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Review

Biotechnological Applications of Products Released by Marine Microorganisms for Cold Adaptation Strategies: Polyunsaturated Fatty Acids, Antioxidants, and Antifreeze Proteins

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Abstract: Marine organisms have developed a series of defense and adaptation strategies, permitting them to live and survive in peculiar environments, ranging from temperate to tropical and polar regions, high to low salinity areas and different light conditions, as well as are constantly exposed to variations induced by climate change and human activities. These defense strategies include the production of molecules and enzymes which may have applications for humans as well. In this review, we summarized the studies on bacterial and microalgal polyunsaturated fatty acids, antioxidants, and antifreeze proteins, which can find applications in different market sectors, such as feed and cosmetic fields. For all the aforementioned compounds, the compound annual growth rate is expected to increase by 5.35–36.3% in the near future, as the market interest toward these products is on the rise. Both industries and researchers are focused on developing mechanisms to reduce production time and costs, improve yields, and discover new proteins.

Keywords: cold environments; adaptation strategies; microalgae; marine bacteria; biotechnological applications; PUFAs; antioxidants; ice-binding proteins

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1. Introduction

Cold environments consist of various habitats, including the poles, permafrost-affected soils, glaciers, lakes, and deep seas. These environments are conventionally considered extreme and inhospitable, and therefore scarcely compatible with life. Temperature represents one of the key factors on which the growth and the biochemical reactions rate of microorganisms depend. Moreover, it increases the viscosity of solvents and solubility of gases, affects solute transport and diffusion, and causes ice formation and osmotic stress, producing negative effects on biological processes [1]. Therefore, the organisms inhabiting these cold places have evolved a series of mechanisms to deal with cold stress and survive in such harsh environments [2]. Although many studies have already been conducted, knowledge about these low-temperature adaptations is still limited. However, the advent of omics techniques has significantly contributed to the understanding of microbial cold adaptation strategies, with the description of biodiversity in terms of species composition and the identification of new key genes associated with the cold tolerance of microorganisms [3].

Strategies adopted by microorganisms in the cold adaptation process include the regulation of cell membrane fluidity, and the synthesis of cold-adapted proteins utilized to maintain regular physiological activities of organisms (e.g., cold shock proteins, ice-binding proteins (IBPs)), energy supply, regulators and metabolic changes, and reactive oxygen species (ROS) [2,4,5]. In particular, the low temperature decreases the fluidity of the cell membrane and the presence of branched-chain and polyunsaturated fatty acids (PUFA) playing an essential role in the process of adaptation of microorganisms to such extreme temperatures. Consequently, the regulation of fatty acid desaturase activity also affects cell

membrane fluidity [2,6,7]. In cold and oxygen-rich environments, the antioxidant defense also represents an important part of the developmental adaptation required to maintain steady-state ROS concentration [4]. In particular, cold-adapted microorganisms present an efficient enzymatic system essential for survival in the presence of ROS represented by antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase, peroxiredoxin, and desaturases, that can neutralize the effects of radicals [8]. In addition to enzymatic antioxidants, several antioxidant molecules are also produced by the microorganisms in cold environments, such as pigments (mostly carotenoids) [9] which, thanks to their peculiar characteristics, can be used in many commercial applications, including food, cosmeceuticals, and pharmaceuticals. Cold-adapted microorganisms survive challenges associated with freezing, also thanks to the production of IBPs, including antifreeze proteins (AFPs) and ice-nucleating proteins (INPs). These molecules bind to ice surfaces modulating the growth of ice crystals that could cause cell death, preventing the freezing of liquids inside the cells. Considering that IBPs are able to control ice crystal growth, they have garnered interest in recent years for many potential industrial applications [10–12].

The ability to withstand the cold natural stress conditions makes the cold-adapted microorganisms valuable for large-scale cultivation and production of certain high-value products. Sampling of these marine microorganisms is relatively simple compared to macroorganisms, and there is also the possibility of culturing them in laboratory conditions at both small and large scale in order to increase biomass production. Marine microorganisms from cold environments are characterized by huge biodiversity and recent studies have continuously described the discovery of new species. This is also correlated by a large diversity of possible bioactivities and compounds they may produce [13]. Recently, scientific interests have been focused not only on the understanding of their biodiversity and physiology, but also on their possible biotechnological exploitation, such as the use of marine molecules in agriculture, pharmaceutical, and bioplastic and biofuel production. In the literature, previous reviews are available on bacteria and microalgae from cold environments. Lauritano et al. [1] reviewed bacterial and microalgal physiological and molecular responses in polar marine environments. Other reviews were specific to only microalgae. For example, some authors mainly focused on molecular studies applied to polar microalgae, also discussing possible links between gene loss/expansion and gene expression variations with biochemical and physiological data [14,15]. Montuori et al. reviewed bioactivity information on cold microalgae or algae exposed to cold stress laboratory conditions [16], while Malavasi et al. focused on extremophile microalgae, including three cryophilic/psychrophilic ones (*Chlamydomonas nivalis*, *Mesotaenium berggrenii*, and *Raphidonema* sp.), also discussing their potential astrobiology exploitation [17]. As summarized by [18], various patents are also available for products derived from polar microalgae but these are mainly related to cosmetic ingredients (i.e., carotenoids and mycosporine-like amino acids) and AFPs for industrial applications and chemical processing. Similarly, marine cold-adapted bacteria were studied in terms of their symbiotic associations [19], life in cold habitats [20], and possible applications [13,21,22].

This review will focus on products from cold-adapted bacteria and microalgae produced as defense strategies in cold habitats which also have potential industrial applications (Figure 1), such as PUFAs, antioxidant systems (including metabolites and enzymes), and AFPs. The reason why we focused our review on bacteria and microalgae is that they are known to allow an environmentally safe and sustainable approach for possible exploitation, the fields of applications are variable (ranging from cosmesis, feed, pharmaceuticals to biofuel production), and the market interest toward these products is expected to increase in the near future. In fact, the global microbial products market size is projected to reach approximately USD 302 billion by 2030, recording a compound annual growth rate (CAGR) of 5.35% from 2022 to 2030 (https://www.precedenceresearch.com/microbial-products-market; accessed on 15 May 2023). Moreover, according to Applied Market Research (https://www.alliedmarketresearch.com/microalgae-market/amp; accessed on 15 January 2023),

the microalgal global market was valued USD 977.3 million in 2020, and is expected to reach USD 1485.1 million by 2028 with a CAGR of 5.4%. In fact, the microalgal industry mainly focuses on PUFA, pigments, or whole microalgal cells, and both industry and scientific communities are looking for new strains or new culturing conditions in order to rapidly produce high amounts of the these compounds [23].

