM	3-(4′-Hydroxyphenyl)-4-phenylpyrrole-2,5-dicarboxylic acid (HPPD-1 and HPPD-2)	Cytotoxic	Seawater	[120]
Halomonas sp. GWS-BW-H8hM	2-Amino-6-hydroxyphenoxazin-3-one2-Amino-8-benzoyl-phenoxazin-3-one2-Amino-8-(4-hydroxybenzoyl)-6-hydroxyphenoxazin-3-one	Antimicrobial, cytotoxic	Seawater	[121]
Pseudoalteromonas carrageenovora IAM 12662	LPS	Antiviral	Erba DHAB	[84,122]
Pseudoalteromonas haloplanktis TAC125	Peptides	Antioxidant	Antarctic coastal sea water	[123]
Pseudoalteromonas mariniglutinosa	n.a.	Antitumor	Erba and Nereus DHABs	[84]
Pseudoalteromonas rava SANK 73390	Thiomarinols A–H and J	Antimicrobial	Seawater	[124,125]
Streptomyces aureoverticillatus (NPS001583)	Aureoverticillactam	Antitumor	Marine sediments	[126]
Streptomyces C42	Champacyclin	Antimicrobial	Deep sea	[127]
Streptomyces CNH-990	Marmycins A and B	Cytotoxic	Seawater	[128,129]
Streptomyces drozdowiczii SCSIO 10141	Marfomycins A, B, and E	Anti-infective	Deep sea	[130]
Streptomyces drozdowiczii NTK 97	Frigocyclinone	Antimicrobial	Antarctica	[131]
Streptomyces Merv 8102	Essramycin	Antimicrobial	Marine animals, plants, and sediments	[132]
Streptomyces niveus SCSIO 3406	Marfuraquinocins	Cytotoxic antimicrobial	Deep sea	[133]
Streptomyces scopuliridis SCSIO ZJ46	Desotamide B	Antimicrobial	Deep-sea sediments	[134]
Streptomyces sioyaensis SA-1758	Altemicidin	Cytotoxic, antimicrobial	Sea mud	[135]
Streptomyces sp. 12A35	Lobophorins H and I	Antimicrobial	Deep sea	[136]

Table 2. *Cont.*

Marine Prokaryotes	Product	Bioactivity	Environmental Sources	Ref.
Streptomyces sp. ART5	Articoside	Cytotoxic,	Arctic deep sea	[137]
Streptomyces sp. CNB-982	Cyclomarins A–C	anti-inflammatory	Marine sediments	[138, 139]
Streptomyces sp. CNQ-418	Marinopyrroles A–F	Antimicrobial, cytotoxic, anti-apoptotic	Deep-sea sediments	[140, 141]
Streptomyces sp. CNQ-85	Daryamides A–C (2E,4E)-7-Methylocta-2,4-dienoic acid amide 26	Antitumor, antifungal	Seawater	[142]
Streptomyces sp. CNR-698	Ammosamides A–D	Cytotoxic	Deep sea	[143–145]
Streptomyces sp. M045	Chinikomycins A and B	Antitumor	Seawater	[146]
Streptomyces sp. MDF-04-17-069	Tartrolon D	Cytotoxic	Marine sediments	[147]
Streptomyces sp. Mei37	Mansouramycins A–D	Antimicrobial, cytotoxic	Marine sediments	[148]
Streptomyces sp. NTK 935	Benzoxacystol	Antiproliferative	Deep sea	[149]
Streptomyces sp. SCSIO 03032	Spiroindimicins A–D	Antitumor	Deep sea	[150]
Streptomyces sp. SCSIO 11594	Dehydroxyaquayamycin, Marangucycline B	Antibacterial, antitumor	Deep sea	[151]
Streptomyces xiamenensis M1-94P	Xiamenmycin C and D	Anti-fibrotic	Deep-sea sediments	[152]
Streptomycete sp.	Piperazimycins A–C	Antitumor	Marine sediments	[153]
Zunongwangia profunda SM-A87	EPS	Antioxidant	Nereus DHAB	[154]

For instance, in Mediterranean DHABs transcripts related to *Streptomyces* have been identified, thus representing an important source of bioactive natural products with clinical or pharmaceutical applications [71,155]. Additionally, *Pseudoalteromonas flavipulchra* recently isolated from the Nereus halocline shows great antimicrobial activity which is associated with the different metabolites and/or enzymes that this species can produce [84,156,157].

An attractive example of the potential of extremophiles in the biomedical field has been provided by *Halobacteroides lacunaris* TB21, which was isolated from Thetis basin [118]. This polyextremophile organism produce a lipopolysaccharide (LPS) analog which can bind to the TLR4/MD-2 complex in HEK 293 hTLR4 cells, exerting an immunostimulant activity [118]. Additionally, *Pseudoalteromonas carrageenovora* isolated from Erba basin sediments is able to produce an LPS whose function is still undescribed [84,122]. This halophilic bacterium can also produce low molecular weight products from carrageenans, which have been reported to hold protective effects against the human immunodeficiency virus, the yellow fever virus, the herpes simplex viruses, the vaccine virus, and the pig fever virus [158]. Another interesting species isolated from the Nereus brine-pool–sea-water interface is *Bacillus halodurans*, which was engineered for the production of the anti-viral therapeutic peptide Enfuvirtide, marketed by Roche under the trademark Fuzeon®, which has given rise to possibilities for pharmaceutical applications [84,110]. In addition, *Zunongwangia profunda*, inhabiting the same interface, produces exopolysaccharides (EPS) with antioxidant properties [154].

Innovative research was carried out for screening the bioactivity of molecules extracted from the Atlantis II, Discovery, Kebrit, Nereus, and Erba DHABs [83,85]. Extracts from 36 isolates were tested on three different human cancer cell lines: HeLa, MCF-7, and DU145 [83,85]. In particular, many extracts from *Halomonas* strains have been found to induce apoptotic and cytotoxic effects. For example, *Halomonas meridiana* collected from Nereus halocline has been observed to prompt apoptosis of MCF-7 cells [85]. Recently, it was shown that the extract of *Pseudoalteromonas mariniglutinosa* collected from Erba and Nereus haloclines also inhibited the growth of MCF-7 cells [84]. *Halomonas* species can produce EPS which have been shown to have pro-apoptotic activity towards human T-leukemia cells and breast cancer MCF-7 cells [159,160]. Other bioactive extracts derived from *Chromohalobacter salexigens*, *Chromohalobacter israelensis*, *Halomonas meridiana*, and *Idiomarina loihiensis* have been found to be able to induce more than 70% mortality in HeLa cancer cells through different caspase-mediated apoptotic pathways [83].

Intriguingly, three extracts belonging to the genus *Salinivibrio* have been found to specifically blocked the growth of fibrosarcoma cells (HT-1080), opening up interesting perspectives for the discovery of new bioactive compounds produced by this genus [84]. The extracts of *Halomonas hamiltonii* and *Alcanivorax dieselolei* have been observed to inhibit the proliferation of BT20 cells, whereas the *Alteromonas macleodii* extracts collected from Nereus and Erba halocline were found to inhibit the cell growth of HCT [84]. *Alteromonas species* are also well known for producing dithiolopyrrolone, a potent antibiotic approved by the Food and Drug Administration and commercialized as Bactroban® (GlaxoSmithKline) [104]. These studies emphasize the wide diversity of brine pool microorganisms capable of producing bioactive molecules, highlighting the incredible potential of DHABs as a source of novel molecules exploitable in the pharmacological industry.

3.2. DHABs as a Reservoir of Polyextreme Enzymes

Today's society is moving toward "white" (i.e., industrial) biotechnology, which is growing for its efficiency from environmental and commercial points of view [161]. For example, natural enzyme catalysis has been utilized for application in a broader range of industrial settings, representing a valuable alternative to its chemical catalysts [162–164]. It is expected that 40% of the industrial application of chemical reactions that require organic solvents harmful to the environment will be replaced by enzymatic catalysis by 2030 [165]. The continuous demand for natural new enzymes that are biocompatible and non-toxic and which have high activity over a wide range of conditions, including temperature, salinity, pH, and metal concentrations, has been scaled up within pharmaceutical,

food, and beverage industrial processes [166]. Hence, extremophilic microorganisms represent important sources of stable and valuable enzymes which are used as biocatalysts in industrial and biotechnological processes [53]. Enzymes from these organisms, which are called "extremozymes" due to their special features, can catalyze chemical reactions under conditions which inhibit or denature the non-extreme forms [167], including high salinity, acidic or basic pH, and high temperatures [168]. Thus, through the use of genetic engineering and/or by bioprospecting of extreme environments it is possible to discover and develop new extremozymes that can be suitable for many industrial processes [164]. Extremophilic bacteria and archaea produce enzymes which can be employed in industrial reactions using either directly living organisms or purified molecules, expanding the ranges of optimal enzyme performance and thus enabling biocatalysis under the enzymatically unfavourable conditions found in industrial processes [169]. Hence, the peculiar characteristics of extremophiles belonging to prokaryotic domains living in DHABs can represent a new source for exploitable enzymes for their capacity to operate under extreme conditions [170,171]. In fact, many of these molecules (e.g., aldehyde dehydrogenase, proteases, cellulases, esterases, ferredoxin oxidoreductase, agarase, amylases, κ-Carragenases, ketoreductases, and cyclodextrin glycosyltransferase) have been commercialized and have applications in different biotechnological areas with considerable benefits for many kinds of industries (Table 3). In particular, they are currently being employed in "red" biotechnology (i.e., biotechnology applied to pharmaceutical and medical fields). Other enzymes such as cellulase, chitinase, esterase, mercuric reductase, and β-glucosidases are exploited in "grey" (i.e., environmental) biotechnology while lipase is used in "blue" biotechnology, being applied to aquatic organisms and β-glucosidases and xylanase in biofuel production.

Table 3. DHAB microbiome as a source of polyextremozymes. The bacterial and archaeal species marked with an asterisk have been isolated from DHABs, whereas the other genera are potentially producers of extremozymes because these have identified from DHABs (but not cultured thus, being isolated from marine and/or other extreme environments).

Enzyme	Biological Source	Specific Adaptations	Function and/or Applications	Ref.
Aldehyde dehydrogenase (EC 1.2.1.3–7)	*Bacillus halodurans* from Nereus interface; Atlantis II Red Sea brine pool; Cytophaga sp. KUC-1 from Antarctic seawater and *Halobacterium salinarum*	Slight halophile; thermo- and psychrophilic	Biotransformation of a large number of drugs and other xenobiotics generates aldehydes as intermediates or as products resulting from oxidative deaminations	[172–175]
Protease (EC 3.4.21–25)	*Salinivibrio costicola** and *Pseudoalteromonas ruthenica** from Erba DHAB. *Bacillus circulans* BM15 and *PseudoAlteromonas sp.* 129-1. *Bacillus sp.* NPST-AK1, *Halobacterium halobium* (ATCC 43214), *Bacillus licheniformis*, *Bacillus halophilus*, *Pseudoalteromonas strain* EB27, *Halomonas meridiana* DSM 5425, *Bacillus sp.* (Ve2-20-91 (HM047794)), and *Bacillus caseinilyticus*	Haloalkaliphilic and thermotolerant alkaline	Protein hydrolysis finds a broad variety of potential applications in diverse biotechnological processes such as in the feed, food, pharmacology (anticancer and antihemolytic activity) and cosmetic (keratin-based preparation) industries, and cleaning processes (e.g., detergent additive)	[176–185]
Cellulase (EC 3.2.1.4)	Cytophaga hutchinsonii, Halorhabdus tiamatea from Shaban DHAB, *Bacillus sp.* SR22 from seawater, *Bacillus sp.*, *Vibrio sp.*, *Rhodococcus sp.*, *Clostridium* and *Streptomyces* from mangrove Halorhabdus utahensis from Great Salt Lake	Halo-alkali tolerant and thermotolerant	Breakdown of cellulose-producing polysaccharides; potential application in the food, animal feed, beer and wine, textile and laundry, and pulp and paper industries, agriculture, biofuel, pharmaceutical industries, and waste management	[186–191]
Chitinase (EC 3.2.1.14), chitin deacetylase (EC 3.5.1.41)	*Bacillus thuringiensis* HBK-51 from soil. *PseudoAlteromonas sp.* DC14, *Vibrio cholerae, Vibrio parahaemolyticus*, and *Arthrobacter sp.* AW19M34-1 from seawater	Halo-alkali tolerant and thermotolerant	Hydrolysis of chitin and hence N-acetyl chitobiose production which in turn can be useful in fermentation research and biomedicine. There have also been applications in the cosmetic and pharmaceutic fields	[192–194]
Esterase (EC 3.1.1.1)	*Zunongwangia profunda** from Atlantis II and Nereus interface and brine pools. *Alcanivorax dieselolei* B5(T) from Erba interface. *Bacillus cereus* AGP-03 from hot spring. *Archaeoglobus fulgidus*	Thermo-halotolerant and metal resistant; cold-active and organic solvent-tolerant	Leather manufacturing, flavor development in the dairy industry, oil biodegradation, and the synthesis of pharmaceuticals and chemicals	[195–200]
Ferredoxin oxidoreductase (EC 1.2.7.1)	*Halorhabdus tiamatea* SARL4BT* from Shaban DHAB. *Desulfovibrio sp.* from Atlantis II DHAB. Methanosarcina barkeri	Low-oxygen tolerant	Oxidation/reduction processes which are applied in the asymmetric oxyfunctionalization of steroids and other pharmaceuticals, synthesis and modification of polymers, oxidative degradation of pollutants, oxyfunctionalization of hydrocarbons, and the construction of biosensors for diverse clinical applications	[189,201,202]

Table 3. *Cont.*

Enzyme	Biological Source	Specific Adaptations	Function and/or Applications	Ref.
Lipase (EC 3.1.1.3)	Idiomarina sp. W33, Halo*Bacillus sp.*, and *Archaeoglobus fulgidus*. *Marinobacter alkaliphilus* ABN-IAUF-1. *Bacillus sp.*, *Arthrobacter sp.*, *Pseudomonas sp.*, and *Psychrobacter sp.* from Antarctic marine sediments. Oceano *Bacillus sp.* PUMB02 from seawater	Halo- alkalitolerant and hyperthermophilic	Hydrolysis of acylglycerols to release fatty acids and lower acylglycerols or glycerol. Lipase enzymes are exploited in the food, beverage, detergent, biofuel production, animal feed, textiles, leather, paper processing, and cosmetic industries	[203–208]
Mercuric reductase (EC 1.16.1.1)	Atlantis II deep-sea brine. *Chromohalobacter israelensis** from Erba and Atlantis II DHABs. *Bacillus firmus** from Discovery DHAB	Extreme halophilic and thermophilic	This enzyme can convert toxic mercury ions into relatively inert elemental mercury. It is very useful in waste-water treatments	[209,210]
Nitrilase (EC 3.5.5.1)	Red Sea Atlantis II brine	Thermostable and heavy metal tolerant	Nitrilase can hydrolyze a single cyano group in dinitriles or polynitriles, yelding cyanocarboxilic acids, which are used in different kinds of industries, including the food and pharmacology industries; also used for bioremediative purposes	[211]
Pullulanase (EC 3.2.1.41)	*Bacillus sp.* and *Streptomyces sp.*	Alkaliphilic	Utilized to hydrolyze the α-1,6 glucosidic linkages in starch, enabling a complete and efficient conversion of the branched polysaccharides into small fermentable sugars during the saccharification process	[212]
Xylanase (EC 3.2.1.8) and β-Xylosidase (EC 3.2.1.37)	*Staphylococcus sp.*, *Arthrobacter sp.*, *Streptomyces* sp., and *Vibrio sp.* XY-214 from seawater. Oceanospirillum linum CL8 and *Halorhabdus utahensis* from Great Salt Lake. *Halorhabdus tiamatea* SARL4BT* from Shaban DHAB. *Pseudoalteromonas mariniglutinosa** from Erba and Nereus DHAB. *Marinimicrobium haloxylanilyticum** from Kebrit DHAB. *Zunongwangia profunda** from Nereus and Atlantis II DHABs. *Halomonas meridiana** from Bannock, Erba, and Nereus DHABs. *Bacillus halodurans** from Nereus interface	Alkali-halotolerant and psychrophilic	Commercial exploitation in the areas of the food, feed, and paper and pulp industries; also used to increase sugar recovery from agricultural residues for biofuel production	[189,213–218]
α-agarase (EC 3.2.1.158) and β-agarase (EC 3.2.1.81)	*Alteromonas macleodii** from Erba, Discovery, and Nereus DHABs. *Alteromonas sp.* GNUM-1, *Alteromonas agarlyticus*, *Alteromonas sp.* strain C-1, *Vibrio sp.* PO-303, *Altermonas sp.* SY37-12, and Cytophaga flevensis from seawater and marine sediments	Moderate halophile	Degradation of agar-degrading bacteria used as oriental food; wide applications in the food industry, cosmetics, and medical fields, and as a tool enzyme for biological, physiological, and cytological studies	[219–221]

Table 3. *Cont.*

Enzyme	Biological Source	Specific Adaptations	Function and/or Applications	Ref.
α-amylase (EC 3.2.1.1)	*PontiBacillus chungwhensis** from Discovery DHAB. Halomonas meridiana* from Nereus, Erba, and Bannock DHABs. *Zunongwangia profunda** from Atlantis II and Nereus DHABs. *Cytophaga sp. HaloBacillus sp.*, *Bacillus sp.* GM8901, *Bacillus sp.* TSCVKK, and *Methanococcus jannaschii. Halobacterium sp.* from hypersaline environment. *Alteromonas haloplanctis* from Antarctic seawater	Moderate halophile and alkali- tolerant; hyperthermophilic	α-amylase has implications in the food, pharmaceutical, and chemical industries; multifunctional amylase exhibits transglycosylation and hydrolysis activities to produce isomaltooligosaccharides, maltooligosaccharides and glucose	[222–232]
β-glucosidases (EC 3.2.1.21)	*Halorhabdus tiamatea* SARL4BT* from Shaban DHAB. *Alteromonas sp.* L82 from the Mariana Trench. Cytophaga hutchinsonii	Low-oxygen tolerant, cold-adapted, and salt-tolerant	β-glucosidases convert cellobiose and short cellodextrins into glucose. β-glucosidases are widely used in the production of biofuels and ethanol from cellulosic agricultural wastes, in the production of wine, and in the flavor industry. They can cleave phenolic and phytoestrogen glucosides from fruits and vegetables for extracting medicinally important compounds and enhancing the quality of beverages	[189,191,233, 234]
κ-Carragenases (EC 3.2.1.83)	*Pseudoalteromonas carrageenovora** from Erba sediments. *Bacillus sp. Alteronomonas sp.*, Cytophaga sp., and *PseudoAlteromonas sp. Pseudomonas sp.*, *Vibrio sp.* NJ-2, and *Vibrio parahaemolyticus* from seawater	Alkali-halotolerant	Production of oligosaccharides with potential applications in the biomedical field, in bioethanol production, in the textile industry, and as a detergent additive	[235–237]
Cyclodextrin glycosyltransferase (EC 2.4.1.19)	Bacillus lehensis* from Discovery DHAB	Alkali-halotolerant	Cyclodextrins produced by this enzyme have broad, non-toxic applications in the pharmaceutical, cosmetic, and food industries	[66,238]

For instance, the production of novel thermoactive and alkali-tolerant α-amylases has been documented for many prokaryotic species such as *Pontibacillus chungwhensis*, *Halomonas meridiana*, and *Zunongwangia profunda* isolated taxa from DHABs. This group of enzymes has a very wide spectrum of industrial application, including in the sugar production, animal nutrition, baking, brewing, and distilling industries, in the production of digestive aids, in the pharmaceutical industries, and in the production of biofuel [239]. Amylases are consistently the most important among the enzymes of industrial interest and are forecasted to reach US$ 6.2 billion by 2020 [240]. For this reason, there is noteworthy attention paid to extremophilic α-amylases that have activity and stability characteristics suitable for the harsh conditions, including extreme salinity (2–4 M NaCl) and elevated temperature (80°C), demanded by industrial activities [241].