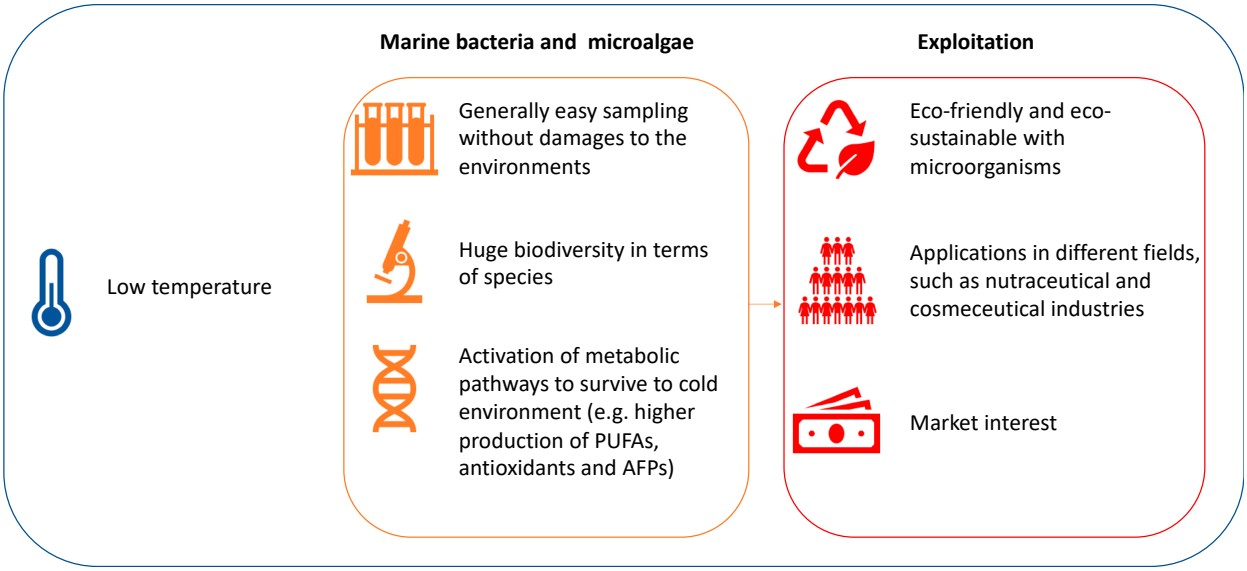

Figure 1. Schematic representation of review subjects: Exploitation of high value molecules from marine bacteria and microalgae from cold environments. PUFAs stand for polyunsaturated fatty acids, while AFPs for antifreeze proteins.

2. Polyunsaturated Fatty Acids (PUFAs) from Cold-Adapted Microalgae and Bacteria

PUFAs are essential to the health and survival of marine organisms. They are a class of straight-chain fatty acids containing two or more double bonds. They include ω-3 and ω-6 (double bond is on the third and sixth carbon atom opposite to the carboxyl group, respectively) [2,24]. The n-3 PUFAs, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Figure 2), are essential compounds for human health and have been shown to possess several bioactivities (e.g., antioxidant and anti-inflammatory) which have positive effects on different human diseases, such as cardiovascular disorders, diabetes, obesity, and cancer [25]. Recent studies also suggested that the immune modulatory properties of EPA and DHA provided in emulsions may be beneficial for SARS-CoV-2-infected patients [26]. In addition to the health field, PUFAs are of interest in aquaculture, feed industry (e.g., Veramaris® Pets and AlgaPrime™) and may find also cosmetic applications [25,27].

The awareness of the huge benefits of PUFAs together with the lack of enzymes necessary for their synthesis in humans has led to an increase in PUFAs' commercial production. In fact, the global PUFAs market is projected to reach around USD 9718 million by 2032, with a CAGR of 5.4% from 2022 to 2032 (https://www.futuremarketinsights.com/reports/polyunsaturated-fatty-acids-market; accessed on 15 May 2023). At present, fish oil is the main commercial source of PUFAs. However, the decrease in the fish population, extensive purification procedures with high costs, together with the fact that fish oil oxidizes easily and, therefore, is often associated with an unpleasant taste and smell, hinder its production [28]. For this reason, the research for new sources of PUFAs is increasing. Microorganisms represent a very promising PUFAs source, as the production is more eco-sustainable and less harmful to the environment, compared to fish oil production. Furthermore, they generally produce single PUFAs in high quantities with high oxidative stability, resulting in reduced purification and production costs [29]. However, various studies are in progress to investigate higher trophic levels (e.g., invertebrates) which have been shown to have metabolic pathways to produce novel and unique PUFAs [30].

Colombo et al. [31] showed how the PUFA content of an organism varies depending on the taxonomic groups studied as well as on the sampling geographic zone (i.e., latitude) and trophic level (a function of diet). In addition, they showed that the contents of omega-3 long-chain (i.e., ≥20 carbons) PUFAs were higher in polar and temperate marine organisms than those from the tropics [31]. PUFA production by microorganisms living in cold environments is thought to be mainly involved in the maintenance of membrane fluidity as cold-adaptation strategy [32].

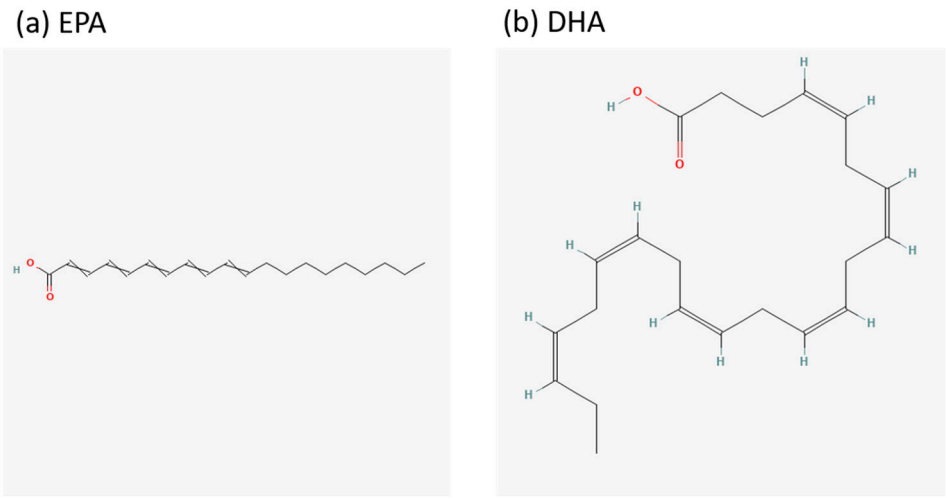

Figure 2. Chemical structure of (**a**) eicosapentaenoic acid (EPA) and (**b**) docosahexaenoic acid (DHA) from the database PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/135002#section=2D-Structure and https://pubchem.ncbi.nlm.nih.gov/compound/445580#section=2D-Structure, respectively; accessed on 16 January 2023).

Bacteria able to produce PUFAs have often been isolated from permanently cold and/or high-pressure environments (e.g., polar or deep sea habitats) [33]. In fact, in cold habitats, the presence of specific fatty acids in the bacterial membrane is essential to allow the transport of nutrients and preserve fluidity. Furthermore, they have also been shown to protect the microbial membrane against oxidative damage [33–35]. Interestingly, an additional advantage of using bacteria compared to other sources for the production of PUFAs is the absence of heavy metals in the bacterial biomass, the stability of the oil obtained [36], and the possibility of genetically manipulating bacteria to obtain increasing PUFAs synthesis [37].

Marine bacteria are among the major microbial producers of PUFAs, including genera of *Shewanella*, *Photobacterium*, and *Vibrio* that mainly produce EPA, and DHA-producing *Colwellia*, *Moritella*, and *Psychromonas*. For example, the Antarctic *Shewanella livingstonensis* Ac10 produces a large quantity of EPA when grown at 4 °C. Moreover, Kawamoto et al. demonstrated that EPA is important for the adaptation of the bacterium at low temperature, as the growth of the Ac10 EPA-deficient mutant was severely slowed down at 4 °C, while it resumed following the addition of EPA into the culture medium [38,39]. Furthermore, mutagenesis studies showed that DHA can only partly substitute the EPA biological function in this bacterium [40]. Similarly, the marine bacterium *Shewanella piezotolerans* WP3 isolated from deep-sea sediments of the Western Pacific Ocean (depth of 1914 m) also contains EPA in the membrane. The loss of EPA led to defects in growth at low temperature (4 °C) and high pressure (20 MPa), suggesting that EPA is important for the adaptation of this bacterium under extreme conditions [41–43]. However, in some bacteria, the inhibition of EPA production can be replaced by a high amount of monounsaturated fatty acids or branched-chain fatty acids, demonstrating that EPA is not essential for the growth at low temperature of some particular species, including *Photobacterium profundum* SS9 [44] and *Shewanella marinintestina* IK-1 [45]. Usui et al. demonstrated that EPA produced by the deep-sea *Shewanella violacea* strain DSS12 collected from the Ryukyu Trench at the depth of

5110 m, escaped the membrane from becoming hyperfluid and preserved the stability of the membrane by avoiding structural changes due to high pressure [42]. Moreover, EPA was essential for cell division under high pressure in the *S. violacea* strain DSS12 [46].

Several strategies have been developed for the production of large quantities of PUFA in bacteria, including the cerulenin treatment, nitrogen supplementation, low temperature, as well as metabolic engineering, as reviewed by [33,47]. For example, DHA production by the psychrophilic deep-sea bacterium *Moritella marina* MP-1 [48] (originally designated *Vibrio marinus* [49]) can be increased nearly eight-fold by modifying the growth media [50]. In particular, the addition of supplemental nitrogen and the acidification of the media at pH 6.0 significantly increased the DHA production in the bacterium, with a final overall titer of 82 ± 5 mg/L in marine broth supplemented with glycerol, yeast extract, and tryptone, compared to 11 ± 1 mg/L in the unsupplemented media [50]. Moreover, a statistical approach incorporating the Plackett–Burman design and response surface methodology (RSM) was also used for the identification and optimization of parameters affecting PUFA production in the Antarctic marine bacterium *Kocuria* sp. BRI 35 [51]. PUFA production maximized in the presence of lower amount of magnesium sulfate (0.9 g/L) and higher concentrations of peptone protease (5 g/L) and glucose (10 g/L) at 15 °C, increasing the production of PUFA per unit biomass from 0.94 mg/g to 11.12 mg/g, thereby showing potential for the commercial PUFA production [51]. Similarly, the Plackett–Burman design was also used to optimize EPA production in *Vibrio cyclitrophicus* isolate 560. Thanks to this method, the EPA amount increased to 7.5 mg/g dry weight and 10% of the total fatty acid [52]. RSM proved that temperature, dissolved oxygen, and pH significantly affected EPA production in the bacterial isolate 717 collected from deep sea core sediment in the Mid-Atlantic Ridge. This strategy allowed for an increase in the production from 9 mg/g biomass to 45 mg/g [36].

Marine bacteria producing PUFAs were summarized in [33]. Despite the huge potential of these molecules, surprisingly only a few are PUFA-producing cold-adapted marine bacteria recently isolated, probably due to the costs and difficulties in sampling in such extreme environments, suggesting that more studies focused on this topic should be conducted. Here, we report marine bacteria that produce PUFAs and can live in low temperatures (Table 1).

Table 1. Cold-adapted marine bacteria producing polyunsaturated fatty acids (PUFAs) studied in the last 20 years. Abbreviations EPA and DHA stand for eicosapentaenoic acid and docosahexaenoic acid, respectively.

Bacteria	Origin	PUFA	Amount	Reference
Shewanella livingstonensis	Antarctic seawater	EPA	5% *	[38]
Shewanella piezotolerans WP2	Deep-sea sediments (1914 m depth), Western Pacific Ocean	EPA	7% *	[41]
Shewanella piezotolerans WP3	Deep-sea sediments (1914 m depth), Western Pacific Ocean	EPA	13% *	[41]
Photobacterium frigidiphilum SL13	Deep-sea sediments (1450 m depth), Pacific Ocean	EPA	6% *	[53]
Moritella marina MP-1	Deep sea	DHA	82 ± 5 mg/L in marine broth supplemented with glycerol, yeast extract, and tryptone, pH 6.0	[50]
Kocuria sp. BRI 35	Antarctic seawater	DHA	11.12 mg/g with 0.9 g/L magnesium sulfate, 5 g/L peptone protease, 10 g/L glucose	[51]
Vibrio cyclitrophicus isolate 560	Deep-sea sediments, Mid-Atlantic ridge	EPA	7.5 mg/g in bioreactor cultivation, 7.9 g/L peptone, 16.2 g/L NaCl, and 6.2 g/L yeast extract	[52]

* Percentage of PUFAs from the total fatty acids.