Interestingly, nitrilases have also been identified in DHABs, and are employed as commercial biocatalysts for the synthesis of plastics, paints, and fibers in the chemical industries and are also employed in the pharmaceutical industries for the manufacturing of (S)-ibuprofen, a widely used non-steroidal anti-inflammatory drug [242]. Moreover, nitrilases can detoxify cyanide present within wastes and degrade herbicides, representing an enzyme of extreme importance in bioremediation [243]. Biotransformation using native organisms as catalysts tends to be insufficient because the amount of nitrilases present as total cellular proteins is very low, and the reaction rate is slow and unstable [244]. Thus, the nitrilase recently identified by metagenomes mining in the Atlantis II DHAB could represent a valuable alternative not only for its thermal stability and tolerance to heavy metals compared to closely related nitrilases but also for the great number of microorganisms which could possess and produce these enzymes [211]. Another example of utilising sequence-based and activity-based metagenomics in mining for potential industrial biocatalysts is the esterase EstATII collected from the Atlantis II basin in the Red Sea, which displays a combination of extremophilic properties [197]. This enzyme is thermophilic (optimum temperature 65 °C) and halotolerant (for up to 4.5M NaCl) and maintains significant activity in the presence of a wide variety of toxic heavy metals, making it a potentially useful biocatalyst [197]. In agreement with this study, O.16 esterase was identified in the Urania basin which shoswed remarkable polyextremophilic properties (i.e., 180× enhanced activity at 2 to 4 M NaCl and functioning at 40 MPa [198]). This enzyme also displayed increased activity when dissolved in 70% ethanol or n-propanol and extraordinarily high enantioselectivity in hydrolysis and transesterification of compounds important in the pharmaceutical, cosmetic, and food industries [198,245]. Thus, DHABs seem to be a suitable habitat for mining esterases which are potentially useful for industrial biotransformation, considering the great size of the lipolytic enzyme market, which is valued at the billion-dollar mark in the world's market [246].

A study conducted on bacterial strains isolated from haloclines of Urania, Bannock, Discovery, and L'Atalante showed that *Bacillus horneckiae* gave highly stereoselective reduction for racemic propyl ester of anti-2- oxotricyclo[2.2.1.0]heptan-7-carboxylic acid (R,S)-1, a key intermediate of the synthesis of D-cloprostenol (chemical analog of prostaglandin [66]). Another isolate of *Halomonas aquamarina* was found to enantioselectively hydrolyze this molecule, indicating the potential of DHAB extremophile microbiome and marine-derived esterases and ketoreductases in stereoselective biocatalysis [66]. The same authors also isolated *Bacillus lehensis* from Discovery DHABs which harness an alkali-tolerant cyclodextrin glycosyltransferase and are able to produce non-toxic products for the pharmaceutical, cosmetic, and food industries.

Overall, DHABs seem to be a suitable habitat for mining novel biocatalyst enzymes which are potentially useful for industrial biotransformations, encouraging further scientific challenges and research for fully realising the potential of DHAB extremozymes.

3.3. DHAB-Derived Prokaryotes: Promising Candidates for Enhanced Bioremediation of Oil Hydrocarbons

Petroleum hydrocarbons are among the most widespread pollutants on our planet and are becoming a severe problem because of their causing harmful damage to the environment and human health [247,248]. Oil pollution can occur in the environment following either catastrophic accidents

(shipping disasters or pipeline failures) or natural oil seepages and biota [249]. Such contaminants can exert carcinogenic, neurotoxic, and mutagenic effects when organisms are exposed to them, significantly impacting the environment [250–252]. For these reasons, many innovative technologies have been developed for the clean-up of oil-polluted areas [247]. One of the most reliable of these is certainly bioremediation, which exploits the metabolic capabilities of microorganisms to break down recalcitrant hydrocarbons into harmless by-products, thus minimising the impact on the environment [253]. It is a more environmentally friendly alternative when compared with classical remediation techniques, which allow the reduction from the environment of a vast array of pollutants [254].

Most petroleum hydrocarbons encountered in the environment can be degraded or metabolized by indigenous bacteria which have developed specific pathways for sustaining their energetic and carbon requirements for living and blooming in the presence of these contaminants [255,256]. Indeed, many studies have focused their attention on hydrocarbon-degrading bacteria in oil-rich environments, including oil spill areas and oil reservoirs [257], and have demonstrated that their abundance is closely related to the respective types of petroleum hydrocarbons and surrounding environmental factors [258–261]. Despite this, many other normal and extreme microorganisms have been isolated and employed as biodegraders for dealing with petroleum hydrocarbons, representing a promising biotechnological alternative for achieving oil-hydrocarbon degradation [9,262]. Because of the particular physiological characteristics of microorganisms isolated from extreme environments, including DHABs, prokaryotes can be employed for enhanced bioremediation of oil hydrocarbons, especially in hypersaline environments [263,264]. For instance, members of the genera *Alcanivorax* and *Marinobacter,* which have been isolated respectively from Erba halocline and Shaban Deep [70,84], are essential marine hydrocarbonoclastic bacteria present in the active phase of oil spills, playing a significant role in the natural remediation of oil-polluted marine environments all over the world [265–267]. Their number increases very quickly after oil spills, although it declines only a few weeks later (see [268] as well as references therein). The outstanding bioremediation capacity of *A. dieselolei* has also been supported by sequencing the genome of the strain KS-293 isolated from surface seawater [269]. Its genome consistently contains multiple genes and enzymes involved in pathways associated with hydrocarbon degradation (linear and branched alkanes) and shows high similarity with *A. dieselolei* strain B5 [270–272]. This strain has been observed to preserve cell integrity under pressures of up to 10 MPa cultured with n-dodecane as a sole carbon source, downregulating 95% of its genes [273,274]. Additionally, Sass et al., 2008 demonstrated that the strain DS-1, closely related to *Bacillus aquimaris*, isolated from the Discovery DHAB, could grow with n-alkanes (n-dodecane and n-hexadecane) in the presence of 12–20% NaCl [275]. Furthermore, *Salinisphaera shabanensis* and *Marinobacter salsuginis* have been isolated from the Shaban Deep, displaying a high capacity to assimilate aliphatic hydrocarbons [70,276]. *S. shabanensis* can be cultured at a wide range of salinity and temperatures (0.2–4.8 M NaCl and 5–42 °C), on a vast array of substrates including n-alkanes (dodecane). *M. salsuginis* is a heterotrophic, facultative anaerobic bacterium capable of fermentation and nitrate reduction [70].

Moreover, other bacteria belonging to the genus *Marinobacteria* and isolated from seawater and Nereus halocline have shown to be efficient for bioremediation purposes for degrading hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs), as revealed by the complete genome sequence of *Marinobacter flavimaris* SW-145 [277–280]. Other strains belonging to the genera *Vibrio*, *Pseudomonas*, *Arthrobacter Pseudoalteromonas*, *Idiomarina*, *Halomonas,* and *Thalassospira* identified in different DHABs have been collected and cultured from marine sediments and are able to grow on PAHs including naphthalene, dibenzothiophene, pyrene, and phenanthrene [21,39,267,281,282].

Even though many oil hydrocarbons can be easily degraded in low salinity marine habitats [267], very little is known about their fate in moderate and in hypersaline environments where microbial activity is enormously inhibited [263,283]. To this purpose, some archaea belonging to the class of *Halobacteria* identified in many DHABs hold great promise and have considerable potential to bioremediate hydrocarbons in high salty environments such as nearshore oil production sites, salt marshes, sabkhas, and other coastal flats, including industrial wastewaters [264,284].

Haloarchaea of the class Halobacteria identified in many DHABs located both in the Mediterranean and the Red Sea [17,44,56,189] can produce PAH-degrading enzymes, which may be exploited to remove aromatic hydrocarbons from the polluted environments safely [285]. In the hypersaline coastal areas of Kuwait, hydrocarbonoclastic haloarchaea, together with *M. flavimaris*, a diazotrophic strain able to grow under 1 M–3.5 M NaCl conditions, have effectively contributed to oil bioremediation [286].

Although more archaeal strains have been isolated, our information on the physiological, biochemical, and genomic basis of hydrocarbon degradation by members of the *Halobacteria* is still extremely scant [263,287]. Such information is crucial for designing novel and more efficient technologies employing haloarchaea for the remediation of contaminated high salinity environments.

4. Conclusions and Future Directions

Polyextremophilic bacteria and archaea are an extraordinary reservoir of novel enzymes and bioactive molecules which can provide important benefits for different biotechnological applications, ranging from medicine to environmental fields. So far, studies on DHABs are limited, and we urgently need to expand data on microbial diversity and ecology of these extreme ecosystems. The uniqueness of these habitats is able to select for highly specialized organisms which show extreme adaptions at morphological, physiological, biochemical, and genetic levels, hinting at a bright future for "blue" biotechnology. In this extensive literature review we have observed that polyextremophiles maintain several high metabolic similarities to other non-extreme prokaryotes. This important issue, which remains to be further investigated, could open new research perspectives on the production of the biomolecules' portfolio of marine microorganisms. Such explorations are expected to provide huge rewards not only in terms of the impact on existing industries for the discovery of new products with beneficial or useful properties, but also in the "blue" economy. This scenario is also perfectly framed within the Sustainable Development Goals of the United Nations, which aims to identify actual solutions for disease outbreaks, climate change, and environmental degradation in order to have safer, cleaner, and more efficient industrial manufacturing processes in order to improve human health and wellbeing from a sustainable development perspective.

Nowadays, "blue" biotechnologies are taking advantage of increasing numbers of "-omics" tools and high-throughput screenings for unveiling the chemical diversity of the extreme environments present in the oceans. These tools are facilitating the identification of prokaryotic metabolic adaptations, which can lead to the production of novel molecules and thus can be exploited for the development of new biotechnologies. To date, novel uncultured species identified in DHABs of the genera *Streptomyces*, *Pseuodalteromonas*, and *Bacillus* seem to hold great potential in producing new bioactive molecules. Among the culturable species identified in DHABs, *Chromohalobacter israelensis, Zunongwangia profunda, Marinobacter flavimaris, Alcanivorax dieselolei, Halomonas meridiana, Alteromonas macleodii,* and *Bacillus halodurans* are promising species for biotechnological applications. Further innovative technologies and studies applied to DHABs will be essential to carry out in-depth investigations and to disentangle microbial assemblages, functions, and metabolites of biotechnological interest from these peculiar systems. Thus, the actual development of DHAB-derived biotechnologies will depend on technological and methodological advancements and the ability of scientists to promote research projects for the study of these ecosystems.

Supplementary Materials: The following information is available online at http://www.mdpi.com/1660-3397/18/2/91/s1, Table S1: Prokaryotic strains isolated from DHABs, Table S2: Prokaryotic strains isolated from DHABs and their closest relatives obtained from phylogenetic analyses.

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A Cold-Adapted Chitinase-Producing Bacterium from Antarctica and Its Potential in Biocontrol of Plant Pathogenic Fungi

Kezhen Liu [1,2], Haitao Ding [2,*], Yong Yu [2] and Bo Chen [2,*]

[1] College of Marine Science, Shanghai Ocean University, Shanghai 201306, China; 18043425667@163.com
[2] MNR Key Laboratory for Polar Science, Polar Research Institute of China, Shanghai 200136, China; yuyong@pric.org.cn
* Correspondence: htding@outlook.com (H.D.); chenbo@pric.org.cn (B.C.); Tel.: +86-21-5871-8663 (H.D.); +86-21-5871-1026 (B.C.)

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Abstract: To obtain chitinase-producing microorganisms with high chitinolytic activity at low temperature, samples collected from Fildes Peninsula in Antarctica were used as sources for bioprospecting of chitinolytic microorganisms. A cold-adapted strain, designated as GWSMS-1, was isolated from marine sediment and further characterized as *Pseudomonas*. To improve the chitinase production, one-factor-at-a-time and orthogonal test approaches were adopted to optimize the medium components and culture conditions. The results showed that the highest chitinolytic activity (6.36 times higher than that before optimization) was obtained with 95.41 U L^{-1} with 15 g L^{-1} of glucose, 1 g L^{-1} of peptone, 15 g L^{-1} of colloid chitin and 0.25 g L^{-1} of magnesium ions contained in the medium, cultivated under pH 7.0 and a temperature of 20 °C. To better understand the application potential of this strain, the enzymatic properties and the antifungal activity of the crude chitinase secreted by the strain were further investigated. The crude enzyme showed the maximum catalytic activity at 35 °C and pH 4.5, and it also exhibited excellent low-temperature activity, which still displayed more than 50% of its maximal activity at 0 °C. Furthermore, the crude chitinase showed significant inhibition of fungi *Verticillium dahlia* CICC 2534 and *Fusarium oxysporum* f. sp. *cucumerinum* CICC 2532, which can cause cotton wilt and cucumber blight, respectively, suggesting that strain GWSMS-1 could be a competitive candidate for biological control in agriculture, especially at low temperature.

Keywords: Antarctica; chitinase; cold-adapted; optimization; antifungal; *Pseudomonas*

1. Introduction

Chitin is a polysaccharide consisting of β-*N*-acetyl-D-glucosamine (GlcNAc) units linked by β-1,4 glycosidic bonds [1]. Chitin is a major resource for the preparation of chitin oligosaccharides, chitosan oligosaccharides and other chitin derivatives, which have tremendous applicable values in the fields of medicine, food, health care and environmental protection [2]. Generally, chitin can be decomposed through physical, chemical or biological approaches [3]. Although physical and chemical methods have been used broadly, both of them have many invincible drawbacks such as low yield, high cost, poor product uniformity and environmental pollution, while a biological method possesses the advantages of mild reaction condition, good yield, high product uniformity and environmental friendliness, especially for the enzymatic method implemented by chitinase [4].

Chitinases, which are capable of hydrolyzing chitin to release GlcNAc and *N*-acetyl chitin oligosaccharides [5], have been found in many organisms, including bacteria [6], fungi [7], plants [8], insects [9] and even humans [10]. Chitinases and chitinase-producing microorganisms have

received considerable attention due to their potential applications in biological control of fungal pathogens [11] and preparation of chitin derivatives [4] in recent years. Although plenty of chitinase-producing microorganisms have been discovered and characterized, such as *Sanguibacter antarcticus* KOPRI 21702 [12], *Basidiobolus ranarum* [13], *Bacillus pumilus* U5 [14], *Chitinolyticbacter meiyuanensis* SYBC-H1 [15], *Paenibacillus* sp. D1 [16], *Serratia Marcescens* XJ-01 [17], *Streptomyces* sp. ANU 6277 [18], *Lysinibacillus fusiformis* B-CM18 [19], *Streptomyces griseorubens* C9 [20], *Streptomyces pratensis* KLSL55 [21], *Humicola grisea* ITCC 10360.16 [22], *Cohnella* sp. A01 [23], *Serratia marcescens* JPP1 [24] and *Stenotrophomonas maltophilia* [25], their chitinolytic activities are still fairly low, especially at low and intermediate temperatures, which leads to the high cost and limited large-scale application of chitinases or chitinase-producing microorganisms. As a rule of thumb, cold-adapted enzymes usually display higher activity than their mesophilic and thermophilic counterparts at the same temperature [26]. Such enzymes can be more easily found in Antarctica, a natural resource pool of cold-adapted microorganisms [27].

To obtain chitinase-producing microorganisms with high chitinolytic activity, samples collected from Fildes Peninsula on King George Island of Antarctica were used as sources for bioprospecting of chitinolytic microorganisms. The production of chitinase of the selected strain was optimized by statistical design. Besides, enzymatic properties and antifungal potential of the extracellular chitinase secreted by the strain were also investigated in this study.

2. Results

2.1. Screening, Isolation and Identification of the Chitinase-Producing Bacterium

Strain GWSMS-1, isolating from marine sediment, produced a clear transparent zone on the colloidal chitin plate (Figure 1a), indicating that it is capable of secreting chitinase to hydrolyze the colloidal chitin around itself. The native-PAGE was conducted to further verify the chitinase activity of the secreted enzyme. As shown in Lane 2 of Figure 1b, a clear band was observed on the gel, implying the presence of chitinase in the crude enzyme secreted by strain GWSMS-1.

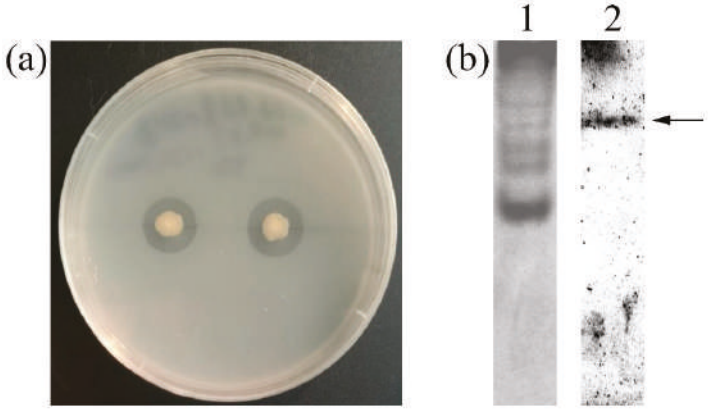

Figure 1. Screening and confirmation of the chitinase-producing bacterium. (**a**) Inoculation of strain GWSMS-1 on colloidal chitin plate. (**b**) Native-PAGE of concentrated crude chitinase secreted by strain GWSMS-1. In lane 1, the gel was stained by Coomassie Brilliant Blue R-250. In lane 2, the gel was stained by Calcofluor White M2R. The proposed chitinase was indicated by an arrow.