Among microorganisms, microalgae have been reported to be a major producer of PUFAs in the marine environment. Microalgae-based products are nowadays used in food or feed supplements. There are in fact a series of species that have been classified as food sources falling into the GRAS (generally recognized as safe) category [54]. Thanks to their properties, several species are considered as providers of health-benefiting molecules that contribute to the well-being of humans and animals [55]. According to the Applied Market Research (https://www.alliedmarketresearch.com/microalgae-market/amp; accessed on 15 January 2023), the ω-3 global market was estimated to be USD 2.49 billion in 2019 and a CAGR of 7% between 2020 and 2027 is expected [56].

Microalgae PUFAs also play key roles as cellular components of membranes, energy storage, and as precursors of various signaling molecules [57,58]. Different studies have shown how microalgal species from cold environments can be a valuable source for PUFA production. Different strains have been evaluated and different culturing volumes (also by using bioreactors) and conditions were tested using the OSMAC (one strain many compounds) approach [59]. Teoh et al. studied the fatty acid profiles of six Antarctic microalgae, named *Chlamydomonas* UMACC 229, *Chlorella* UMACC 234, *Chlorella* UMACC 237, *Klebsormidium* UMACC 227, *Navicula* UMACC 231, and *Stichococcus* UMACC 238, cultivated under different temperature regimes ranging from 4 to 30 °C [60]. They found that the variations in fatty acid profiles depended on the species. PUFAs were predominant in *Chlamydomonas* UMACC 229, while *Navicula* UMACC 231 was the only species found to produce EPA. In particular, they also observed that the percentage of PUFA, especially EPA, in *Navicula* UMACC 231 decreased as the culture temperature increased.

In 2018, *Nannochloropsis salina* (CCMP1776) from North Atlantic Ocean was also shown to produce a high quantity of PUFAs (102.93 ± 1.90 mg/g *N. salina* dried biomass) and, in particular, EPA at 5 °C (91.26 ± 3.81 mg/g *N. salina* dried biomass), while triacylglycerols with EPA at all three acyl positions were higher at 15 °C [61]. In 2019, Schulze et al. studied eight cold-adapted microalgal strains belonging to different genera, i.e., *Chlamydomonas*, *Chlorella*, *Tetraselmis*, *Pseudopleurochloris*, *Nannochloropsis* and *Phaeodactylum*, and evaluated their capability of producing fatty acids in different conditions of light (50–100 µmol s^{-1} m^{-2}) and temperatures (8–15 °C) [23]. In particular, they found that, the Arctic species *Chlamydomonas* sp. (RCC 2488) had the highest PUFA productivity (65 mg L^{-1} d^{-1}) at low temperature/light, while *Pseudopleurochloris antarctica* (SAG 39.98) had the highest eicosapentaenoic acid productivity (7.6 mg L^{-1} d^{-1}).

In the same year, Suzuki et al. [62] studied the cold-adapted microalga *Koliella antarctica* SAG 2030 (Trebouxiophyceae) and investigated various growth conditions (i.e., nitrogen and phosphorus starvation, salinity, and light intensity) to identify the best conditions to optimize PUFA production in bubble-tube and flat-plate photobioreactors. The authors found that nitrogen and phosphorus starvation induced triacylglycerol (TAG) accumulation, of which PUFAs accounted for 30.3–45.8%. The highest EPA content of 6.7 mg g^{-1} dry weight was observed in control treatments. Recently, Morales-Sánchez et al. also studied PUFA production by the polar marine microalga *Chlamydomonas malina* (RCC2488) [7]. In order to identify the best culturing condition to obtain high quantities of the products of interest, they cultured the species at 8 °C, at different salinities (0–80 ppt) and light intensities (70–500 µmol photons m^{-2} s^{-1}), as well as with different nitrogen concentrations (presence or absence of $NaNO_3$) using bubble column and flat-panel photobioreactors. Results showed that the highest PUFA (85.4 mg L^{-1} day^{-1}) productivity was obtained with the salinity of 17.5 ppt, light intensity of 250 µmol photons m^{-2} s^{-1}, and nitrogen-replete conditions. Overall, these studies suggested species-dependent PUFA production, with an increase at lower temperatures (*Chlamydomonas* sp. and *N. salina* were among the species which showed the highest PUFA production) (summarized in Table 2).

Table 2. Cold-adapted microalgae polyunsaturated fatty acids (PUFAs) production. EPA abbreviation stands for eicosapentaenoic acid.

Microalgal Name	Code	Culturing Information	Results on PUFA Production	Reference
Chlamydomonas *Chlorella* *Chlorella* *Klebsormidium* *Navicula* *Stichococcus*	UMACC 229 UMACC 234 UMACC 237 UMACC 227 UMACC 231 UMACC 238	Temperatures tested 4–30 °C	PUFAs were predominant in *Chlamydomonas*, while *Navicula* was the only species found to produce EPA which decreased with increasing culture temperature.	[60]
Nannochloropsis salina	CCMP1776	Temperatures tested 5–15 °C	Highest PUFAs (102.93 ± 1.90 mg/g *N. salina* dried biomass) and EPA (91.26 ± 3.81 mg/g *N. salina* dried biomass) concentrations at 5 °C.	[61]
Chlamydomonas sp. *MALINA* *Chlamydomonas* sp. *CEFAS* *Chlorella stigmatophora* *Tetrasemis chuii Butcher* *Tetraselmis* sp. *Pseudopleurochloris antarcti-ca* *Nannochloropsis granulata* *Phaeodactylum tricornutum*	RCC 2488 RCC 2607 RCC 661 SAG 1.96 RCC 2604 SAG 39.98 RCC 2478 RCC 641	Different conditions of light (50–100 µmol s^{-1} m^{-2}) and temperatures (8–15 °C)	*Chlamydomonas* sp. RCC 2488 had the highest PUFA productivity (65 mg L^{-1} d^{-1}) at low temperature/light, while *Pseudopleurochloris antarctica* had the highest EPA productivity (7.6 mg L^{-1} d^{-1}).	[23]
Koliella antarctica	SAG 2030	Nitrogen and phosphorus starvation, variation in salinity and light intensity	Nitrogen and phosphorus starvation induced triacylglycerol accumulation, of which PUFAs accounted for 30.3–45.8%. The highest EPA content of 6.7 mg g^{-1} dry weight was observed in control treatment.	[23]
Chlamydomonas malina	RCC 2488	8 °C, salinities (0–80 ppt), light intensities (70–500 µmol photons m^{-2} s^{-1}), presence or absence of $NaNO_3$	Conditions for the highest PUFA (85.4 mg L^{-1} day^{-1}) productivity were 17.5 ppt, 250 µmol photons m^{-2} s^{-1}, nitrogen repletion.	[7]

3. Antioxidants from Cold-Adapted Microalgae and Bacteria

Free radical production occurs continuously in all organisms because it is a part of normal cellular functioning. In low quantities, free radicals are rapidly converted to less reactive forms, but, if produced in high quantities, originating from endogenous or exogenous sources, they can induce damages to nucleic acids and proteins [63]. To detoxify free radicals, cells possess various defense systems characterized by molecules (e.g., glutathione, carotenoids, polyphenols, and vitamins) or enzymes (e.g., catalase, superoxide dismutase, and glutathione peroxidase). Generally, superoxide dismutase and catalase represent a first defense against oxygen toxicity, while the other enzymes together with the antioxidant compounds provide a unique defense system to prevent the negative effects due to ROS. Antioxidant molecules and enzymes prevent free radical-induced cell damages by avoiding the radicals formation, scavenging them, or by promoting their degradation [64]. According to Applied Market Research (https://www.alliedmarketresearch.com/anti-oxidants-market; accessed on 17 January 2023), the global antioxidants market size in 2020 was USD 3437.3 million and is projected to reach USD 7376.4 million by 2031 (with a CAGR of 6.9% from 2022 to 2031). Antioxidants may find applications in different fields, ranging from food and feed, personal care products, pharmaceuticals, fuel, lubricant, plastic, rubber, and latex additives [65] (https://www.alliedmarketresearch.com/anti-oxidants-market; accessed on 17 January 2023). Marine organisms have been shown to have the capability of producing a plethora of antioxidant molecules and enzymes. In particular, marine microorganisms from cold environments can be a potential source of natural antioxidants as adaptive responses to oxidative stress [1,8,66].

3.1. Metabolites

Several classes of microbial molecules have shown to exert antioxidant and scavenging activities. Marine microorganisms are a rich source of terpenes, terpenoids, and their derivatives that present different chemical structures with several bioactivities, including antioxidant activity [67]. Among them, carotenoids act as strong antioxidant compounds,

playing a fundamental role in the photo-oxidative protection process of the cells. They are chemical quenchers of singlet oxygen, acting as effective ROS scavengers, used as nutraceutical ingredients and/or cosmeceutical compounds for UV radiation protection [68]. The current nutraceutical and cosmeceutical markets based on marine products are also focused on carotenoids, due to their high market demand and value. The global carotenoid market is expected to reach USD 2.7 billion by 2031, registering a CAGR of 3.9% from 2022 to 2031 (https://www.alliedmarketresearch.com/carotenoids-market; accessed on 15 May 2023).

The synthesis of carotenoid pigments in cold-adapted marine bacteria has been demonstrated by genome mining studies in the orange-pigmented bacterium *Marisediminicola antarctica* ZS314T, isolated near the Chinese Antarctic Station [69]. Moreover, in the yellow and orange-pigmented bacteria *Cellulophaga fucicola* 416 and *Zobellia laminaria* 465, respectively, isolated from Antarctic sponges, zeaxanthin, β-cryptoxanthin and β-carotene were identified [70,71], together with two isomers of zeaxanthin found only in *C. fucicola* [70] and phytoene isolated only in *Z. laminarie* [71]. All pigments showed very high antioxidant activity, suggesting a possible use in the food and feed colorants [70,71]. A promising bacterial source of natural carotenoids is represented by the red-orange Arctic *Rhodococcus* sp. B7740, collected from 25 m deep seawater, in which rare aromatic carotenoids, such as synechoxanthin and isorenieratene, together with the common β-carotene were identified [72]. Isorenieratene is used in the smear cheese industry [73], and represents a very promising compound in potential food and medicine applications, due to its higher stability than common dietary carotenoids, such as β-carotene and lutein, in model gastric condition after ingestion [72]. It showed an excellent scavenging activity of both singlet oxygen and hydroxyl radicals able to prevent UV-B-induced DNA damage due to the auto-oxidation process [74].

In *Rhodococcus* sp. B7740, an isoprenoid quinone, a dehydrogenated menaquinone with eight isoprene units, was also isolated in high concentrations. It showed higher antioxidant activity and antiglycation capacity versus ubiquinone Q10 and MK4, suggesting potential applications in medicine [75].

Polar *Fragilariopsis pseudonana*, *Chaetoceros neogracile*, *Stellarima microtrias* and *Porosiara pseudodenticular* were studied and evaluated for the production of the antioxidant compounds, polyphenols and tocopherols [76]. Polyphenols include a group of approximately 8000 known compounds, divided into different classes based on their basic chemical structure [77]. Tocopherols are the major form of vitamin E [78]. The study found that methanol extracts of *Fragilariopsis pseudonana* had higher antioxidant activity (via ABTS assay) than other algae, and the polyphenol contents were higher as well [76]. In 2008, Janknegt et al. tested 15 microalgal species, belonging to three groups, i.e., Antarctic microalgae, temperate diatoms, and temperate flagellates, exposing them to simulated surface irradiance including ultraviolet radiation [79]. Analyzed species were the Antarctic *Chaetoceros brevis*, *Chaetoceros dichaeta*, *Chaetoceros* sp., *Nitzschia frigida*, and *Pyramimonas* sp.; the temperate diatoms *Odontella sinensis*, *Navicula salinarum*, *Navicula pelliculosa*, *Nitzschia ovalis*, *Thalassiosira weissflogii*; and the temperate flagellates *Tetraselmis suecica*, *Prorocentrum micans*, *Fibrocapsa japonica*, *Emiliania huxleyi*, and *Porphyridium purpureum*. They observed that antioxidant responses were highly species-specific. However, they found that the glutathione redox status was higher in the Antarctic than in the temperate species [79]. In 2012, Goiris et al. tested 32 microalgal biomass samples for possible antioxidant activity by using three antioxidant assays, total phenolic and carotenoid content. They found that the antioxidant activity varied strongly between species and was also dependent on the growth conditions and extraction solvent used. The highest antioxidant capacities were observed for the microalgae *Tetraselmis suecica*, *Botryococcus braunii*, *Neochloris oleoabundans*, *Isochrysis* sp., *Chlorella vulgaris*, and *Phaeodactylum tricornutum* [80].

A recent study explored the capability of nineteen species of Nordic microalgae to produce different bioactive compounds, including carotenoids or polyphenols [81]. Species were *Chlorococcum* sp. MC1, *Coelastrella* sp. 3–4, *Coelastrum astroideum* W10, *Coelastrum microporum* FNY-1, *Desmodesmus opoliensis* SQ2, *Desmodesmus* sp. RUC-2, *Desmodesmus* sp.