Strain GWSMS-1 was classified into genus *Pseudomonas* by molecular identification using 16S-rDNA sequencing. To understand the evolutionary relationship between *Pseudomonas* sp. GWSMS-1 and its phylogenetically related species, a 16S rDNA-based phylogenetic analysis was conducted using a total of thirty-one 16S rDNA gene sequences retrieved from EzBioCloud web server [28]. The 16S

rDNA of strain GWSMS-1 showed the highest similarity (99.79%) with *Pseudomonas guineae* LMG 24016 [29], a psychrotolerant bacterium also isolated from Antarctica (Figure 2). Since these two strains occupied a distinct position in genus *Pseudomonas*, it is suggested that they might experience a similar evolutionary journey to adapt to the extreme environment of Antarctica.

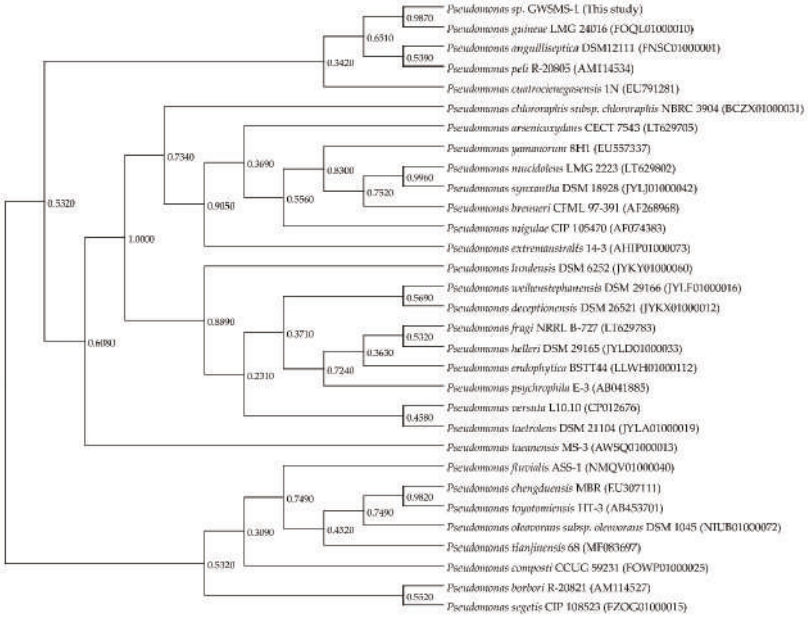

Figure 2. Phylogenetic analysis based on 16S rDNA sequences of *Pseudomonas* sp. GWSMS-1 and its phylogenetically related species. The GenBank accession number is provided following the species name.

2.2. One-Factor-at-a-Time Optimization

The change of chitinolytic activity of *Pseudomonas* sp. GWSMS-1 during the fermentation process was monitored to determine the fermentation time for chitinase production with the highest activity. As shown in Figure 3, the chitinolytic activity could be detected in the fermentation broth after 24 h of cultivation, and it achieved its maximum on the sixth day. It is worth mentioning that the chitinolytic activity (solid circle) increased with the increase in protein concentration (empty circle) in the first six days, but decreased sharply with increased consumption of chitin in later days. It is proposed that the chitinase of strain GWSMS-1 is an inducible enzyme, which could only be produced in the presence of chitin with high enough concentration. Therefore, fermentation broth cultivated for 6 days was used for measuring the chitinolytic activity in further study.

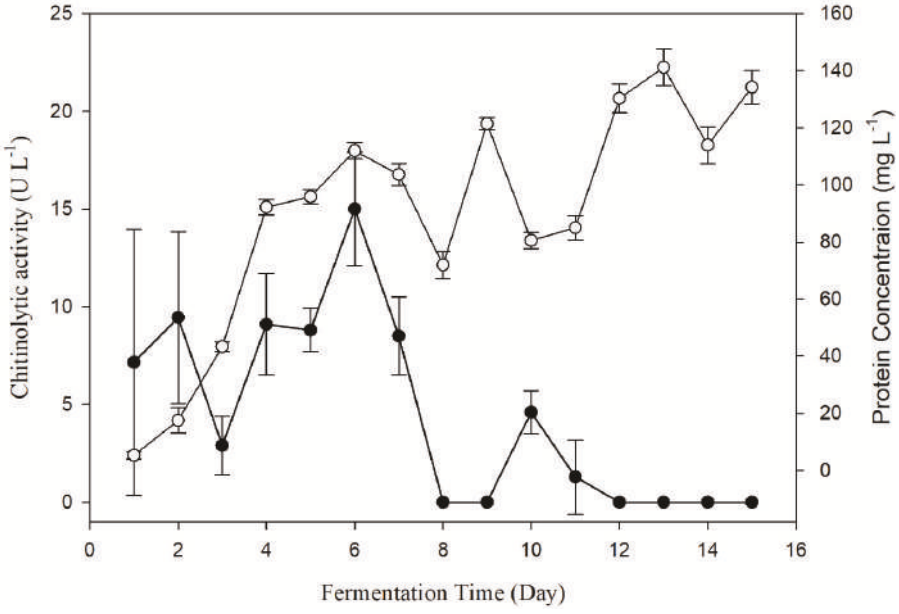

Figure 3. Changes in the chitinolytic activity of *Pseudomonas* sp. GWSMS-1 during the fermentation process. Chitinolytic activity and protein concentration are represented as solid and empty circles, respectively.

The results of carbon source selection showed that the carbon source exerted a significant influence on the chitinolytic activity, which was undetectable when glycerol was used as carbon source, while the strain produced the highest amount of chitinolytic activity when glucose was used as carbon source (Figure 4a) with a concentration of 10 g L^{-1} (Figure 4c). Nitrogen source test results displayed that the organic nitrogen sources had a better effect on chitinolytic activity than those of inorganic nitrogen sources (Figure 4b). The highest chitinolytic activity was determined from the fermentation broth when peptone was employed as the nitrogen source with a concentration of 2 g L^{-1} (Figure 4d). In addition, different chitin concentrations also affected the production of chitinase by *Pseudomonas* sp. GWSMS-1, and the highest apparent yield of the enzyme was observed when the chitin concentration was 10 g L^{-1} (Figure 4e). The fermentation condition optimization showed that the optimum temperature, pH and shaking speed for the production of chitinolytic enzymes were determined as 20 °C (Figure 4f), 7.0 (Figure 4g) and 100–150 rpm (Figure 4h), respectively.

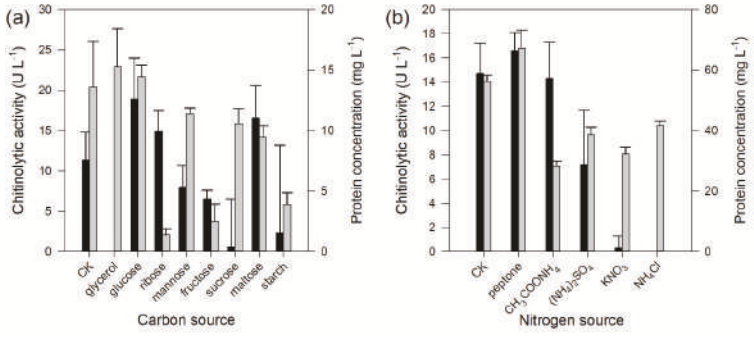

Figure 4. *Cont.*

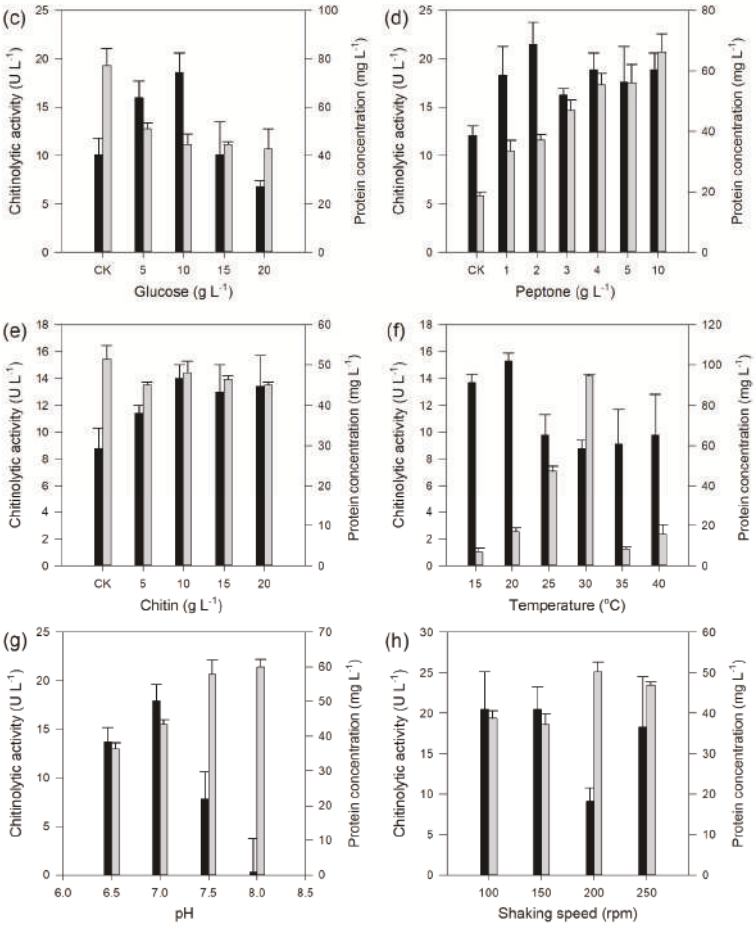

Figure 4. Chitinase production of *Pseudomonas* sp. GWSMS-1 optimized by the one-factor-at-a-time method. Effects of (**a**) carbon source, (**b**) nitrogen source, (**c**) glucose concentration, (**d**) peptone concentration, (**e**) chitin concentration, (**f**) temperature, (**g**) pH and (**h**) shaking speed on the chitinase production. Chitinolytic activity and protein concentration are represented as black and grey bars, respectively.

2.3. Orthogonal Design

With the aim of obtaining more chitinase secreted by strain GWSMS-1, the medium components were further optimized by orthogonal design. The results showed that the apparent highest chitinolytic activity of 72.16 U L^{-1} was obtained with the seventh combination (Table 1). Further analysis of the data implied that the desired highest activity would be achieved when the concentrations of glucose, peptone, colloid chitin and magnesium ions are 15 g L^{-1}, 1 g L^{-1}, 15 g L^{-1} and 1 mM, respectively. Subsequently, an additional experiment was performed to verify this combination, which was not included in the orthogonal test. Finally, the chitinolytic activity was determined as 95.41 U L^{-1} with the above combination, which was higher than the apparent highest activity (72.16 U L^{-1}) observed in the orthogonal test. In variance analysis, $F_{0.01} = 6.23$ is used as a reference value, and $F > 6.23$ means a significant effect of the factor. According to the variance analysis of the orthogonal test showed in

Table 2, all these four factors involved in the optimization showed significant effects on the yield of chitinase at the $p = 0.01$ level, and peptone and chitin were the most significant factors.

Table 1. Orthogonal design and the responding chitinolytic activity.

No.	(A) Glucose (g L^{-1})	(B) Peptone (g L^{-1})	(C) Chitin (g L^{-1})	(D) Mg^{2+} (mM)	Chitinolytic Activity (U L^{-1})
1	5	1	5	1	52.25 ± 3.73
2	5	2	10	5	16.17 ± 2.16
3	5	3	15	10	22.64 ± 6.26
4	10	1	10	10	18.66 ± 3.73
5	10	2	15	1	51.01 ± 2.15
6	10	3	5	5	7.46 ± 6.47
7	15	1	15	5	72.16 ± 7.77
8	15	2	5	10	37.32 ± 3.73
9	15	3	10	1	6.22 ± 2.16
K1	91.07	143.08	97.04	109.49	
K2	77.14	104.51	41.06	95.80	
K3	115.71	36.33	145.81	78.63	
k1	30.36	47.69	32.35	36.50	
k2	25.71	34.84	13.69	31.93	
k3	38.57	12.11	48.60	26.21	
Range	12.86	35.58	34.91	10.29	
Factor order	B > C > A > D				
Optimization combination	A3	B1	C3	D1	

Table 2. Analysis of Variance (ANOVA).

Source	Sum of Square	Degrees of Freedom	Mean Square	F-value	*p*-value
Glucose	762.85	2	381.42	17.42	<0.01
Peptone	5843.69	2	2921.84	133.43	<0.01
Chitin	5495.72	2	2747.86	125.49	<0.01
Mg^{2+}	478.03	2	239.02	10.92	<0.01
Error	394.16	18	21.90		
Total	39843.59	27			

Therefore, the final medium for chitinase production of *Pseudomonas* sp. GWSMS-1 was determined as follows (L^{-1}): glucose 15 g, peptone 1 g, colloidal chitin 15 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, KH_2PO_4 0.3 g, $K_2HPO_4 \cdot 3H_2O$ 1 g.

2.4. Temperature and pH-Dependent Enzymatic Properties of Chitinase

The crude chitinase showed chitinolytic activity in a wide temperature range with the maximum catalytic activity at 35 °C. Furthermore, the enzyme also exhibited excellent low-temperature activity, which still displayed more than 50% of its maximal activity at 0 °C (Figure 5a). A generally accepted hypothesis is that high low-temperature activity of cold-adapted enzymes evolved to facilitate binding and conversion of the substrate at low temperatures, which is consistently accompanied by weak thermal stability on account of the intrinsic structural flexibility of the enzymes, which is supposed to be a result of evolutionary pressure [30]. The crude enzyme was only stable at low temperature and was rapidly inactivated with increasing temperature (Figure 5b). The crude chitinase had a high chitinolytic activity between pH 4.0–5.0, with an optimum catalytic activity at pH 4.5 (Figure 5c), indicating that the chitinase might be an acidic enzyme. The pH stability of the crude chitinase exhibited a similar pattern to that of the activity response to pH, which was stable in the pH range of 4.0 to 5.0 and rapidly deactivated under other pH values (Figure 5d).

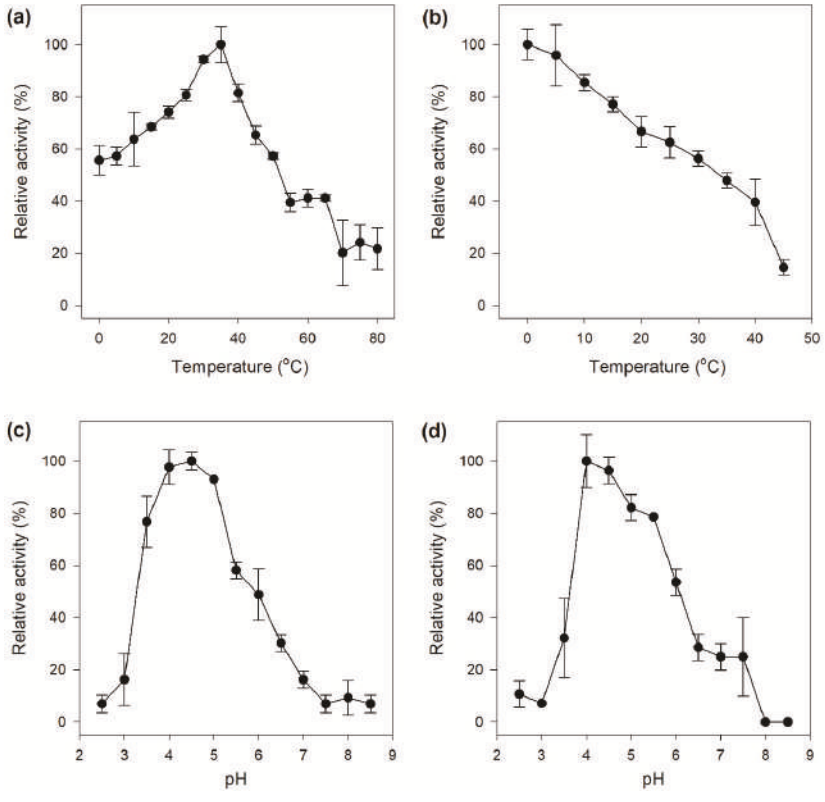

Figure 5. Enzymatic properties of the crude chitinase. (**a**) Optimal temperature; (**b**) temperature stability; (**c**) optimal pH; (**d**) pH stability.

2.5. Antifungal Activity

As a key component of fungal cell wall, chitin is essential for fungal pathogens to maintain their cell structure integrity. Considering that chitinase is capable of degrading chitin to decompose the fungal cell wall, it is indispensable to evaluate the antifungal potential of strain GWSMS-1. However, since strain GWSMS-1 is not one of GRAS (generally regarded as safe) strains, it cannot be applied to the medical field without any safety tests, whereas the requirement is less stringent for agricultural application. Therefore, five common phytopathogenic fungi were selected to evaluate the potential application in biocontrol of strain GWSMS-1. As shown in Figure 6, the crude enzymes significantly inhibited the phytopathogenic fungi *Verticillium dahlia* CICC 2534 and *Fusarium oxysporum* f. sp. *cucumerinum* CICC 2532 and slightly inhibited *Aspergillus niger* CICC 2039 and *Penicillium macrosclerotiorum* CICC 40649, even after incubation for 7 days, while not showing any inhibition toward *Alternaria brassicicola* CICC 2646 during the entire incubation.

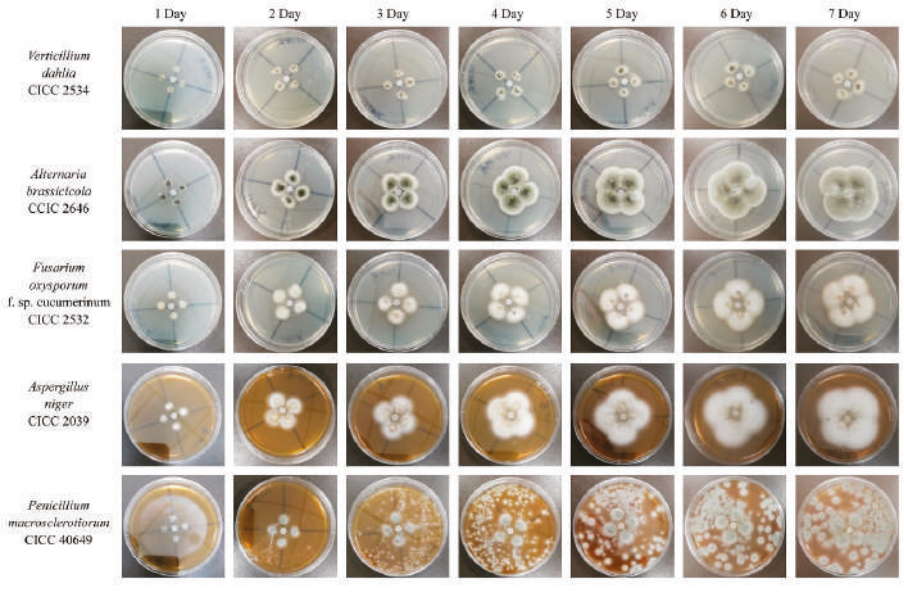

Figure 6. Antifungal activity of the chitinase secreted by *Pseudomonas* sp. GWSMS-1.

3. Discussion

In this study, a chitinase-producing strain GWSMS-1 was isolated from marine sediment near the China's Great Wall Station in Antarctica and characterized as a member of genus *Pseudomonas*. Statistical optimization of the chitinase production, the enzymatic properties and the antifungal activity of the chitinase was conducted for better evaluating the application potential of this strain.

Enzyme production is one of the most important limitations for the large-scale application of enzymes, which significantly affects the usage cost. Generally, the yield of an enzyme is optimized from two aspects: medium indigents and culture conditions. In this study, the optimum culture conditions, including temperature, pH and shaking speed, were determined as 20 °C, 7.0 and 150 rpm, respectively, which shared similar conditions except for temperature with other strains reported previously (Table 3). It is obvious that the optimum temperatures for the secretion of chitinase by different strains are associated with their optimum growth temperature. Dissolved oxygen level, represented by shaking speed, has little effect on chitinase production among different strains. Another noticeable difference in culture conditions among different chitinase-producing strains is the fermentation time, which ranged from 1 to 8 days (Table 3). Comparing with mesophilic microorganisms, *Pseudomonas* sp. GWSMS-1 has a relatively long fermentation time to achieve its maximum yield of chitinase as a cold-adapted microorganism. However, some mesophilic and thermophilic strains, such as *Streptomyces griseorubens* C9 [20], *Bacillus pumilus* U5 [14] and *Humicola grisea* ITCC 10360.16 [22], also showed similar fermentation periods to GWSMS-1, which might be due to their intrinsic regulation of metabolism. In the further study, strain GWSMS-1 could be genetically modified by metabolic engineering to reduce the fermentation time in order to make the fermentation process economical.

Table 3. Summary of the optimized liquid fermentation conditions of chitinase-producing microorganisms.

Strains	Source	Method	Component [a] (g L^{-1})	Condition	Yield (Final/ Initial)
Pseudomonas sp. GWSMS-1 (this study)	Sediments, Antarctic	OFAT OD	Colloidal chitin: 15.0 Glucose: 15.0 Peptone: 1.0 $MgSO_4{\cdot}7H_2O$: 0.25 KH_2PO_4: 0.3 $K_2HPO_4{\cdot}3H_2O$: 1.0	Temperature: 20 °C pH: 7.0 Rotary speed: 150 rpm Time: 6 days	6.36
Sanguibacter antarcticus KOPRI 21702 [12]	Sea sand, Antarctic	OFAT PBD RSM	Chitin: 2.0 Glycerol: 10.0 Peptone: 5.0 Yeast extract: 1.0 $Fe(C_6H_5O_7)$: 0.01 NaCl: 23.0 $MgCl_2$: 2.5 Na_2SO_4: 3.24 $CaCl_2$: 1.8 $NaHCO_3$: 0.16	Temperature: 25 °C pH: 6.5 DO: 30% Time: 3 days	7.5
Basidiobolus ranarum [13]	Frog excrement	RSM	Colloidal chitin: 15 Lactose: 1.25 Malt extract: 0.25 Peptone: 0.75	Temperature: 25 °C pH: 9.0 Rotary speed: 200 rpm Time: 5 days	7.71
Bacillus pumilus U5 [14]	Soil, Iran	PBD RSM	Chitin: 4.760 Yeast extract: 0.439 $MgSO_4{\cdot}7H_2O$: 0.0055 $FeSO_4{\cdot}7H_2O$: 0.019	Temperature: 30 °C pH: 6.5 Rotary speed: 150 rpm Time: 8 days	1.20
Chitinolyticbacter meiyuanensis SYBC-H1 [15]	Soil, China	PBD RSM	Chitin: 3.8 Inulin: 3.55 Urea: 3.1 $(NH_4)_2SO_4$: 0.64 $MgSO_4{\cdot}7H_2O$: 0.5 $FeSO_4{\cdot}7H_2O$: 0.02 KH_2PO_4: 0.7 K_2HPO_4: 0.3	Temperature: 30 °C pH: 7.0 Rotary speed: 200 rpmTime: 4 days	15.5
Paenibacillus sp. D1 [16]	Effluent, India	PBD RSM	Chitin: 3.75 Yeast extract: 0.6 5Urea: 0.33 $MgSO_4$: 0.30 K_2HPO_4: 1.17	Temperature: 30 °C pH: 7.2 Rotary speed: 180 rpm Time: 3 days	2.56

Table 3. *Cont.*

Strains	Source	Method	Component [a] (g L^{-1})	Condition	Yield (Final/ Initial)
Serratia Marcescens XJ-01 [17]	Fishing field, China	OFA TOD	Colloidal chitin: 7.5 $(NH_4)_2SO_4$: 5 $MgSO_4{\cdot}7H_2O$: 0.5 KH_2PO_4: 2.4 $K_2HPO_4{\cdot}3H_2O$: 0.6	Temperature: 32 °C pH: 8.0 Rotary speed: 180 rpm Time: 32 h	N.M. [c]
Streptomyces sp. ANU 6277 [18]	Soil, India	OFAT	Colloidal chitin: 10.0 Starch: 2.0 Yeast extract: 4.0 KH_2PO_4: 2 $MgSO_4{\cdot}7H_2O$: 1 $FeSO_4{\cdot}7H_2O$: 0.1	Temperature: 35 °C pH: 6.0 Time: 2.5 days	N.M.
Lysinibacillus fusiformis B-CM18 [19]	Chickpea rhizosphere	OFAT RSM	Colloidal chitin: 5.50 Starch: 0.55 Yeast extract: 0.55 NaCl: 4.5 NH_4Cl: 1.0 $CaCl_2$: 0.1 $MgSO_4$: 0.12 KH_2PO_4: 3.0 Na_2HPO_4: 6.0	Temperature: 32.5 °C pH: 7.0 Rotary speed: 150 rpm Time: 2–5 days	56.1
Streptomyces griseorubens C9 [20]	Soil, Algeria	PBD RSM	Colloidal chitin: 20.0 Yeast extract: 0.25 Data syrup: 4.7 K_2HPO_4/KH_2PO_4: 1.81	Temperature: 40 °C pH: natural Rotary speed: 150 rpm Time: 7 days	26.38
Streptomyces pratensis KLSL55 [21]	Soil, India	OFAT	Colloidal chitin: 15 Fructose: 12.5 KNO_3: 5 Mn^{2+}: 0.5	Temperature: 40 °C pH: 8.0 Rotary speed: 160 rpm Time: 2 days	14.3
Humicola grisea ITCC 10360.16 [22]	Desert soil, India	PBD RSM	Chitin: 7.49 Colloidal chitin: 4.91 Yeast extract: 5.5 KCl: 0.19 NH_4Cl: 1.0 $MgSO_4{\cdot}7H_2O$: 0.2 KH_2PO_4: 0.68 K_2HPO_4: 0.87	Temperature: 45 °C pH: 6.5 Rotary speed: 150 rpm Time: 8 days	1.43

Table 3. *Cont.*

Strains	Source	Method	Component [a] (g L^{-1})	Condition	Yield (Final/ Initial)
Cohnella sp. A01 [23]	Wastewater, Iran	OFAT OD	Colloidal Chitin: 15 NH_4NO_3: 5 KH_2PO_4: 0.7 NaCl: 1.7	Temperature: 60 °C pH: 6.5 Rotary speed: 180 rpm Time: 3 days	N.M.
Serratia marcescens JPP1 [24]	Peanut hulls, China	PBD RSM	Colloidal chitin: 12.7 Glucose: 7.34 Peptone: 5.0 $(NH_4)_2SO_4$: 1.32 $MgSO_4{\cdot}7H_2O$: 0.5 K_2HPO_4: 0.7	N.M.	2.1
Stenotrophomonas maltophilia [25]	Soil, India	PBD RSM	Colloidal chitin: 4.94 Maltose: 5.56 Yeast extract: 0.62 KH_2PO_4: 1.33 $MgSO_4{\cdot}7H_2O$: 0.65	N.M.	N.M.

OFAT, one-factor-at-a-time; OD, orthogonal design; PBD, Plackeet–Burmann Design; RSM, response surface methodology; N.M., not mentioned in the corresponding study. [a] The trace elements added into the medium were omitted.

Since only a few strains, such as *Thermococcus chitonophagus* [31], *Microbispora* sp. V2 [32] and *Metarrhizium anisopliae* [33], can utilize chitin as the sole carbon source, most of the chitinolytic microorganisms cannot produce chitinolytic enzymes with chitin as the sole carbon source. Therefore, it is necessary to add additional carbon sources that are feasible to utilize by these strains through co-metabolism (Table 3). The additional carbon source mainly provides energy for cell growth and proliferation as the primary matrix, while chitin is decomposed and utilized as the secondary matrix. In general, glucose is the best carbon source for enzyme production, and strain GWSMS-1 is no exception. However, other small molecules such as lactose and glycerol showed a better effect on chitinase secretion than glucose in some cases (Table 3). Unlike the variety of carbon source preferences of different strains, almost all the studies showed that the organic nitrogen source is better for enzyme production than the inorganic nitrogen source (Table 3), which inhibited the synthesis of chitinase during fermentation; organic nitrogen was better for strain GWSMS-1 as well.

The potential application in biocontrol of fungi of the cold-active chitinase secreted by strain GWSMS-1 was evaluated using five common plant pathogens. The crude chitinase showed significant inhibition on fungi *Verticillium dahlia* CICC 2534 and *Fusarium oxysporum* f. sp. *cucumerinum* CICC 2532 which can cause cotton wilt and cucumber blight, respectively, suggesting that strain GWSMS-1 would be a competitive candidate for the biological control in agriculture.

4. Materials and Methods

4.1. Chemicals, Agents and Media

Chitin, potassium ferricyanide and *N*-acetyl-D-glucosamine (NAG) were available from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals of analytical grade were purchased from Sangon Biotech (Shanghai, China).

Colloidal chitin was prepared according to Souza et al. [34] as follows: five grams of chitin powder was added to 60 mL of concentrated HCl slowly and incubated overnight, with vigorous stirring at room temperature. The mixture was added to 200 mL precooling ethanol and incubated at room temperature overnight with vigorous stirring. The precipitate was harvested by centrifugation at 5000 *g* for 20 min at 4 °C. The colloidal chitin was washed with sterile distilled water to neutral and stored in the dark at 4 °C.

Potassium ferrocyanide solution was prepared by dissolving 0.5 g potassium ferrocyanide in 1 liter of 0.5 M Na_2CO_3 buffer and stored in a dark environment.

The initial liquid medium consisted of peptone (2 g L^{-1}), glucose (1 g L^{-1}), colloid chitin (5 g L^{-1}), $FeSO_4 \cdot 7H_2O$ (0.01 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}), KH_2PO_4 (0.3 g L^{-1}) and $K_2HPO_4 \cdot 3H_2O$ (0.917 g L^{-1}), while the solid medium contained agar at a concentration of 1.5%.

4.2. Screening and Characterization of Chitinase-Producing Microorganisms

Samples of seal and penguin feces, soil and marine sediment, collected from Fildes Peninsula (60°20′S–60°56′S, 44°05′W–46°25′W) in Antarctica, were used as sources for bioprospecting of chitinase-producing microorganisms. Strains which formed transparent zones on the colloidal chitin plate at 15 °C were selected for further study. The isolates were characterized by 16S rDNA sequencing. The 16S rDNA gene was amplified using genomic DNA as templates, with universal primer pairs 27F (5′-AGAGTTTGATCMTGGCTCAG-3′ (27F) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′).

The PCR products were ligated with the pMD19-T vector and transformed into *E. coli* DH5α competent cells for sequencing. The nucleotide sequence of the 16S rDNA gene was subject to BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) to find homologous sequences. Multiple sequence alignment was conducted using the software Clustal X 2.0 [35]. The phylogenetic tree was constructed using the neighbor-joining method [36] in MEGA 6.0 [37], with a bootstrap test of 1000 replicates.

4.3. Preparation of Crude Chitinase

The fermentation broth was centrifuged at 8000× *g* for 10 min, and the supernatant was concentrated by using a 10 kDa ultrafiltration centrifuge tube. The eluate was then filtered through a 0.22 μm filter and stored at −20 °C for further experiments.

4.4. Native-PAGE and Active Staining of Chitinase

To obtain enough crude chitinase for native-PAGE, the crude chitinase was further concentrated as follows: the crude enzyme was mixed with appropriate amount of colloidal chitin and incubated at 4 °C for 2 h; the mixture was washed twice with 50 mM of Tris-HCl (pH 8.0), then the concentrated chitinase was eluted by using 50 mM of acetate buffer (pH 4.0) and dialyzed by using 50 mM of Tris-HCl (pH 8.0). The native-PAGE was performed using 4% stacking gel and 12% separating gel with 0.5% colloidal chitin added. The gel was stained by Coomassie Brilliant Blue R-250 and Calcofluor White M2R [38] to verify the chitinase activity of the crude enzyme.

4.5. Chitinase Activity Assay

Chitinase activity was determined by measuring the amount of NAG generated from colloidal chitin using a potassium ferrocyanide solution, according to Taiji et al. [39]. An appropriate amount of crude extracellular chitinase secreted by strain GWSMS-1 was mixed with colloidal chitin (1%, *m*/*v*) suspended in 50 mM phosphate buffer at pH 6.0. The mixture of enzyme and substrate was incubated at 30 °C for 2 h, then treated at 100 °C for 5 min to inactivate the enzyme. Subsequently, the reaction solution was centrifugated at 10,000× *g* for 5 min to remove the precipitate, and 0.05 mL of supernatant was mixed with 1.45 mL potassium ferrocyanide solution. The absorbance of the mixture at 420 nm was measured after treating at 100 °C for 15 min and cooling to room temperature. The NAG concentration was calculated based on the standard curve obtained under the same condition. One unit of chitinase activity was defined as the amount of enzyme required to produce 1 μmol of NAG per minute at 30 °C in a phosphate buffer at pH 6.0.

4.6. One-Factor-at-a-Time Optimization

To obtain maximum extracellular chitinase secreted by strain GWSMS-1, the one-factor-at-a-time method was adopted to optimize the medium composition including carbon and nitrogen sources, carbon, nitrogen and chitin concentration, as well as the culture conditions including fermentation time, temperature, pH and shaking speed (Table 4). All experiments were performed in triplicate.

Table 4. Factors and variables of one-factor-at-a-time optimization.

Factors	Variables
Time (days)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15
Carbon source	glycerol, glucose, ribose, mannose, fructose, sucrose, maltose, starch
Nitrogen source	peptone, CH_3COONH_4, $(NH_4)_2SO_4$, KNO_3, NH_4Cl
Glucose (g L^{-1})	5, 10, 15, 20
Peptone (g L^{-1})	1, 2, 3, 4, 5, 10
Chitin (g L^{-1})	5, 10, 15, 20
Temperature (°C)	15, 20, 25, 30, 35, 40
pH	5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0
Shaking speed (rpm)	100, 150, 200, 250

4.7. Orthogonal Design

Based on the results of the single factor test, the medium composition was further optimized by orthogonal design. The orthogonal test employed a four-factor and three-level orthogonal table L_9 (3^4) to optimize concentrations of glucose, peptone, chitin and magnesium ions (Table 5). All experiments were performed with three replicates.

Table 5. Levels of orthogonal design.

Levels	Glucose (g L^{-1})	Peptone (g L^{-1})	Chitin (g L^{-1})	Mg^{2+} (mM)
1	5	1	5	1
2	10	2	10	5
3	15	3	15	10

4.8. Temperature and pH-Dependent Enzymatic Properties of Crude Chitinase

Generally, the activity and stability of enzymes can be determined by pH denaturation and thermal denaturation [40]. The optimal temperature of the crude chitinase was determined by assaying the activity at different temperatures ranging from 0 to 80 °C with 5 °C intervals at pH 6.0. The thermal stability was determined by measuring the residual activity after treating the crude enzyme at different temperatures from 0 to 45 °C with 5 °C intervals at pH 6.0 for 30 min. The optimum pH for the crude chitinase was determined by measuring the activity in acetate buffer (pH 2.5–3.5), citric acid buffer (pH 4.0–5.5), phosphate buffer (pH 6.0–7.5), Tris-HCl buffer (pH 8.0–9.0) and glycine–NaOH buffer (pH 9.5–11.0) at 30 °C. The pH stability was assayed by measuring the residual activity after incubating the crude enzyme in the buffers mentioned above at 30 °C for 30 min.

4.9. Antifungal Activity Assay

The antifungal activity of the extracellular chitinase secreted by strain GWSMS-1 was investigated by hyphal extension inhibition. Hyphal extension inhibition assay was estimated by the paper disk method. Filter papers with a 6 mm diameter were immersed in the concentrated crude enzyme solution for 5 min. A piece of soaked filter paper was placed at the center of the petri dishes containing potato dextrose agar (PDA). The mycelium of the test fungi was inoculated around the filter paper and incubated at 20 °C for 7 days for the mycelia to grow. The heat-inactivated crude enzyme was used as a control. Fungi used in this study were purchased from China Center of Industrial Culture Collection (CICC) (Beijing China), including *Verticillium dahlia* CICC 2534, *Alternaria brassicicola* CICC 2646, *Fusarium oxysporum* f. sp. *cucumerinum* CICC 2532, *Aspergillus niger* CICC 2039 and *Penicillium macrosclerotiorum* CICC 40649.