2–6, *Ettlia pseudoalveolaris* FNY-2, *Haematococcus pluvialis/lacustris* HP, *Monoraphidium* sp. B1–2, *Scenedesmus obliquus* 13–8, *Scenedesmus* sp. B2–2, *Scotiellopsis reticulata* UFA-2, *Chlorella saccharophila* (*Chloroidium saccarophilum*) RNY, *Chlorella sorokiniana* 2–21–1, *Chlorella sorokiniana* B1–1, *Chlorella vulgaris* 13–1, *Chlorella vulgaris* LNY and *Micractinium* sp. P9–1. The authors found that some of these strains were able to produce high amounts of carotenoids (over 12 mg·g^{-1} dry weight) and phenolic compounds (over 20 mg GAE·g^{-1} dry weight) and thus they were chosen for an additional high light-cold stress experiment (500 µmol·m^{-2}·s^{-1} and 10 °C). *Chlorococcum* sp. (MC1) and *Scenedesmus* sp. (B2–2) produced higher concentrations of carotenoids and phenolic compounds, showing higher antioxidant capacity upon stress.

Raw extracts of cold-adapted species also demonstrated antioxidant properties. In particular, Ingebrigtsen et al. tested five pelagic marine diatoms, *Attheya longicornis*, *Chaetoceros socialis*, *Chaetoceros furcellatus*, *Skeletonema marinoi* and *Porosira glacialis*, from the North Atlantic Ocean for possible bioactivities when cultured in different conditions of light and temperature [82]. They found antioxidant activity (using the ferric-reducing ability of plasma FRAP assay) for two fractions of the extract of *C. socialis* cultivated at low temperature–high light, two fractions of the extract of *P. glacialis* and all the three tested extracts of *S. marinoi*. However, the compounds responsible of the observed activity have not been identified yet. Subsequently, Ingebrigtsen et al. also tested field mixed samples collected from three locations along the coast of northern Norway and Spitsbergen using two antioxidant assays: the cellular antioxidant assay and the cellular lipid peroxidation assay. They found various levels of bioactivity in all the samples, suggesting that, in addition to single species culturing in laboratory conditions, complex marine natural samples can be an excellent source of bioactivities as well [83].

3.2. Cold-Adapted Enzymes

With the beginning of the study of marine microorganisms isolated from cold environments, many enzymes with potential market applications previously inaccessible have been identified. It has been shown that these cold-adapted enzymes can be used in various biotechnological sectors, from agricultural production to industrial processes, food chemistry [8,84], synthetic biology, and biomedical uses [85,86].

Microorganisms adapted to low temperatures produce enzymes with unique catalytic properties compared to their mesophilic counterparts, including higher catalytic efficiency, greater flexibility, and lower thermal stability. Therefore, cold marine environments are interesting research areas for enzyme discovery for several industrial applications where such characteristics are desirable. They are excellent biotechnological tools because they (i) are cost-effective as only lower quantities are required, thanks to the higher catalytic efficiency at low temperatures, (ii) catalyze the desired reactions at temperatures where unwanted chemical reactions are slowed and bacterial contamination is reduced, (iii) can be generally inactivated by moderate heat, thanks to their thermolability, thereby avoiding the use of harmful chemical reagents, and (iv) satisfy the need to reduce energy consumption, thus reducing environmental impacts [8].

Among the enzymes that catalyze reactions of industrial interest, many oxidoreductases have been described in microorganisms adapted to cold to defend cells against the negative effects of superoxide radicals.

Oxidoreductases have been also identified in cold-adapted bacteria. An example is the Arctic bacterium *C. psychrerythraea* 34H that possesses an accurate antioxidant defense system, thanks to the presence of three copies of the catalase gene and two superoxide dismutase genes, one of which contains nickel [87]. Moreover, studies carried out on *C. psychrerythraea* strain 34H have shown the presence of a phenylalanine hydroxylase, an oxidoreductase with high-catalytic efficiency at 10 °C, high thermostability and low affinity for the substrate, likely due to the high flexibility of the active site [88]. The characterization of the recombinant form of glutathione reductase isolated from the Antarctic *C. psychrerythraea* showed activity also at moderate temperatures when overexpressed in *E. coli*, suggesting potential industrial uses in the protection of cells and tissues from

oxidative stress [89]. The genome sequence of Antarctic marine bacteria *Pseudoalteromonas haloplanktis* TAC125 revealed the presence of two genes encoding an iron superoxide dismutase (sodB; PSHAa1215) and one catalase-encoding gene (katB), with a possible homologue PSHAa1737 [90]. Crystallographic studies on the Fe-superoxide dismutase showed high catalysis at low temperature with increased flexibility of the active site compared to their mesophilic homologues, without modifying the overall structure [91]. Moreover, a thioredoxin system consisting of thioredoxin and thioredoxin reductase, important for maintaining the reduced state of cytoplasmic proteins, was also identified in *P. haloplanktis* TAC125. Cold-active iron-containing superoxide dismutases were isolated and characterized from the psychrophilic bacteria *Marinomonas* sp. NJ522 [92] and *Pseudoalteromonas* sp. ANT506 [93] collected from Antarctic sea-ice, and *Psychromonas arctica* adapted to survive at subzero temperatures [94]. In the bacterium *Pseudoalteromonas* sp. ANT506, a glutathione peroxidase was also isolated. The biochemical characterization of the recombinant enzyme proved that the maximum catalytic temperature and pH value were 30 °C and pH 9.0, respectively. Moreover, when the temperature was reduced to 0 °C, the enzyme maintained 45% of the maximum activity [95]. Similarly, a cold-adapted typical 2-Cys peroxiredoxin isolated from the Antarctic psychrophilic bacterium *Psychrobacter* sp. ANT206 showed the optimum temperature for activity at 30 °C and also demonstrated good thermolability, pH stability, and salt tolerance. Moreover, it demonstrated possible pharmaceutical applications as it could protect the DNA from oxidative damage at 25 °C [96]. A catalase isolated from *Bacillus* sp. N2a (BNC) obtained from Antarctic seawater showed high catalytic efficiency at low temperatures allowing the bacterium to scavenge H_2O_2 efficiently [97].