5. Conclusions

In this study, a cold-adapted chitinase-producing strain GWSMS-1 was isolated from marine sediment and characterized as *Pseudomonas*. Strategy coupling of the one-factor-at-a-time and the orthogonal test was employed to optimize the chitinase production of the strain. The optimized production was about 6.36 times higher than that before optimization. Based on the biochemical characterization, the crude chitinase was determined as a typical cold-active enzyme, which exhibited excellent low-temperature activity at 0 °C. In addition, it also showed significant inhibition of two plant pathogens, suggesting that strain GWSMS-1 would be a competitive candidate for the biological control in agriculture, especially in high latitudes.

Author Contributions: H.D. and B.C. conceived and designed the experiments; K.L. performed the experiments; H.D., K.L., and Y.Y. analyzed the data; H.D., Y.Y. and B.C. contributed reagents/materials/analysis tools; H.D. and K.L. wrote the paper.

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Review

Properties and Applications of Extremozymes from Deep-Sea Extremophilic Microorganisms: A Mini Review

Min Jin [1,2], Yingbao Gai [1], Xun Guo [1], Yanping Hou [1] and Runying Zeng [1,2,*]

[1] State Key Laboratory Breeding Base of Marine Genetic Resource, Third Institute of Oceanography, Ministry of Natural Resources, Xiamen 361000, China; jinmin@tio.org.cn (M.J.); Gaiyingbao@tio.org.cn (Y.G.); guoxun0528@163.com (X.G.); Houyanping@tio.org.cn (Y.H.)

[2] Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Zhuhai 519000, China

* Correspondence: zeng@tio.org.cn; Tel.: +86-592-2195323

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Abstract: The deep sea, which is defined as sea water below a depth of 1000 m, is one of the largest biomes on the Earth, and is recognised as an extreme environment due to its range of challenging physical parameters, such as pressure, salinity, temperature, chemicals and metals (such as hydrogen sulphide, copper and arsenic). For surviving in such extreme conditions, deep-sea extremophilic microorganisms employ a variety of adaptive strategies, such as the production of extremozymes, which exhibit outstanding thermal or cold adaptability, salt tolerance and/or pressure tolerance. Owing to their great stability, deep-sea extremozymes have numerous potential applications in a wide range of industries, such as the agricultural, food, chemical, pharmaceutical and biotechnological sectors. This enormous economic potential combined with recent advances in sampling and molecular and omics technologies has led to the emergence of research regarding deep-sea extremozymes and their primary applications in recent decades. In the present review, we introduced recent advances in research regarding deep-sea extremophiles and the enzymes they produce and discussed their potential industrial applications, with special emphasis on thermophilic, psychrophilic, halophilic and piezophilic enzymes.

Keywords: deep sea; extremophilic microorganisms; extremozyme; thermophilic enzyme; psychrophilic enzyme; halophilic enzyme; piezophilic enzyme

1. Deep-Sea Extremophilic Microorganisms: A Novel Source of Extremozymes

Nearly three-quarters of the Earth's surface area is covered by ocean, the average depth of which is 3800 m, implying that the vast majority of our planet comprises deep-sea environments. The deep sea is one of the most mysterious and unexplored environments on the Earth, and it supports diverse microbial communities that play important roles in biogeochemical cycles [1]. The deep sea is also recognised as an extreme environment, as it is characterised by the absence of sunlight and the presence of predominantly low temperatures and high hydrostatic pressures, and these environmental conditions become even more challenging in particular habitats, such as deep-sea hydrothermal vents with their extremely high temperatures of >400 °C, deep hypersaline anoxic basins (DHABs) with their extremely high salinities and abysses of up to 11 km depth with their extremely high pressures.

Deep-sea extremophiles are living organisms that can survive and proliferate in deep-sea environments that have extreme physical (pressure and temperature) and geochemical (pH, salinity and redox potential) conditions that are lethal to other organisms. The majority of deep-sea extremophiles belong to the prokaryotes, which are microorganisms in the domains of Archaea and Bacteria [2,3]. These extremophilic microorganisms are functionally diverse and widely distributed in taxonomy [4],

and they are classified into thermophiles (55 °C to 121 °C), psychrophiles (−2 °C to 20 °C), halophiles (2–5 M NaCl or KCl), piezophiles (>500 atmospheres), alkalophiles (pH > 8), acidophiles (pH < 4) and metalophiles (high concentrations of metals, e.g., copper, zinc, cadmium and arsenic) according to the extreme environments in which they grow and the extreme conditions they can tolerate. Many deep-sea extremophiles tolerate more than one extreme condition, and thus are polyextremophiles [5]. These extreme conditions are generally harmful to the majority of organisms, but extremophilic microorganisms are able to survive and thrive in them due to their highly flexible metabolisms and the unique structural characteristics of their biomacromolecules [6,7].

In the past few decades, deep-sea extremophilic microorganisms have attracted the attention of researchers searching for novel bioactive substances such as enzymes that can be used in the major sectors of industry worldwide [8]. The diverse temperatures, salinities, pHs and pressures that are provided by nature in extreme deep-sea environments can be utilised to search for novel and potentially robust enzymes that are more suitable for industrial applications [9], and it has been found that the extremozymes that are produced by deep-sea extremophilic microorganisms have a wide variety of industrial applications due to their high activities and great stabilities under extreme conditions. Indeed, the stability and enzymatic activities of extremozymes make them valuable alternatives to ordinary biotechnological processes, bestowing them with considerable economic potential in the agricultural, feed, food, beverage, pharmaceutical, detergent, leather, textile, pulp and biomining industries [10].

Although a lot of enzymes have been identified to date worldwide, the majority of which have been evaluated for industrial applications, the enzyme market remains inadequate in meeting industrial demands [11] largely due to many of the enzymes that are presently available being unable to tolerate industrial conditions [12]. The industrial process demands biocatalysts that can resist a range of harsh conditions, including temperature, pH, salinity and pressure, while exhibiting high conversion rates and reproducibilities [13]. Furthermore, it is important that the enzymes that are used in technologies are compatible with ecological processes [14]. While only a few extremozymes are presently being produced and used at the industrial level, the development of novel industrial processes based on these enzymes is being promoted by advances in deep-sea extremophile and extremozyme research, the growing demand for novel biocatalysts in industries, breakthroughs in deep-sea sampling techniques and the rapid development of new molecular and omics technologies, such as metagenomics, proteomics, protein engineering, gene-directed evolution and synthetic biology [15]. Thus, the discovery of enzymes with novel enzymatic activities and improved stability remains a priority in enzyme research [10].

2. Strategies for Discovering Extremozymes in Deep-Sea Environments

The classic method that is used to discover novel extremozymes from deep-sea microorganisms is the cultivation of microorganisms followed by screening for the desired enzymes. However, while numerous extremozymes with promising properties for industrial applications have been isolated from deep-sea environments using this method, approximately 99.9% of those environmental microorganisms cannot be cultivated using traditional laboratory techniques [16], meaning that the discovery of many useful extremozymes would not be possible using this method alone. Metagenomic technologies have been developed to bypass the requirement for the isolation or cultivation of microorganisms, and they could prove to be a powerful tool for discovering novel genes and enzymes directly from uncultured microorganisms [17]; indeed, metagenomes have been successfully employed to search for extremozymes from deep-sea environments, overcoming the bottlenecks associated with the uncultivability of extremophiles [18].

Metagenomic analyses are based on the direct isolation of genomic DNA from environmental samples, and they are either sequence based (i.e., putative enzymes are obtained based on their conserved sequences) or function based (i.e., functional enzymes are obtained based on the expressed features such as a specific enzyme activity) [19]. In the sequence-based approach, the colony hybridisation technique is used for screening metagenomic clones using an oligonucleotide primer or probes for the target gene, and the desired gene may also be amplified by polymerase chain reaction

(PCR) using specific or degenerate primers and subsequently cloned into suitable expression vectors. Besides, the desired gene sequences can sometimes be directly retrived from metagenomics data after proper bioinformatic annotations, and then be synthesized de novo and codon-optimized if required. This sequence-based technique leads to the discovery of novel sequences that are similar to existing known sequences, and this provides the possibility of finding enzymes efficiently [20]. However, the ability to identify specific enzymes using this method depends on existing bioinformatic analyses, thus many novel or unknown activities can be overlooked [21].

The common function-based metagenomic strategies include enzyme activity-based screening performed in culture plates; for example, the use of the starch-iodine staining test for detecting amylase activity. Functional metagenomics has some advantages over the sequence-based approach because the identification of genes according to their functions rather than their sequences eliminates the possibility of incorrect annotations or obtaining similar sequences of gene products with different or multiple functions. Furthermore, functional screening is more suitable for identifying novel genes encoding novel enzymes because it does not rely on gene sequence information [22]. The primary disadvantage of this screening method is that gene expression may fail due to difficulties in promoter recognition, low translation efficiency, lack of specific cofactors in certain expression hosts, protein misfolding and post-translational modification defects of the desired proteins. However, all these issues can be solved using vectors with a wide host range that enables expression in a variety of hosts, vectors that are adapted to a large insert size and Rosetta strains of *Escherichia coli*, which contains transfer ribonucleic acid (tRNA) for rare amino acid codons [17,23]. Consequently, the function-based metagenomic approach is now the most frequently used technique for screening for novel extremozymes from the deep sea [24–27].

The application of enzymes in industrial processes sometimes fails due to the presence of undesirable properties and a lack of stability and robustness [28]. However, molecular approaches can be used to engineer natural proteins and develop more effective extremozymes with enhanced stabilities and activities for industrial purposes. The enhancement of the stability of enzymes can prove to be very beneficial because it would enable them to maintain high activity for prolonged periods of time under challenging physicochemical conditions, which would be a useful characteristic for numerous industrial processes. One method that can be used to stabilise proteins is protein engineering [29,30], which has become a powerful approach for altering or improving enzymatic characteristics in the past two decades. Protein engineering is divided into the following two methods: (1) directed evolution, where a random mutagenesis is applied to a protein [31]; and (2) rational protein design, where knowledge of the structure and function of the protein is exploited to modify its characteristics [32]. Both these methods have been successfully applied to increase the activity, selectivity and thermostability of proteins.

3. Properties and Applications of Extremozymes Isolated from Deep-Sea Extremophilic Microorganisms

Hundreds of industrial processes and products benefit from the use of enzymes that have been isolated from microorganisms. However, the majority of the enzymes that are presently on the market are produced using mesophilic enzymes, which are often inhibited under the extreme conditions of several industrial processes [10]. In addition, the stability of the biocatalyst is important for reducing costs because enzymes that are sufficiently stable to withstand the industrial conditions can be used for repeated cycles of the biocatalytic process, and hence aid in reducing expenditures. Thus, the exploration of deep-sea extremophilic microorganisms provides an opportunity for obtaining extremozymes that are stable under a variety of different conditions, which may be attractive in industrial processes. Furthermore, enzymes that catalyse reactions under non-physiological conditions and/or with non-natural substrates can also be found in deep-sea environments [33]. Consequently, deep-sea extremozymes have received increasing attention for their applications in various industrial processes owing to their adaptability to harsh physical and chemical conditions [11].

3.1. Deep-Sea Thermophilic Enzymes

Deep-sea thermophiles have been one of the most studied groups of extremophiles over the past four decades [34,35]. These microorganisms are able to grow at high temperatures of 41 °C–120 °C [36,37], therefore, they produce extremozymes with high-temperature resistance. These thermophilic enzymes use a variety of mechanisms to tolerate extreme temperatures, possessing electrostatic interactions and physical properties that allow them to maintain their activity. In general, thermophilic enzymes have similar three-dimensional structures to their mesophilic counterparts but have many more charged residues on their surfaces and different amino acid contents. In addition, thermophilic enzymes usually have shorter loops, thereby inhibiting nonspecific interactions that are induced by their increased flexibility at high temperatures [38,39]. Thermophilic enzymes also have increased number of bisulphide bonds formed between two cysteine residues, which enhances their structural rigidity and, thus, resistance to unfolding at high temperatures [40,41].

To date, thermophilic enzymes have attracted the most attention among the various types of extremozymes because enzymes that are adapted to higher temperatures have several important advantages to industrial processes. High temperatures not only significantly increase the solubility of many reagents, particularly polymeric substrates, but also reduce the risk of contamination, which would result in unfavourable complications. Moreover, high temperatures also promote faster reactions, maintain a low viscosity and increase solvent miscibility [42]. Thermophilic enzymes are usually capable of accepting proteolysis and extreme conditions, such as the presence of organic solvents, denaturing agents and high salinity, making them attractive in the biorefinery, paper and bleaching, and first- and second-generation biofuel industries [43]. A large number of enzymes from deep-sea thermophilic microorganisms have been characterised to date (Table 1) [35], and thermophilic proteases, lipases and polymer-degrading enzymes, in particular, have found their way into industrial applications [11].

Table 1. Representative thermophilic enzymes from deep-sea microorganisms.

Source	Habitat	Enzyme	Thermostability	References
Bacillus sp. JM7	deep-sea water	keratinase	50 °C (70%, 1 h)	[44]
Pyrococcus furiosus	deep-sea vents	prolidase	100 °C (100%, 12 h)	[45]
Geobacillus sp. EPT9	deep-sea vents	lipase	80 °C (44%, 1 h)	[46]
Geobacillus sp. 12AMOR1	deep-sea vents	monoacylglycerol lipase	70 °C (half-life 1 h)	[47]
Flammeovirga Sp. OC4	deep-sea water	β-Agarase	50 °C (35%, 144 h)	[48]
Flammeovirga pacifica	deep-sea water	β-Agarase	50 °C (100%, 10 h)	[49]
Microbulbifer strain JAMB-A7	deep-sea sediment	β-Agarase	50 °C (half-life 502 min)	[50]
Flammeovirga pacifica	deep-sea water	α-amylase	60 °C (81%, 20 min)	[51]
Geobacillus sp. 4j	deep-sea sediment	α-amylase	80 °C (half-life 4.25 h)	[52]
Fosmid library	deep-sea vents	cellulase	92 °C (half-life 2 h)	[24]
Flammeovirga pacifica	deep-sea water	arylsulfatase	50 °C (70%, 12 h)	[53]
Staphylothermus marinus	deep-sea vents	amylopullulanase	100 °C (half-life 50 min)	[54]
Geobacillus sp. MT-1	deep-sea vents	xylanase	65 °C (half-life 50 min)	[55]

The diversity of deep-sea thermophiles makes them valuable in the search for novel thermostable proteolytic enzymes [56,57], which are attractive for use in the detergent, food and feed industries. Jin et al. [44] reported the purification and characterisation of a thermostable and alkali-stable keratinase Ker02562 from *Bacillus* sp. JM7, which was isolated from the deep sea. This enzyme was shown to be stable at 50 °C and in extreme alkaline environments (pH 10–13) and so may have significant applications in the detergent industry, as the enzymes that are used in detergent additives need to be able to withstand temperatures of 40–60 °C and an alkaline pH (pH 9.0–11.0) [58]. Other thermophilic proteases with important industrial applications include thermolysin, which is used in the synthesis of dipeptides, DNA-processing enzymes and pretaq protease, which is used to clean up DNA before PCR amplification [59]. For example, the proline dipeptidase named prolidase, which was identified from the archaeon hyperthermophile *Pyrococcus furiosus* isolated from deep-sea vents and volcanic

marine mud in Italy and specifically cleaves dipeptides with proline at the C-terminus and a nonpolar residue (Met, Phe, Val, Leu, Ala) at the amino terminus, is by far the most thermostable example of a prolidase known to date, with a temperature optimum above 100 °C and no loss of activity after 12 h at this temperature [45].

Industrial and biotechnological processes also require thermostable lipases for use in processes such as grease esterification, hydrolysis, transesterification, interesterification and organic biosynthesis. Zhu et al. [46] cloned and characterised a thermostable lipase from the deep-sea hydrothermal field thermophile *Geobacillus* sp. EPT9 and found that the recombinant lipase was optimally active at 55 °C and pH 8.5 and exhibited good thermostability, retaining 44% residual activity after incubation at 80 °C for 1 h. A thermostable monoacylglycerol lipase (GMGL) has also been identified from the thermophilic bacterium *Geobacillus* sp. 12AMOR1, which was isolated from a deep-sea hydrothermal vent site in the Arctic [47]. GMGL is active on monoacylglycerol substrate but not diacylglycerol or triacylglycerol, and recombinant GMGL shows the highest hydrolysis activity at 60 °C and pH 8.0 and has a half-life of 60 min at 70 °C. These thermostable lipases have considerable potential for applications in the food, detergent, cosmetics, perfumery, pharmaceutical, pulp and paper, and chemical industries [60].

Deep-sea thermostable polymer-degrading enzymes, such as agarases, amylases and cellulases, are another group of industrially important biocatalysts that have received much interest. Agarases catalyse agar hydrolysis and have been successfully utilised to produce agar-oligosaccharides, which possess a variety of biological and physiological functions that are beneficial to human health and thus have potential applications in the food and nutraceutical industries [14,61–63]. Since the gelling temperature of agar is approximately 40 °C, thermostable agarases are required for the efficient recovery of DNA from agar gel and are also advantageous for the industrial production of oligosaccharides from agar. Hou et al. reported the expression and characterisation of a novel thermostable and pH-stable β-agarase AgaP4383 from the deep-sea bacterium *Flammeovirga pacifica* WPAGA1 [49]. Phenotypic and genomic analyses revealed that *F. pacifica* WPAGA1 is capable of degrading and metabolising complex polysaccharides and can grow on the red alga *Gracilaria lemaneiformis* as a sole carbon source [64,65], and that AgaP4383 exhibits endolytic activity on agar degradation, producing neoagarotetraose and neoagarohexaose as the final products. AgaP4383 also exhibits good thermostability, with no loss of activity after incubation at 50 °C for 10 h [63]. Recently, a novel thermostable and pH-stable β-agarase Aga4436 was reported from another deep-sea bacterium in the genus *Flammeovirga*, *Flammeovirga* Sp. OC4, which also shows high activity and stability at high temperatures [48]. These favourable properties of AgaP4383 and Aga4436 could make them attractive for use in the food and biotechnology industries.

Thermostable amylolytic enzymes are one of the most interesting groups of enzymes for industrial processes, as they are important for the hydrolysis of starch at high temperatures, promoting the reactions and reducing the risk of contamination [66]. Several thermostable amylases have been reported from deep-sea microorganisms [51,52,67], some of which have been developed into products. For example, Fuelzyme®, a product from Verenium Corporation (San Diego, CA, USA), utilises an alpha-amylase from the thermophile *Thermococcus* sp., which was isolated from a deep-sea hydrothermal vent. Fuelzyme® operates in extremely high temperatures (>110 °C) and at an acidic pH (4.0–6.5), making it suitable for mash liquefaction during ethanol production, releasing dextrins and oligosaccharides with lower molecular weights and better solubilities [67]. However, Fuelzyme® and Spezyme® (DuPont-Genencor Science, Wilmington, DE, USA) are only presently used in the production of biofuel. It has been proposed that the combined use of these commercially available amylases and other *Bacillus* amylases will increase the efficiency of industrial starch processing and will be suitable for downstream applications [68].

Thermophilic enzymes are also widely used in industrial lignocellulolytic processes, with thermostable cellulases having applications in the food, animal feed, textile, and pulp and paper industries [7]. Functional screening of fosmid expression libraries derived from deep-sea hydrothermal vents identified an extreme cellulase that was active and thermostable at 92 °C. This enzyme showed endolytic activities against a variety of linear 1,4-β-glucans, such as phosphoric

acid swollen cellulose, carboxymethyl cellulose, lichenan and β-glucan. Other industrial important thermostable polymer-degrading enzymes have also been identified and characterised from deep-sea environments, such as amylopullulanases, arylsulphatases and xylanases [53–55].

3.2. Deep-Sea Psychrophilic Enzymes

The deep sea is primarily a cold environment, and the majority of the water is present at 5 °C. Consequently, this biome harbours an abundance of cold-adapted psychrophiles, which have a restricted range of temperature for growth, and are extremophiles that are adapted to moderate or extreme cold and have been shown to achieve active metabolism at −25 °C and conduct DNA synthesis at −20 °C [69]. Psychrophiles are divided into the following two types according to their growth temperatures: eurypsychrophiles (formerly psychrotolerant microorganisms), which comprise the majority of isolates from the deep sea and have a broad temperature range and tolerate warmer environments, and stenopsychrophiles (formerly true psychrophiles), which cannot grow at temperatures above 20 °C [70]. Psychrophiles have developed several mechanisms that allow them to thrive in icy environments, including the production of cold-induced cold-shock proteins and RNA chaperones, enhanced tRNA flexibility, enhanced membrane fluidity for maintaining the semi-fluid state of the membranes, and the production of cold-active secondary metabolites, enzymes, pigments and antifreeze proteins [71,72]. The most common adaptive characteristic of psychrophilic enzymes is their high reaction rate at low temperatures, which is generally achieved by their flexible structures and low stabilities [73]. From a structural perspective, psychrophilic proteins have a higher content of α-helix than β-sheets, which is recognised as an essential feature for maintaining flexibility even at low temperatures [7]. Because cold-active enzymes maintain a high catalytic rate at low temperatures through the augmentation of the solvent connection and structural flexibility [73,74], they are capable of binding more tightly to the solvent, in a way similar to that of salt-adapted enzymes [75].

Enzymes that are adapted to low temperatures possess several features that are favourable for industrial applications [76], and have been used in industries as diverse as food processing, molecular biology and fine chemical synthesis [77,78]. Cold-active enzymes bring potential benefits to the food and feed industries, where it is crucial to avoid spoilage as this may result in a change in nutritional value and flavour of the original thermosensitive substrates and products [77,79]. Cold-adapted enzymes are also useful for molecular biology because of the need to use enzymes in sequential reactions and to inactivate enzymes once they have accomplished their functions. To this end, heat-labile enzymes have great potential, as heat inactivation can be performed at temperatures that do not cause the melting of double-stranded DNA (dsDNA), eliminating the need for additional chemical extraction steps [77]. The detergent, biofuel production and pulp and paper industries are also interested in cold-active hydrolases, such as proteases, lipases, amylases and cellulases, as these enzymes can provide economic benefits by reducing energy consumption and production costs [80,81].

Deep-sea psychrophiles are a promising source of industrially important cold-active enzymes (Table 2). Cold-active cellulose-degrading enzymes, such as glucosidases, are useful in the textile, beverage and biofuel industries. The first cold-active and alkali-stable β-glucosidase was isolated from the deep-sea bacterium *Martelella mediterranea* and showed favourable characteristics, being able to retain more than 50% of its maximum enzymatic activity at 4 °C and 80% of its maximum enzymatic activity at pH 11 for 11 h [82]. The deep-sea-sediment–dwelling bacterium *Exiguobacterium oxidotolerans* also produces a cold-active β-glycosidase, which maintains 61% of its maximum activity at 10 °C and in a pH range of 6.6–9.0 [83]. Several cold-adapted xylanases that have been isolated from the deep sea, such as from *Zunongwangia profunda* [84] and *Flammeovirga pacifica* [85], have potential uses in the food industry, such as as additives to wheat flour to improve dough handling and the quality of the baked products. Numerous cold-adapted amylases have also been isolated from deep-sea microorganisms over the past few years that have potential applications in the detergent, textile and food industries [86–88]; for example, Jiang et al. [89] reported a cold-adapted alpha-amylase from the deep-sea bacterium *Bacillus* sp. dsh19-1, which shows maximum activity at 20 °C. Cold-active

lipases and esterases are important catalysts in the chemical, pharmaceutical, cosmetic, food, laundry detergent and environmental remediation industries and can be isolated from deep-sea microorganisms and metagenomic libraries derived from deep-sea samples [77]. For example, Chen reported a novel psychrophilic esterase Est11 from the deep-sea bacterium *Psychrobacter pacificensis*, which is highly active and stable at 10 °C and 5 M NaCl [90]. Furthermore, incubation with ethanol, isopropanol, propanediol, dimethyl sulphoxide (DMSO), acetonitrile and glycerol were shown to have remarkable positive effects on Est11 activity, indicating that this cold-active, halo-tolerant and organic solvent-resistant esterase may be useful in harsh industrial processes [90].

Table 2. Representative psychrophilic enzymes from deep-sea microorganisms.

Source	Habitat	Enzyme	Activities at Low Temperatures	References
Martelella mediterranea	deep-sea water	β-glucosidase	50% at 4 °C	[82]
Exiguobacterium oxidotolerans	deep-sea sediment	β-glycosidase	61% at 10 °C	[83]
Zunongwangia profunda	deep-sea sediment	xylanase	38% at 5 °C	[84]
Flammeovirga pacifica	deep-sea water	xylanase	50–70% at 10 °C	[85]
Luteimonas abyssi	deep-sea water	α-amylase	36% at 10 °C	[87]
Zunongwangia profunda	deep-sea sediment	α-amylase	39% at 10 °C	[88]
Pseudomonas strain	deep-sea sediment	α-amylase	50% at 5 °C	[91]
Wangia sp. C52	deep-sea sediment	α-amylase	50% at 25 °C	[92]
Bacillus sp. dsh19-1	deep-sea sediment	α-amylase	35.7% at 4 °C	[89]
Psychrobacter pacificensis	deep-sea water	esterase	70% at 10 °C	[90]
Metagenomic libraries	deep-sea sediment	esterase	100% at 10 °C	[25]
Metagenomic libraries	deep-sea sediment	esterase	38% at 15 °C	[26]
Metagenomic libraries	deep-sea sediment	lipase	most active below 30 °C	[93]
Pseudoaltermonas sp. SM9913	deep-sea sediment	serine protease	60% at 20 °C	[94]
Planococcus sp. M7	deep-sea sediment	protease	45% at 10 °C	[95]

3.3. Deep-Sea Halophilic Enzymes

Halophiles are capable of thriving in high salt concentrations, which can be found in DHABs or deep-sea hypersaline anoxic lakes. These extreme habitats have been discovered on the sea floor in different oceanic regions, such as the Red Sea, the eastern Mediterranean Sea and the Gulf of Mexico. In a DHAB, the dissolved evaporitic deposits are trapped in the sea floor sediments, forming very stable brine and a sharply stratified chemocline in the water column. The brines that are enclosed in these basins are characterised by hypersalinity (5–10 times the concentration of sea water), a high pressure (approximately 35 MPa), a lack of oxygen and highly reducing conditions, and an absence of light, making them one of the most extreme environments on the Earth, which has allowed these habitats to remain isolated for thousands of years [7,96]. Since the discovery of the first Mediterranean DHAB termed 'Tyro' in 1983, six more DHABs have been discovered termed 'l'Atalante', 'Bannock', 'Discovery', 'Medee', 'Thetis' and 'Urania' [97], all of which are sources of anaerobic halophilic microorganisms.

Halophilic microorganisms can be classified into the following three categories according to the optimal salt concentration for growth: (i) slight halophiles, which are capable of developing at 200–500 mM NaCl; (ii) moderate halophiles, which can develop at 500–2500 mM NaCl; and (iii) extreme halophiles, which can develop at 2500–5200 mM NaCl [98]. Certain halophiles are also thermostable and tolerant to a wide range of pHs, and halophiles have high metabolic diversity, comprising anoxic phototrophic, fermenter, aerobic heterotrophic, sulphate reducer, denitrifying and methanogenic organisms [99].

Halophiles have developed different adaptive strategies to survive the osmotic pressures that are induced by the high NaCl concentrations in the environments they inhabit. Some extremely halophilic bacteria use a type of 'salt-in' strategy to balance the osmotic pressure of the environment, whereby they accumulate inorganic ions (K^+, Na^+, Cl^-) in the cytoplasm [100]. However, moderate halophiles have distinct adaptations that allow them to biosynthesize and/or accumulate large amounts of specific organic osmolytes in the cytoplasm. These accumulated osmolytes act as osmoprotectants and help to maintain osmotic balance and low salt concentrations in the cytoplasm without interfering

with the normal cellular metabolism [101]. Halophiles also produce enzymes that are active and stable in the presence of salts, with the enzymes employing different adaptation mechanisms and exhibiting very high stability at low water activity as well as in the presence of organic solvents and high salt concentrations [35,102]. Structural analyses have revealed that the major differences between non-halophilic and halophilic proteins occur on the surfaces of the molecules. Halophilic enzymes contain a greater percentage of certain amino acid residues, such as serine and threonine, a higher proportion of aspartic and glutamic acids, a lower percentage of lysine, and a higher occurrence of amino acids with low hydrophobic characters than non-halophilic enzymes, which allow a higher number of salt bridges to be created and cooperation with electrostatic interactions [103]. The stability of these enzymes relies on the negative charge of the acidic amino acids on the protein surface, the hydration of the protein surface due to the carboxylic groups that are present in aspartic and glutamic acids, and the occurrence of hydrophobic groups in the presence of high salt concentrations. In addition, the negative surface charges are believed to be important for the solvation of halophilic proteins and the prevention of denaturation, aggregation and precipitation [101,104]. Deep-sea halophilic enzymes provide great opportunities for the food, detergents, textile, bioremediation and biosynthetic industries [102], with their industrial potential lying in their activity and stability not only at high salt concentrations but also in the presence of organic solvents [105,106]. Moreover, several deep-sea halophilic enzymes are also active and stable at high or low temperatures [89,90]. These unique properties make deep-sea halophilic enzymes attractive wherever enzymatic conversion needs to occur under challenging physical and chemical conditions, such as at extreme salt concentrations and temperatures and in the presence of organic solvents.

Numerous halophilic enzymes isolated from the deep sea have been cloned and characterised to date (Table 3), with examples of industrially important halophilic enzymes including polysaccharide-hydrolysing enzymes, such as amylases and xylanases [10,102,107]. For instance, two cold-adapted and salt-tolerant alpha-amylases have been reported from the deep-sea bacteria *Bacillus* sp. dsh19-1 and *Zunongwangia profunda*, which are among the very few known alpha-amylases that can tolerate both cold and saline conditions [88,89]. The high activities and stabilities of these halophilic amylases in harsh conditions make them desirable for industrial applications, particularly for the treatment of waste water containing high salt concentrations and starch residues. In addition, a novel psychrophilic and halophilic β-1,3-xylanase (Xyl512) was recently characterised from the deep-sea bacterium *Flammeovirga pacifica* strain WPAGA1, and it was found that a high-saline concentration (1.5 M NaCl) could alter the optimum temperature and pH of Xyl512, as well as significantly improve its overall activity by two-fold compared with an absence of NaCl, which would meet the food industry's demands for low temperatures and high concentrations of salt [85]. Some deep-sea halophilic enzymes are lipolytic, such as esterases, which have particularly great potential in the production of biodiesel, polyunsaturated fatty acids and food [108,109]. Five esterase genes have been identified from a metagenome expression library derived from the DHAB Urania, some of which were highly active in high-salinity conditions and were able to function in polar solvents, making them suitable for use in the chemical, pharmaceutical and biofuel industries [110]. In addition, research regarding deep-sea extremophiles has identified and characterised other industrially important halophilic enzymes, such as proteases, mercuric reductase and the first reported DNA polymerase to exhibit halophilic and thermophilic features [111–113].

Table 3. Representative halophilic enzymes from deep-sea microorganisms.

Source	Habitat	Enzyme	Activities at High Saline Concentrations	References
Zunongwangia profunda	deep-sea sediment	α-amylase	93% activity at 4 M NaCl	[88]
Bacillus sp. dsh19-1	deep-sea sediment	α-amylase	60.5% activity at 5 M NaCl	[89]
Zunongwangia profunda	deep-sea sediment	xylanase	near 100% activity at 5 M NaCl	[84]
Flammeovirga pacifica	deep-sea water	xylanase	maximum at 1.5 M NaCl	[85]
Emericellopsis sp. TS11	deep-sea sponge	xylanase	maximum at 2 M NaCl	[114]
Metagenomic libraries	deep-sea brine	esterase	maximum at 3–4 M NaCl	[110]
Fosmid library	deep-sea sediment	esterase	maximum at 3.5 M NaCl	[27]
Psychrobacter pacificensis	deep-sea water	esterase	maximum at 5 M NaCl	[90]
Pseudoalteromonas spp.	deep-sea sediment	protease	maximum at 2 M NaCl	[111]
Metagenomic libraries	deep-sea brine	mercuric reductase	maximum at 4 M NaCl	[112]
candidate division MSBL1 archaeon SCGC-AAA261G05	deep-sea brine	DNA polymerase	maximum at 0.5 M NaCl	[113]

3.4. Deep-Sea Piezophilic Enzymes

The hydrostatic pressure can reach 70–110 MPa in the deepest parts of the oceans, making these environments highly challenging. These deep-sea habitats host a group of extremophiles known as piezophiles, which survive and thrive under conditions of extremely high hydrostatic pressure [115,116]. Piezophiles can be classified into the following two categories based on their pressure requirements for growth: 1) piezophilic microorganisms, which exhibit optimal growth at pressures above atmospheric pressure; and 2) piezotolerant microorganisms, which can grow at atmospheric pressure and high pressures but do not require high pressures for optimal growth [117]. High pressure plays a selective role on living organisms by affecting cellular structures and processes, such as cell motility and division. Consequently, the ability to live under extreme pressures requires substantial physiological adaptations that involve modifications to gene regulation and the cellular structure. The adaptative mechanisms of piezophiles have not yet been fully clarified but are known to involve a reduction in cell division, the production of compatible osmolytes and polyunsaturated fatty acids, a switch in the flexibility state, and the formation of multimeric and antioxidant proteins [118–121]. Lauro et al. have also described the occurrence of extended helices in the 16S ribosomal RNA (rRNA) genes for adaptation to high pressures [122].

So far, very little research has been conducted on deep-sea piezophilic enzymes [117,123], and many more experiments and computational studies on different enzymes from a variety of piezophiles are required to advance our understanding [120]. However, piezophilic proteins have shown high efficiency in several industrial processes [5], with particular applications for food production, where high pressures are employed for the processing and sterilisation of food materials [124]. For example, piezophilic α-amylase has been shown to produce trisaccharide instead of maltobiose and tetrasaccharide from maltooligosaccharide at high pressures with little energy, which is a useful reaction in the food processing industry [35,117]. In addition, a peptidase from *Pyrococcus horikoshii* demonstrates stability at high pressures and thus may be useful for food processing [35,125]. Deep-sea piezophiles often produce polyunsaturated fatty acids, such as omega-3 polyunsaturated fatty acids, to stabilise the cell membrane under high pressure. This increase in unsaturated fatty acids creates highly disordered phospholipid bilayers, which renders the membrane resistant against high pressure [116]. Thus, the lipid biochemistry of piezophilic microorganisms is a very interesting topic, and the enzymes that participate in these metabolic pathways under high pressures may have great potential for industrial applications [126]. Piezophilic endonucleases may also have potential in the biotechnology industry. For example, the 'star activity' that is exhibited by *Eco*RI and other restriction endonucleases under high-osmotic-pressure conditions, whereby they lose some specificity to their recognition sequences, can be reversed by piezophilic endonucleases operating under a hydrostatic pressure of 50–75 Mpa [117,127].

4. Conclusions and Prospects

Deep-sea extremophiles are emerging as an important source of novel industrially robust extremozymes. The biodiversity of deep-sea extremophiles and the evolutionary adaptations of their derived extremozymes to the harsh conditions that are found in deep-sea ecosystems has facilitated the selection of more robust biocatalysts, which have special properties that are not found in any other prokaryotes. These extremozymes will be beneficial for novel biocatalytic processes, allowing them to be more efficient, specific, accurate and environmentally friendly. Due to their special features and enormous potential in industrial applications, the number of studies on deep-sea extremophiles and their extremozymes has greatly increased over the last two decades. However, there are still only a few extremophiles available and only a minor fraction of the deep-sea extremophiles has been exploited for extremozymes to date. This limited exploitation of extremozymes is largely due to the special nutritional requirements and challenging growth conditions of deep-sea extremophiles, which make their isolation and maintenance difficult. Fortunately, however, the rapidly increasing number of extremophilic genomes and metagenomes that can now be easily obtained by next-generation sequencing technologies offers an ever-expanding resource for the identification of new extremozymes from non-cultivable deep-sea extremophiles. In addition, although enzyme engineering techniques have been established, the enzyme optimisation process is still a limiting factor for the development of new extremozyme-inspired industrial processes. Thus, the simultaneous development of protein engineering technologies will assist the further modification and improvement of biocatalytic features, which will increase the application of deep-sea extremophiles in industry. Moreover, important advances in our knowledge of the genetics, physiology, metabolism and enzymology of deep-sea extremophiles are expected, which will enable us to better understand the applications of their biocatalysts. Thus, the advancement of modern molecular techniques and deep-sea sampling approaches in the future will allow deep-sea extremozymes to have significant impacts on a wide range of industries.