Regarding microalgae, in 2008, Park et al. evaluated the effects of low temperature-induced stress exposure on the protein profile of the Antarctic microalga *Chaetoceros neogracile* using a proteomic approach. In particular, they cultured the alga at 4° and then cooled it to 0 °C [98]. After cold exposure, they found changes in the antioxidant enzymes superoxide dismutase, glutathione reductase, and glutathione S-transferase, suggesting that they are a part of *C. neogracile* survival mechanisms at low temperatures. Wang et al. studied the effects of UV-B radiation on antioxidant enzymes in the Antarctic sea ice microalgae *Chlamydomonas* sp. ICE-L [99]. They found that superoxide dismutase, peroxidase, and catalase activities were higher at high UV-B radiation intensity (70 μW cm^{-2}) than in the control. On the contrary, the ascorbate peroxidase activity was stable under the UV-B radiation enhancement stress exposure. Poong et al. [100] also studied the effects of short-term ultraviolet radiation stress in the Antarctic microalga *Chlorella* sp. using a transcriptomic approach. They performed RNA-sequencing of *Chlorella* sp. cultivated at ambient versus elevated UVR conditions (irradiation with two overhead white fluorescent tubes with irradiance of 50 μmol photons m^{-2} s^{-1}, two UV-A lamps with irradiance of 7.57 W m^{-2} and one UV-B lamp with irradiance of 1.29 W m^{-2} for 2 h at 4 °C). They found, between the differentially expressed genes, transcripts involved in the regulation of antioxidative mechanisms. In particular, superoxide dismutase and catalase were found to be down-regulated in response to UVR stress exposure. Recently, another transcriptomic study was performed for two Antarctic green algae (*Chlamydomonas* sp. ICE-L and *Tetrabaena socialis*) by Zhang et al. [101] in order to investigate their adaptation strategies to cope with extreme cold conditions (cultured at −5 °C and 5 °C). Results revealed considerable shared positively selected genes and showed that they possess multiple protective mechanisms, including genes related to antioxidants, such as carotenoid and antioxidant enzymes. In particular, they found ZDS1 gene encoding zeta-carotene desaturase involved in carotenoids synthesis and CAT2 gene encoding Catalase 2 which can protect cells from hydrogen peroxide.

4. Ice-Binding Proteins from Cold-Adapted Microalgae and Bacteria

IBPs are macromolecules present in organisms that survive at low temperatures. They are characterized by the peculiar ability to interact specifically with ice crystals, and are involved in freeze injuries protection, affecting ice nucleation, growth, and re-

crystallization. Among them, AFPs are capable of protecting organisms from damage caused by freezing, by binding irreversibly to ice crystals and inhibiting their growth through the adsorption–inhibition mechanism [102], causing thermal hysteresis (TH) and ice recrystallization inhibition (IRI). AFPs have been identified in different cold-adapted organisms, including fish [103,104], insects [105,106], plants [107,108], and microorganisms, such as bacteria [109], fungi [110,111], yeasts [112–114], and microalgae [115,116], and are involved in their survival at subzero temperatures. They were first discovered in the Antarctic Notothenioidei [117], but considering the high cost of purification and the problems encountered with the large-scale recombinant production of these fish proteins, they were instead researched on other organisms [118]. For this reason, the discovery of AFPs synthesized by microorganisms adapted to live in ice-filled habitats garnered great interest.

In general, AFPs share a common function of binding to ice crystals, although there are important diversities in their amino acid sequences, molecular weights, structures, and antifreeze activities [119]. The latter depends mainly on differences in their ability to bind ice surfaces to prevent ice growth [120,121]. However, some sequence characteristics were common in AFPs identified from different species, including Cys, Ala-Ala, and Trp-Gly content, and amino acid distribution correlated with the propensity for disturbance [122].

Another extremely interesting type of IBPs are the ice-nucleating proteins (INPs), which promote ice formation at high subzero temperatures (−2 °C to −10 °C). They have been found in plants, insects, and bacteria, and help in freezing survival by controlling the position of ice in the organisms, thus maintaining the ice extracellularly and saving the cytosol from freezing [11]. INPs with higher activity are generally bacterial proteins. In particular, these proteins are widespread in several Gram-negative, pathogenic, and epiphytic bacteria [123,124]. However, little is known about these proteins, although several INP-producing bacteria have been identified. Furthermore, these proteins tend to misfold and aggregate during expression and purification processes due to their large size [12].

The use of IBPs in industrial processes involving ice growth control, e.g., in improving cryopreservation, ice-templating strategies, and gas hydrate inhibition, as reviewed by [10], offers enormous technological advantages. For example, AFPs properties, including the depression of the freezing point of water or protection from recrystallization during storage, make these proteins useful in commercial applications, such as in food industry [125], agriculture [108], cryosurgery, and cryopreservation of cells [126], tissues [127], and organs [128]. According to Markets and Markets (https://www.marketsandmarkets.com/Market-Reports/antifreeze-protein-market-264931272.html?gclid=CjwKCAjwpayjBhAnEiwA-7ena_WGXCnB0NXxpqCtkatYjpFmnRYd3vczUojzH86V2TH1pIvjbQkJhxoCFKoQAvD_BwE; accessed on 22 May 2023), the global market for AFPs has been valued at USD 5.75 million in 2021 with a CAGR of 36.3% from 2021 to 2026. Moreover, INPs are presently used for artificial snow production [129], and could be used for other interesting applications, including frozen foods and beverage industries [130], microfluidic devices [131], cell surface display [132], and climate control [133].

However, there are only a few studies in the literature describing IBPs produced from bacteria to date, and their possible applications have only been established in the laboratory. A hyperactive AFP was identified in the Gram-negative bacterium *Colwellia* sp. strain SLW05 collected from sea ice on the west side of the Antarctic Peninsula [134]. Studies on the recombinant AFP showed TH activity of approximately 4 °C at 0.14 mM, and induced rapid growth of ice crystals in the hexagonal direction [135]. Its hyperactivity is probably due to binding to multiple planes of ice by a compound ice-binding site without repetitive sequence motifs, located at a flat surface of the β-helix. Interestingly, an IBP gene is missing from the genome of *C. psychrerythraea* 34H isolated from presumably ice-free Arctic marine sediments. Similarly, hypothetical IBPs are not present in the genomes of Antarctic marine bacteria *Actinobacterium* PHSC20C1 or *Pseudoalteromonas haloplanktis* TAC125, suggesting a role related to survival in an icy environment for these proteins [134].

An IBP was isolated from the bacterium *Marinomonas primoryensis* collected from the Antarctic saline lakes in the Vestfold Hills [136] that originated from the sea during the Last Glacial Maximum about 10,000 years ago. For this reason, these lakes are ice-covered and characterized by salt water (approximately half as saline as seawater) [137]. The protein was originally thought to be a hyperactive and exceptionally large (1.5 MDa) AFP with unusual properties, including Ca^{2+}-dependence, and it demonstrated over 2 °C of freezing point depression at 0.5 mg mL^{-1} [136]. Subsequently, a new role has been assigned to the protein: an ice adhesin that transiently binds the organism to ice, rather than preventing its growth or recrystallization. Probably, the attachment to ice could keep the bacterium close to nutrients and oxygen in the phototrophic zone [138,139]. Moreover, its X-ray crystallographic structure elucidated the ice-binding mechanism, demonstrating that anchored clathrate waters bind the protein to ice [140].