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Article

Biochemical and Genomic Characterization of the Cypermethrin-Degrading and Biosurfactant-Producing Bacterial Strains Isolated from Marine Sediments of the Chilean Northern Patagonia

Patricia Aguila-Torres [1,*,†], Jonathan Maldonado [2,3,4,†], Alexis Gaete [2,3], Jaime Figueroa [5], Alex González [6], Richard Miranda [7], Roxana González-Stegmaier [5,8], Carolina Martin [1] and Mauricio González [2,3,*]

1 Laboratorio de Microbiología Molecular, Escuela de Tecnología Médica, Universidad Austral de Chile, Puerto Montt 5504335, Chile; carolina.martin@uach.cl
2 Laboratorio de Bioinformática y Expresión Génica, Instituto de Nutrición y Tecnología de los Alimentos, Universidad de Chile, Santiago 7810000, Chile; jomaldon@gmail.com (J.M.); alex.ignacio@live.com (A.G.)
3 Center for Genome Regulation, Santiago 7810000, Chile
4 Laboratorio de Biología de Sistemas de Plantas, Departamento Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago 8331150, Chile
5 Instituto de Bioquímica y Microbiología, Facultad de Ciencias, Universidad Austral de Chile, Valdivia 5090000, Chile; jefigueroa@uach.cl (J.F.); rgstegmaier@gmail.com (R.G.-S.)
6 Laboratorio de Microbiología Ambiental y extremófilos, Departamento de Ciencias Biológicas y Biodiversidad, Universidad de los Lagos, Osorno 5290000, Chile; alex.gonzalez@ulagos.cl
7 Escuela de Ingeniería Civil Industrial, Universidad Austral de Chile, Puerto Montt 5500000, Chile; rmiranda@spm.uach.cl
8 Laboratorio Medicina Traslacional, Instituto Clínico Oncológico, Fundación Arturo López Pérez, Santiago 8320000, Chile
* Correspondence: patricia.aguila@uach.cl (P.A.-T.); mgonzale@inta.uchile.cl (M.G.); Tel.: +56-65-2277118 (P.A.-T.); +56-2-29781440 (M.G.)
† These authors have contributed equally to this work.

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Abstract: Pesticides cause severe environmental damage to marine ecosystems. In the last ten years, cypermethrin has been extensively used as an antiparasitic pesticide in the salmon farming industry located in Northern Patagonia. The objective of this study was the biochemical and genomic characterization of cypermethrin-degrading and biosurfactant-producing bacterial strains isolated from cypermethrin-contaminated marine sediment samples collected in southern Chile (MS). Eleven strains were isolated by cypermethrin enrichment culture techniques and were identified by 16S rDNA gene sequencing analyses. The highest growth rate on cypermethrin was observed in four isolates (MS13, MS15a, MS16, and MS19) that also exhibited high levels of biosurfactant production. Genome sequence analyses of these isolates revealed the presence of genes encoding components of bacterial secondary metabolism, and the enzymes esterase, pyrethroid hydrolase, and laccase, which have been associated with different biodegradation pathways of cypermethrin. These novel cypermethrin-degrading and biosurfactant-producing bacterial isolates have a biotechnological potential for biodegradation of cypermethrin-contaminated marine sediments, and their genomes contribute to the understanding of microbial lifestyles in these extreme environments.

Keywords: cypermethrin; biosurfactants; biodegradation capacities; marine sediments

1. Introduction

Los Lagos region (S41°85′39′′ W73°48′32′′), located at the Chilean Northern Patagonia, has a high-density salmon farming industry with an extensive history of cypermethrin usage [1,2]. Cypermethrin [cyano-(3-phenoxyphenyl)methyl]-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopro-pane-1-carboxylate is a synthetic pyrethroid pesticide used in agriculture and aquaculture [3], classified as a possible human carcinogen by the Environmental Protection Agency with moderate–acute toxicity according to World Health Organization [4,5]. This compound has an impact on marine ecosystems, affecting the biodiversity of fish and aquatic invertebrates [2,6]. Given its bioaccumulative effect and its further biomagnification in the food chain, the presence of this compound in sediment and soil water is of great concern [7]. As shown in a previous report [2], a high concentration of the pyrethroid cypermethrin, with values ranging from 18.0 to 1323.7 ng g^{-1}, was observed in marine sediments in the Northern Patagonia, the same region from which our bacterial isolates were obtained. These habitats are extreme environments with a wide range of temperatures, ranging from 4 to 20 °C, and salinity (range 32‰–33‰), and also a low nutrient availability, as is the case for nitrate (NO_3) and phosphate (PO_4).

Bioremediation has become an essential tool for removing these pollutants through biological methods, taking advantage of the degradative capabilities of microorganisms for cleaning contaminated environments [8,9]. The most recommended procedure is to use the local genetic resources, whose activity can be modified by modifying nutrients, water, air, and biosurfactants [9,10]. Natural degradation of cypermethrin is carried out by microorganisms that could produce the hydrolysis of the ester linkage, resulting in 3-phenoxybenzoic acid [11]. Diverse microorganisms isolated from soil have been reported to degrade cypermethrin, such as *Pseudomonas*, *Serratia*, *Streptomyces*, *Rhodobacter*, *Stenotrophomonas*, *Sphingomonas*, and *Bacillus* [12–17]. However, studies reporting cypermethrin-degrading bacterial strains obtained from marine environments are less frequent (i.e., *Cellulophaga lytica* DAU203) [18], especially from marine sediments.

Biosurfactant production increases the bioavailability of pollutants for microorganisms by increasing cell surface hydrophobicity [19]. This, in turn, improves the binding of organic compounds to the cell membrane, their adsorption, and ultimately, their biosorption [19,20]. Different microorganisms produce a diversity of biosurfactants [21]. However, there are only a few reported biosurfactant-producing microorganisms that have proven useful in cypermethrin biodegradation processes [4,22]. Therefore, the aims of this study were the identification and characterization of cypermethrin-degrading and biosurfactant-producing bacterial strains isolated from marine sediments in extreme environments, as is Chilean Northern Patagonia, and determining the presence of cypermethrin biodegradation pathway genes, integrating biochemical and genomics approaches.

2. Results

2.1. Isolation and Identification of Marine Bacterial Strains

Eleven cypermethrin-degrading and biosurfactant-producing bacteria were isolated from cypermethrin-polluted sediment sampled at Manao Bay, Ancud, Chiloé (Figure 1a). Strains were isolated after three subcultures by enrichment with cypermethrin, with concentrations ranging from 50 to 200 mg L^{-1} (Figure 1b). According to Gram-staining analyses, five isolates were Gram-negative and six were Gram-positive. Strains were cocci, bacilli, and rod-shaped (Table S1). All strains were catalase-positive, and five of them were motile. Four strains were capable of growth on 50 mg L^{-1} of cypermethrin as the sole carbon and energy source, while reaching the highest cell concentration measured by $OD_{600\ nm}$. These strains were selected for further analysis. The eleven marine bacterial strains (MS) obtained were compared using 16S rRNA gene identity; the results are summarized in Figure 1c. The phylogenic analysis showed that strains MS8, MS10, MS15a, and MS19 are closely related to *Pseudomonas*. MS1, MS11, MS12, MS13, MS14, and MS16 strains are closely related to *Rhodococcus*, and MS4 belongs to *Serratia* (Figure S2, Figure 1c).

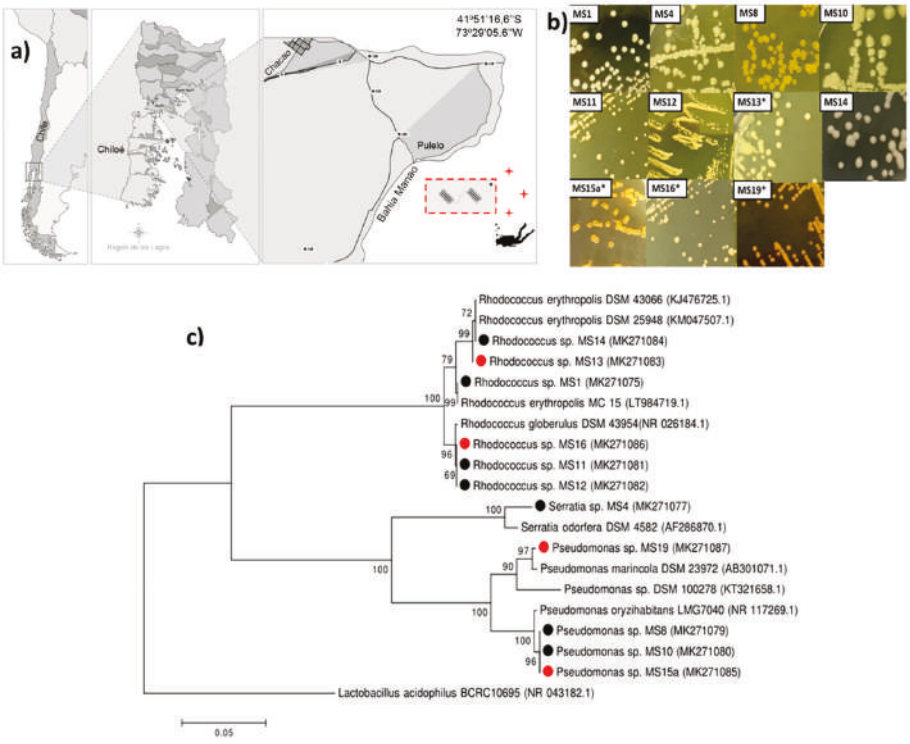

Figure 1. Map of sampling site, geographical position, colony morphologies, phylogeny, and biodiversity of cypermethrin-degrading and biosurfactant-producing bacterial strains isolated from the Chilean Northern Patagonia samples. (**a**) Map of the sampling sites in the Manao Bay: samples were collected from marine sediments at 25 m depth. The asterisk indicates salmon farming center. Crosses mark the three sampling sites. (**b**) Colony morphology, in TSA medium, of cypermethrin-degrading and biosurfactant-producing bacterial strains isolated by enrichment using cypermethrin as the sole carbon and energy source. (**c**) Phylogenetic tree of cypermethrin-degrading and biosurfactant-producing bacterial strains isolated from the Northern Patagonia; black circles indicate cypermethrin-degrading and biosurfactant-producing isolated bacterial strains. Red circles indicate strains selected for genomic analysis.

2.2. Surfactant-Producing Bacteria

The emulsion index (E_{24}) produced by each of the eleven bacterial strains was quantified in order to identify the bacterial strain with the highest capacity of biosurfactant production. All the strains grown in the ETMS medium were capable of emulsion production, which was observed after the addition of diesel petroleum. Strains MS13, MS15a, MS16, and MS19 showed the highest emulsion indexes (>60%) among all the bacterial strains in these conditions (Table S1).

2.3. SEM Studies

The morphologies of selected marine bacteria grown until the mid-exponential phase were studied by scanning electron microscopy (Figure 2). MS13, MS15a, MS16, and MS19 strains showed cell pleomorphism with a rod, bacilli, and coccoid shapes. Cells usually occurred in pairs. In strain MS16, anchor-like appendages forming bridges from one cell to the next were observed (Figure 2).

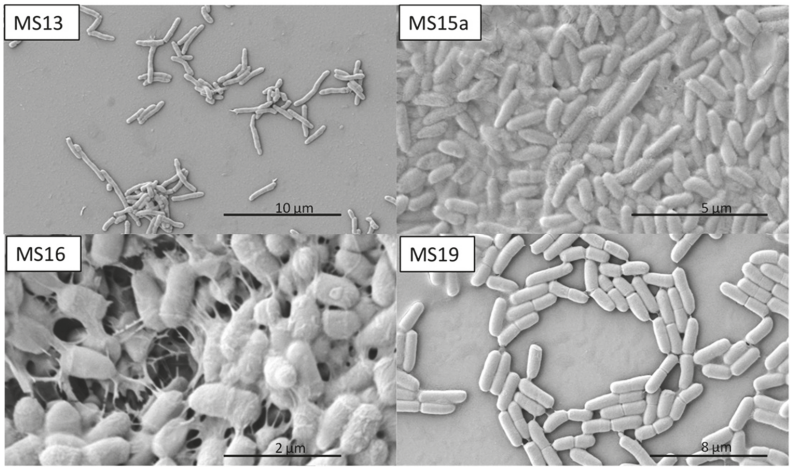

Figure 2. Scanning electron microscopy of cypermethrin-degrading and biosurfactant-producing bacterial strains. Cells tended to occur in pairs. The formation of inter-cellular bonds is highlighted in strain MS16.

2.4. Genome Characterization of Selected Strains

To further investigate the cypermethrin catabolic pathway in selected marine bacterial strains, the whole genome was sequenced. The assembled genomes of *Rhodococcus* sp. MS13 and *Rhodococcus* sp. MS16 consisted of 6,460,280 bp and 6,970,856 bp, and 23 and 37 scaffolds, respectively; the average G + C content was 62% for both strains (Table 1). The assembled genomes of *Pseudomonas* sp. MS15a and *Pseudomonas* sp. MS19 consisted of 5,249,999 bp and 4,496,051 bp, and 27 and 30 scaffolds, respectively; the average G + C content was 65% and 57%, respectively (Table 1). The four sequenced genomes represent high-quality assemblies with N50 indexes over 290Kb, BUSCO completeness indexes over 96%, single copy proportions of BUSCO markers over 94%, and near 0% fragmented markers (Table 1).

Table 1. Genome properties and features.

Property	*Rhodococcus* sp. MS13	*Rhodococcus* sp. MS16	*Pseudomonas* sp. MS15a	*Pseudomonas* sp. MS19
Genome size (bp)	6,460,280	6,970,856	5,249,999	4,496,051
N50 (bp)	557,702	745,857	371,249	290,555
G+C content	62%	62%	65%	57%
DNA scaffolds	23	37	27	30
Total genes	5964	6424	4849	4116
RNA genes	62	58	72	67
tRNA genes	54	52	59	56
Pseudogenes	58	158	65	39
Protein-coding genes	5844	6208	4712	4010
Complete BUSCOs	97%	96%	98%	100%

2.5. Identification of Secondary Metabolites and Detection of Genes Involved in Cypermethrin Catabolism in MS13, MS15, MS16, and MS19 Marine Bacterial Strains, and Comparison with Reference Strains

In order to establish catabolic and anabolic capabilities of the four sequenced strains, an analysis of the biosynthetic coding capacity (BGC) using antiSMASH was performed, and the *estA*, *pytH*, and *laccase* genes encoding the cypermethrin catabolic pathway were identified. BGC's analysis showed a differential pattern of bioactive compounds in all sequenced strains. Figure 3 describes the number of BGC detected in all studied strains compared with their respective reference strains using antiSMASH

version 5.0. Of the four studied strains, *Rhodococcus* sp. MS13 had the highest number of gene clusters, with 17 BGC, followed by *Rhodococcus* sp. MS16, with 15 BGC. *Pseudomonas* sp. MS15a and MS19 had seven and five BGC, respectively. Compared with strains of the genus *Pseudomonas*, *Rhodococcus* showed a greater number of non-ribosomal peptide synthetases (NRPS). Regarding specific NRPS compounds, *Rhodococcus* sp. MS13 and *Rhodococcus* sp. MS16 presented siderophores, antifungals, and antibiotics, whereas *Pseudomonas* only had the first two. Figure 3 also describes the presence of genes involved in the cypermethrin degradation pathway and the biosurfactant compounds that MS13, MS15, MS16, and MS19 produce. Overall, laccases and carboxylesterases were detected in all analyzed genomes, but genes encoding esterases were identified only in *Pseudomonas* sp. strain (MS15a and MS19) genomes.

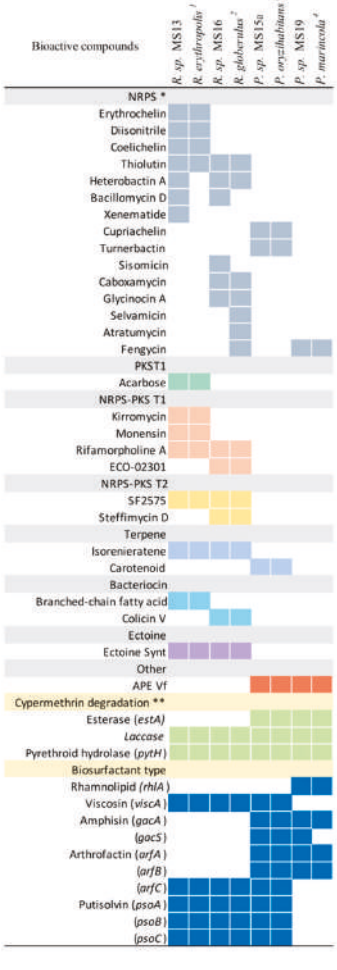

Figure 3. Biosynthetic gene clusters for secondary metabolites and cypermethrin degradation genes in isolated bacteria and reference strains. [1] Soil contaminated with fuel. [2] Rhizosphere of plants grown in soil contaminated with polychlorinated biphenyls (PCBs). [3] Groundwater of pea rhizoplane (*Pisum sativum* L.). [4] Deep-sea in the Fiji Sea. * Secondary metabolites identified through antiSMASH5.0 database and ** *in silico* analysis using a search by homology approach.

The proposed cypermethrin degradation pathway for MS13, MS16, MS15a, and MS19 isolates is described in Figure 4. Genomic analysis suggests that the cypermethrin degradation pathway could be carried out by esterase, carboxylesterase, and laccase.

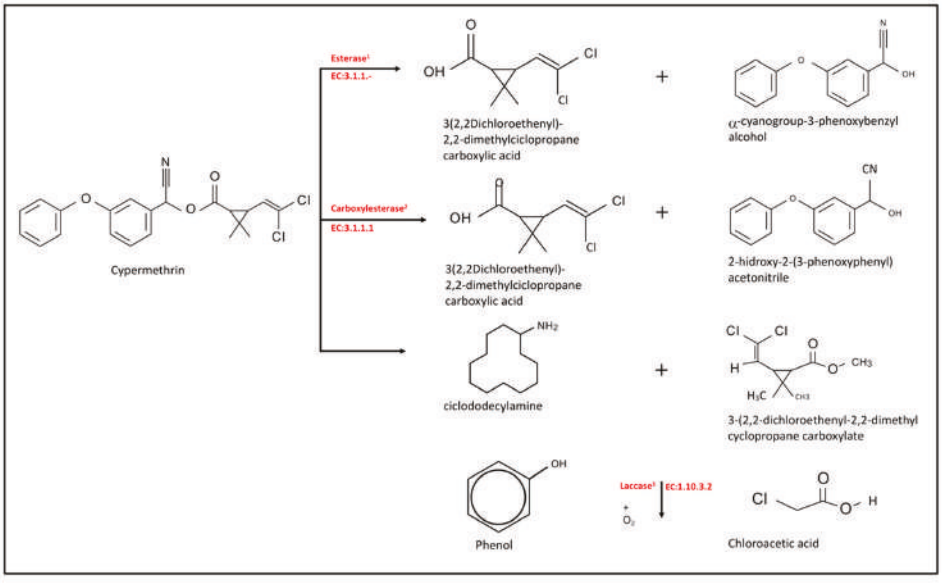

Figure 4. Proposed pathway of cypermethrin biodegradation based on the presence of esterase, laccase, and carboxylesterase-encoding genes in the genomes of MS13, MS16, MS15a, and MS19 isolates. Adapted from [1]Bhatt et al. [23], [2]Zhan et al. [24], and [3]Gangola et al. [17].