An IBP (*Efc*IBP) was identified in the metagenome of the marine bacterial community associated with a cold-adapted ciliate *Euplotes focardii* collected from the coastal sediments of Terra Nova Bay, Antarctica. The recombinant protein showed freeze–thaw stability, moderate cryo-protection of proteins and whole bacterial cells, and remarkable IRI activity, even at very low concentrations, suggesting an involvement of the protein in the survival of the whole-cell consortium [141].

Regarding microalgae, Kang and Raymond showed that an IBP from the sea ice diatom *Navicula glaciei* Vanheurck was able to reduce freeze–thaw damage to red blood cells [142]. Subsequently, Janech et al. [143] identified the source of *N. glaciei* ice-binding activity as a 25 kDa protein and suggested that *Fragilariopsis cylindrus* Grunow can express a similar protein. Thereafter, various studies focused on the psychrophilic diatom *Fragilariopsis cylindrus*, dominant in polar environments and adapted to extremely low temperatures and high salinities. Krell et al. [144] studied expressed sequence tags (EST) and looked for genes putatively involved in acclimation to salt stress in this diatom. They found four full-length open reading frames (ORFs) with high similarities to IBPs. Bayer-Giraldi et al. [145] studied *F. cylindrus* and *Fragilariopsis curta* and showed that both had genes coding AFPs. They also showed that the expression of specific AFPs increased under stress conditions typical for sea ice. Protein phylogeny showed a broad distribution of AFPs, not only in polar organisms, suggesting that these proteins may also have other functions. One year later, Bayer-Giraldi et al. [146] studied the differential protein expression of *F. cylindrus* under cold stress. Recombinant AFP had freezing point depression comparable to the activity of other moderate AFPs, enhanced by high salinity. In addition, it induced strong inhibition of recrystallization at 1.2 and 0.12 μM at low and high salinity, respectively. Data suggested binding of AFPs to multiple faces of the ice crystals. Very recently, Eickhoff et al. also investigated the ice nucleation activity of the diatom *F. cylindrus* using a microfluidic device, showing an increase of up to 7.2 °C in the ice nucleation temperatures for seawater containing the algae when compared to pure seawater [147].

Raymond et al. [148] reported four isoforms of another extracellular IBP from the Antarctic green alga *Chlamydomonas* cf. sp. strain CCMP681. These proteins showed strong recrystallization inhibition activity and had the ability to slow the drainage of brine from sea ice, and also had repeating TXT motif, typical of ice binding in insect antifreezes.

Two AFPs were also identified as produced by the Antarctic marine microalga *Pyramimonas gelidicola* (Chlorophyta) [115]. In particular, two transcripts were identified as coding AFPs, and both cDNA were cloned and expressed in *E. coli*. The corresponding proteins were found to show antifreeze properties based on the measurement of TH and morphological changes to single ice crystals. Authors also performed in silico protein structure predictions, showing that they fold as a right-handed β-helix flanked by an α-helix matching those of their homologs in fungi and bacteria. Another AFP was found in the marine diatom *Chaetoceros neogracile* (Bacillariophyta; Cn-AFP). In this case also, it was expressed in *E. coli* and antifreeze activity was determined [149]. The authors demonstrated an increase in Cn-AFP transcripts when the cells were subjected to freezing stress. Subsequently, Gwak et al. also demonstrated that its expression was rapidly stimulated

by high light stress [116]. The protein was localized to the intracellular space near the chloroplast membrane, thanks to immunogold labeling experiments and the ice-binding sites of Cn-AFP were studied via protein-folding simulation and site-directed mutagenesis [116]. Later, Kim et al. [150] identified another AFP in *Chaetoceros neogracile* and named it Cn-*iso*AFP which had 74.6% protein similarity with Cn-AFP. Cn-*iso*AFP transcription levels increased upon exposure to freezing (−20 °C), thermal (10 °C), or high light (600 µmol photon m^{-2} s^{-1}) stress. Finally, homology modeling and site-directed mutagenesis suggested that the activity was related to the flatness of B-face maintained via hydrophobic interactions [150].

Recently, the Arctic green alga *Chloromonas* sp. KNF0032 was studied using a transcriptomic approach [151]. The study found six *Chloromonas* IBP genes (CmIBPs) and tested the biological functions of three representative CmIBPs (CmIBP1, CmIBP2, and CmIBP3) using in vitro analysis and transgenic plant system, reporting CmIBP1 as the one with the most effective IRI activity. Recently, Bayer-Giraldi et al. also summarized methods to determine IBP activity (TH and recrystallization inhibition) and for protein activity characterization (ice pitting assay and determination of the nucleating temperature) [152].

5. Conclusions

Marine organisms have been reported to be valuable producers of compounds with possible applications for humans. Currently, there are 14 marine-derived drugs on the market, other are in clinical trials and other compounds are also used in the nutraceutical and cosmeceutical fields. Considering that various environments, such as the extreme ones, have been less investigated compared to the more easily reachable sites, many other bioactive compounds may be discovered and entered into the market. The current review is focused on specific compound classes produced by cold-adapted bacteria and microalgae as defense strategies in polar habitats, which also have potential industrial applications. In particular, this review reports PUFAs, antioxidant systems (including metabolites and enzymes) and AFPs which have been garnered the attention of the scientific community and industries. In recent years, PUFAs, especially EPA and DHA, have been frequently studied due to their beneficial effects on human health. They have been generally isolated from fishes or seaweeds [25], but due to overfishing, stock reduction, and climate changes, new sources are necessary and thus the attention now has also shifted to marine microorganisms. Moreover, the antioxidant defense is a fundamental aspect of evolutionary adaptations to cold, to cope with the increase in ROS levels in the cold waters, characterized by the increased oxygen concentration correlated to low temperature. For this reason, marine microorganisms represent an important source of natural antioxidants. Psychrophilic microorganisms are also capable of synthesizing IBPs, special proteins that modulate the growth of ice crystals, allowing them to survive in cold environments.

Industries and researchers are looking for alternative microorganism species, growth conditions, and technologies to increase the production of these important compounds at lower prices. Our review highlights that bacteria and microalgae biotechnology can be a very promising field for exploiting the natural production of high-value molecules from cold environments to fulfill the people needs.

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