3. Discussion

Since 1997, *Caligus rogercresseyi* has been recorded as the most serious parasite in the salmon industry in Northern Patagonia, Chile [1]. Several products have been used to keep sea lice under control in Chile. Cypermethrin [2] is one of them. As a consequence of the extended exposure time to the drug, Northern Patagonia presents high concentrations of cypermethrin in marine sediments [2]. In this scenario, some microorganisms may have acquired the cypermethrin catabolic pathway as a mechanism to use this organic carbon source in an environment with low availability of carbon and hard environmental conditions.

In this study, cypermethrin-degrading and biosurfactant-producing bacterial strains isolated from marine sediment in Northern Patagonia were studied, along with their phenotypic, biochemical, and genomic properties, and their potential to bioremediate cypermethrin-polluted sediments. Based on 16S rRNA gene sequences analyses, bacterial isolates were related to the genera *Pseudomonas*, *Rhodococcus*, and *Serratia*, suggesting a lower abundance of bacterial genera associated with marine sediments in our sampling sites. Phylogenetic analyses indicate that these isolates are highly related to the reference strains *Rhodococcus erythropolis*, *Rhodococcus* sp., *Rhodococcus globerulus*, *Serratia odorifera*, *Pseudomonas oryzihabitans*, *Pseudomonas* sp., and *Pseudomonas marincola*. Isolates from the genus *Rhodococcus* were the most abundant. These results are consistent with those reported by Choi et al. [25], which indicated that *Actinobacteria* and *Gammaproteobacteria* were very prominent families in bacterial communities inhabiting the surfaces of such marine sediments [25].

Overall, clear morphological differences were observed between the eleven isolated strains; and all isolates were able to grow on cypermethrin as the sole carbon source and showed emulsion activity (biosurfactants). However, four of them (MS13, MS15a, MS16, and MS19) reached the maximum

measurable growth on cypermethrin (measured by turbidity—optical density: OD_{600}). Emulsions were also achieved in the cell-free extracts of these strains, indicating that the emulsifying activities were extracellular. Therefore, strains MS13, MS15a, MS16, and MS19 were selected for further characterization towards their application for cypermethrin bioremediation.

Biosurfactants are amphipathic molecules with emulsifying and high surface activity; this turns them into attractive agents for bioremediation [26]. Strains MS13, MS15a, MS16, and MS19 were isolated from cypermethrin-contaminated sediment and showed high potential as biosurfactant-producing bacteria, reaching emulsion indexes of 77%, 60%, 79%, and 71% respectively. Techaoei et al. [27] reported biosurfactant-producing bacteria from soil samples that showed emulsifying activities ranging from 8% to 63% emulsification [27]. Borah and Yadav [28] reported that E24 tested with kerosene, crude oil, and engine oil reached lower values than the ones reported here; namely, 55%, 29%, and 20% emulsion indices, respectively [28]. Previously, it has been described that members of the *Rhodococcus* and *Pseudomonas* genera showed degrading activity of cypermethrin in polluted soils [10], along with *Serratia*, in in vitro assays [4], but to our knowledge, there are no studies on cypermethrin degradation in marine sediments. In this study we determined that, of all obtained isolates, four strains grew successfully in high cypermethrin concentrations, two *Rhodococcus* and two *Pseudomonas*.

Therefore, these bacteria are good candidates for biodegradation of cypermethrin-contaminated marine sediments. Electron microscopy showed that marine strains usually occur in pairs and form bridges via a net-like structure. It has been reported that genera *Rhodococcus* and *Pseudomonas* have nocardioform, that is, rod-shaped or coccoid elements [29,30].

The results of the assembled genome of *Rhodococcus* sp. MS13 and *Rhodococcus* sp. MS16 are comparable with a bacterial strain isolated from marine sediment in Comau fjord, North Patagonia, by Undabarrena [31]. The isolated marine bacterium was *Rhodococcus* sp. H-CA and its complete chromosome has 6.19 Mbp with a content of 62.45% G + C. However, the presence of a *Rhodococcus* sp. RHA1 (RHA1) strain has also been reported, which has the largest bacterial genome sequenced to date, comprising 9,702,737 bp (67% G + C) [29]. Regarding the assembled genomes of *Pseudomonas* sp. MS15a and *Pseudomonas* sp. MS19, our genome data was comparable with strain *Pseudomonas* sp. S.C.T. isolated from marine sediment, whose genome was sequenced and assembled with 4.79 Mbp and 62.5% G + C content [32].

Rhodococcus sp. MS13 and *Rhodococcus* sp. MS16 have great numbers of biosynthetic clusters compared to the other two isolated bacteria; they also have the largest genomes—6.5 and 6.9 Mb. Both strains have great numbers of biosynthetic gene clusters in their respective genomes. These data are similar to those obtained by Ceniceros et al. [33], and Undabarrena et al. [31], which explored the metabolic capacities of the genus *Rhodococcus* and sequence the genome of *Rhodococcus* sp. H-CA8f [31,33]. The strains with the fewest gene clusters are *Pseudomonas* sp. MS19, which has the smallest genome size, 4.5 Mb, together with *Pseudomonas* sp. MS15, with seven gene clusters. The most represented BGCs correspond to non-ribosomal peptide synthetases (NRPS). These results show the biosynthetic potentials of the studied strains.

In relation to NRPS, the strains studied presented siderophore, antifungal, and antibiotic bioactive compounds. Siderophores play an essential role in bacterial metabolism regarding iron uptake, since it has been described that there is a low concentration of iron in seawater (0.01–2 nM) [34,35]. It is interesting to note that *Rhodococcus* sp. MS13 contained more NRPS than *Rhodococcus erythropolis* PR4. One of them, glycinocin A, from *Rhodococcus* sp. MS16, generates lipopeptides, which can act like biosurfactants or antibiotics [34]; this strain showed the highest E24 index.

The cypermethrin degradation pathway depends on three enzymes: esterase, carboxylesterase, and laccase [36–38]. Laccase is an enzyme involved in the oxidation of aromatic compounds, which should play a role in cypermethrin biodegradation. Gangola et al. [17] described a novel cypermethrin degradation pathway in *B. subtilis* [17]: *pytH* gene encodes a pyrethroid-hydrolyzing carboxylesterase capable of degrading a variety of pyrethroids, including cypermethrin, suggesting that these strains could be good cypermethrin-degrading candidates [36], although chemical analyses are yet to

be done; cypermethrin and its intermediate metabolites should be analyzed because sometimes toxic intermediates metabolites such as 3-phenoxybenzoic acid exist. This one metabolite is a toxic intermediate of cypermethrin degradation, a recalcitrant chemical [13].

Cypermethrin is first degraded by esterases into 3-(2,2-dichloroethenyl)-2,2-dimethylciclopropane carboxylic acid and α-cyanogroup-3-phenoxybenzyl alcohol; the latter is transformed into 3-PBA [23,36]; cypermethrin can also to be degraded by carboxylesterases through hydrolysis of the carboxyl ester bond, resulting in 3-(2,2-dichloroethenyl)-2,2-dimethylciclopropane carboxylic acid and 2-hydroxy-2-(3-phenoxyphenyl) acetonitrile [24,36]. The former is converted into CO_2 [36]. To selectmicroorganisms good for bioremediation is necessary so that the cypermethrin-degrading strain can transform pyrethroid into non-toxic intermediate metabolites. These bacteria first could be used for in situ treatment of bioaugmentation in an environmentally-sustainable strategy to reduce cypermethrin levels in cypermethrin-contaminated sediment. According to Chun et al. [37], the stimulation of PCB-dechlorinating and degrading microorganisms with electron-donors/-acceptors addition contributed to the degradation of PCBs in sediment.

There is an approach that made the biodegradation of β-cypermethrin and 3-PBA more efficient—using a coculture of *Bacillus licheniformis* B-1 and *Aspergillus oryzae* M-4 [38].

The enzyme laccase uses molecular oxygen and a phenolic substrate for degrading cypermethrin [17,24,39]. *Laccase* and *pytH* genes were present in all strains, but the *estA* gene was present only in *Pseudomonas* sp., MS15a and *Pseudomonas* sp. MS19 (*in silico*). Amplification of cypermethrin-degrading genes in the selected bacterial strains revealed that the *pytH* and *laccase* genes were present in all studied strains (data not shown). Kubicki et al. [40], have described some biosurfactants produced by marine microorganisms [40]. Our results suggest that both *Rhodococcus* sp. synthesize viscosin, arthrofactin, and putisolvin; *Pseudomonas* sp. M15a synthetizes viscosin, amphisin, and putisolvin; and *Pseudomonas* sp. MS19 synthesizes rhamnolipid, amphisin and arthrofactin; this indicates that our studied strains have a variety of surface-active metabolites.

In summary, these four cypermethrin-degrading and biosurfactant-producing bacterial strains isolated from marine sediments have great biotechnological potential for biodegradation of cypermethrin-contaminated marine sediments.

4. Materials and Methods

4.1. Sampling Site

Sediment samples were collected in April 2018 from cypermethrin-contaminated sediments ($n = 9$). These samples were obtained in triplicate from three sampling sites, near a salmon farm in the Manao Bay, Ancud, located in the district of Chiloé (S41°51′16.6″ W73°29′05.6″), at 25 meter depth (Figure 1a). Samples were transported and stored at 4 °C until analyses.

4.2. Culture Conditions

Bacteria were cultivated in Bushnell–Haas (BH) broth medium (composition per L: 1 g KH_2PO_4; 1 g K_2HPO_4; 1 g NH_4NO_3; 0.2 g $MgSO_4$; 0.02 g $CaCl_2$; and 0.05 g $FeCl_3$ at pH 7.0), containing 50 mg L^{-1}cypermethrin (Merck®) as the sole carbon source. Cypermethrin-degrading and biosurfactant-producing bacterial strains were grown in minimal ethanol salts medium (ETMS) (composition per L: 22.2 g $K_2HPO_4 \times 3H_2O$; 7.26 g KH_2PO_4; 0.2 g $MgSO_4 \times 7H_2O$; 0.4 g $(NH_4)_2SO_4$) and 25 mL of absolute ethanol (Merck®) [41]. Strains were also cultivated in Trypticase Soy Agar (TSA) (Difco®) and Trypticase Soy Broth (TSB) (Difco®). Cultures were grown at 28 °C in a gyratory shaker (model 3016A, Labtech) at 140 rpm. Cell growth was determined by optical density (O.D.) at 600 nm.

4.3. Isolation of Bacterial Strains from Cypermethrin-Contaminated Marine Sediments

Using enrichment culture techniques, several bacterial species were isolated (Figure 1b). To do this, a 5 g sample of marine sediment was added to a 250 mL Erlenmeyer flask containing 50 mL of sterilized

BH enrichment medium with an initial concentration of 50 mg L^{-1} of cypermethrin and incubated at 28 °C in a rotary shaker at 140 rpm for five days. After that, 5 mL of enrichment culture was inoculated into 50 mL of fresh BH medium containing 100 mg $L^{-1}$of cypermethrin. Later, the enrichment culture was incubated five days in fresh enrichment medium containing 200 mg L^{-1} of cypermethrin.

The final culture was serially diluted and spread on B.H. agar plates containing 50 mg L^{-1} cypermethrin for incubation at 28 °C for five days. To inhibit eukaryotic cell growth, 100 and 200 mg L^{-1} of cycloheximide (Sigma, St. Louis, MO, USA) were added to the first and second enrichments, respectively [11]. Plating was repeated until obtaining pure cultures and these were checked through Gram-staining using an Eclipse Ni-U (Nikon, Tokyo, Japan) optical microscope with 1000× magnification (Table S1). Isolates were stored in glycerol (15%) and kept at −80 °C until use. Strains (Table S2) were deposited in GenBank (MK271075, MK271077, MK271079, MK271080, MK271081, MK271082, MK271083, MK271084, MK271085, MK271086, and MK271087).

4.4. 16S rDNA Gene Sequencing

A colony of each isolated strain was obtained from the TSA medium, transferred to the TSB medium, and grown to OD_{600nm} = 0.6. Cells were harvested by centrifugation, and genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to manufacturer's recommendations. Universal primers 27F (5′-AGA GTT TGA TCA TGG CTC AG-3′) and 1492R (5′-CGG TTA CCT TGT TAC GAC TT-3′) were used to amplify 16S rDNA [42]. Master Mix containing *Taq* DNA polymerase (Promega, Madison, WI, USA) was used for PCR-amplification. PCR thermal cycling conditions for the amplification of 16S rDNA genes were: initial denaturation for 4 min at 94 °C, followed by 30 cycles of 30 s at 94 °C denaturation temperature; 1 min at 58 °C as annealing temperature; 1 min at 72 °C as extension temperature; and a final extension of 7 min at 72 °C. PCR products were visualized through agarose gel electrophoresis. PCR products from 16S rRNA amplification were sequenced by Macrogen (Seoul, Korea), using the primer 800R (5′-TAC CAG GGT ATC TAA TCC-3′).

4.5. Determination of Emulsion Index (E_{24})

Emulsion activity was measured by mixing 2 mL of a cell-free supernatant obtained from cells grown in test tubes up to stationary phase (OD_{600nm} = 3.0) with 2 mL of petroleum diesel. The mixture was vortexed at high speed for 2 min. After vortexing, the tubes were left resting for 24 h and the emulsion layer was measured. The emulsion index (E_{24}) was calculated by dividing the height of the emulsion layer by the total height and multiplying that by 100 [43].

4.6. Scanning Electron Microscopy (SEM)

To study the bacterial morphologies of the isolated marine strains, cells were grown in TSB medium to stationary phase (OD_{600nm} = 3.0) and observed by scanning electron microscopy. For this, cells were prepared for SEM, washed with phosphate-buffered saline, and fixed with 2.5% glutaraldehyde at room temperature. Cells were gradually dehydrated in a series of ethanol dilutions and subsequently dried. Samples were then coated with a layer of palladium and examined under 15 kV accelerating voltage in an LEO-420 field emission scanning electron microscope (LEO electron microscopy, Carl Zeiss, S.M.T., Oberkochen, Germany).

4.7. Phylogenetic Analysis

Taxonomy was primarily assigned using sequences of 16S rDNA obtained by BLAST for comparison against the NCBI Non Redundant database. The sequences of strains under study and of type strains were aligned using Mega 6.0 program (Philadelphia, PA, USA) from a region of 600 bp approximately, and a phylogenetic tree was built. Phylogenetic distance between sequences was calculated with the neighbor-Joining algorithm and a bootstrap of 1000. Bootstrap values greater

than 50% are shown in Figure 1c. *Lactobacillus acidophilus* 16S rDNA sequence was used as outgroup to root the tree.

4.8. Genome Sequencing and Sequence Information

Genome sequencing of MS13, MS15a, MS16, and MS19 bacterial strains was performed using Illumina Hiseq sequencing technology (Mr. DNA, Shallowater, TX, USA). Libraries were prepared using KAPA HyperPlus Kits (Kapa Biosystems, Wilmington, MA, USA) by following the manufacturer's user guide. The concentration of DNA was evaluated using the Qubit® dsDNA H.S. Assay Kit (Life Technologies, Madison, WI, USA) and the average library size was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Libraries were end-sequenced for 500 cycles. *De novo* assembly was carried out using C.L.C. Genomics Workbench version 11.0.1 under quality-filtered reads (Q ≥ 20; no more than two ambiguities; final read length ≥500 bp), with length and similarity cutoffs of 60% and 90%, respectively, and minimum contig length of 5000 bp. Genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline version 4.6, released in 2013 and approved on May 16th, 2019 [44]. Gene mining and genomic contexts were visualized using the RAST server [45]. Completeness of genome assemblies was evaluated with BUSCO software version 3.0.2 (Lausanne, Switzerland) and the bacteria subset version odb10 [46].

4.9. Identification of Secondary Metabolites, and Detection of Genes Involved in Cypermethrin Catabolism in MS13, MS15a, MS16, and MS19 Bacterial Marine Strains and a Subsequent Comparison with Reference Strains

Biosynthetic gene clusters (BGC) were determined through AntiSMASH bacterial version 5.0 [33] using the assembled genomes of selected isolated strains. The comparison was made with reference strains using NCBI information. Differences (discolored) or similarities (colored) of BGCs were established manually. To identify cypermethrin catabolic genes, specifically *est*, *pytH*, and *laccase* genes, *in silico* analyses were performed with a search by homology approach using BLAST and the following sequences as probes: AAB61674.1 (*est*), AEV51797.1 (*est*), AEY11370.1 (*pytZ*), RKM76030.1 (*laccase*), ERS87108.1 (*laccase*), ASO18034.1 (*pytH*), and KHF66595.1 (*pytH*). For genomic neighborhoods, five genes upstream and downstream to each *est*, *pytH*, and *laccase* gene were identified through the nucleotide and protein BLAST database (NCBI, Bethesda, MD, USA).

5. Conclusions

This study describes a taxonomic identification and biochemical characterization of cypermethrin-degrading and biosurfactant-producing bacterial strains isolated from a unique and extreme marine ecosystem in the Chilean Northern Patagonia. The availability of MS13, MS15a, MS16, and MS19 genome sequences offers new opportunities for systems metabolic engineering that could be useful for biodegradation of cypermethrin-polluted sediments by biosurfactants-producing microorganisms.

Supplementary Materials: The following information is available online at http://www.mdpi.com/1660-3397/18/5/252/s1. Table S1: Biochemical characteristics of cypermethrin-degrading and biosurfactant-producing bacterial strains isolated from the Chilean Northern Patagonia. Table S2: Identification of cypermethrin-degrading and biosurfactant-producing bacterial strains isolated from the Northern Chilean Patagonia by comparative sequence analyses.

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