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# Biotechnological applications of psychrophiles

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Low temperature environments are numerous on Earth and have been successfully colonized by cold-loving organisms termed psychrophiles. Cold-adapted microorganisms can be used as cell factories for the production of unstable compounds as well as for bioremediation of polluted cold soils and wastewaters. Furthermore, their biomolecules, mainly proteins and enzymes characterized by a high catalytic activity and pronounced heat-lability, have already found useful applications in various domains such as molecular biology, medical research, industrial food or feed technologies, detergents or cosmetics.

**Keywords:** psychrophiles; bioremediation; cold-active enzymes; heat inactivation; biomolecules



## Introduction

Amongst the various organisms thriving in extreme environments on Earth, psychrophiles (cold-loving organisms) are the most abundant in terms of biomass, diversity and distribution. If a psychrophile is defined as an organism living permanently at temperatures close to the freezing point of water in thermal equilibrium with the medium (without entering the debate on classification), this definition includes *de facto* a large range of representatives from all three domains: *Bacteria*, *Archaea* and *Eukarya* (including yeasts, algae, marine invertebrates or polar fish). Such biodiversity partly correlates with a less detrimental effect of low temperatures on cellular structures as compared with high temperatures or extreme pH for instance, but it also reflects the fact that most Earth's biotopes are cold and have been successfully colonized by diverse organisms [1–6]. Extreme psychrophiles have been traditionally sampled from Antarctic and Arctic polar regions, assuming that low temperatures persisting over a geological time-scale have promoted deep and efficient adaptations to freezing conditions. More recently, permafrost representing more than 20% of terrestrial soils has revealed an unsuspected biodiversity in cryopegs, e.g. salty water pockets that have remained liquid for about 100,000 years at –10°C [7]. High altitude mountains, glaciers or natural caves are additional sources of cold-adapted organisms. However, the largest psychrophilic reservoir is provided by oceans, covering 70% of our planet, that have a constant temperature of 4°C below a depth of 1000 m, irrespective of the latitude. Furthermore, deep sea sediments, previously considered as abiotic, apparently host a considerable microbial biomass that remains almost uncharacterized as a result of technical difficulties in sampling and culturing [8].

Such high abundance of psychrophiles evidently offers a huge potential for biomining using culture-based techniques, recombinant protein expression or metagenomic approaches [9,10]. Incidentally, the first cold-adapted enzymes from Antarctic bacteria that have been cloned, sequenced and expressed in a recombinant form were lipases, subtilisins and α-amylase, i.e. well-known representatives of industrial enzymes. This illustrates that besides the fundamental research on biocatalysis in the cold, the biotechnological potential of psychrophilic enzymes was already put into perspective in the early 1990s. Since then, numerous possible applications based on psychrophiles have been described and patenting in this field is increasingly growing [11]. By contrast, the number of known or proven current applications remains modest. It should be stressed that confidentiality accompanying commercial products frequently obscures the possible psychrophilic origin of compounds and, accordingly, the number of current applications is certainly much higher than those summarized below. We present here an overview of the biotechnological uses of psychrophiles and of their

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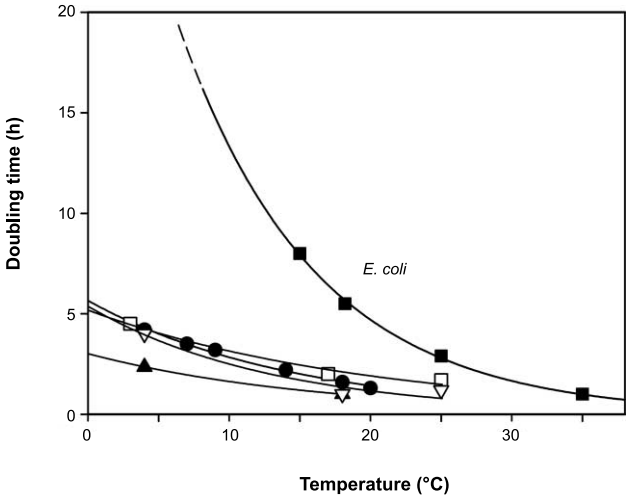


Figure 1. Temperature-dependence of growth (expressed by the generation time) of *E. coli* RR1 (filled squares) and of representative psychrophilic Gram-negative bacteria (left curves) displaying high growth rates at low temperatures (data from various Antarctic isolates of *Pseudoalteromonas haloplanktis*).



biomolecules using selected examples. Previous reviews should be consulted for a complete coverage of this topic [6,12–17].

## Basics of psychrophiles in biotechnology

While the growth of most microorganisms is stopped or at least severely inhibited in a refrigerator, psychrophiles actively divide at these temperatures. As shown in Figure 1, some wild-type psychrophilic bacteria display doubling times at 4°C comparable to that of fast-growing *E. coli* RR1 laboratory strains grown at 37°C. The latter fail to grow exponentially below 8°C [18] whereas psychrophilic bacteria maintain doubling times as low as 2–3h at 4°C. This is primarily achieved by a weak effect of low temperatures on the growth rates as compared with mesophiles. This efficiency is mainly determined by cold-adaptation of the enzymatic machinery (see below). Although contamination of cold-room facilities by psychrophilic or psychrotolerant species is a concern for food spoilage, their growth properties can be advantageously exploited in man-driven operations. Psychrophilic strains, as cell factories, can be grown at tap water temperatures, avoiding heating of fermentation units, or at lower temperatures to produce heat-labile compounds and aggregation-prone proteins. A promising issue also lies in their use in wastewater or soil bioremediation during winter in temperate regions or as bio-additives to relieve pollution (e.g. oil spill) in cold ecosystems that have an inherent lower propensity to recover.

Microbial adaptation to low temperatures of course requires a vast array of metabolic and structural adjustments at nearly all organization levels of the cell, that begin to be understood thanks to the availability of genome sequences and of proteomic approaches. Five genomes from Arctic and Antarctic bacteria and Archaea have been published but some more are already completed. Proteomic analyses have been reported for an Antarctic bacterium, three Siberian permafrost bacteria and an Antarctic archaeon [5,6]. A survey of these data shows that the main up-regulated functions for growth at low temperatures are protein synthesis (transcription, translation), RNA and protein folding, membrane homeostasis, antioxidant activities and regulation of specific metabolic pathways. However, the few common features shared by all these psychrophilic genomes and proteomes have suggested that cold adaptation superimposes on pre-existing cellular organization and, accordingly, the adaptive strategies may differ between various microorganisms.

In contrast to these variable cellular adjustments, most enzymes from psychrophiles are cold-active and this peculiarity provides the basis for the main physiological adaptation to low temperatures. Indeed, cold-active enzymes allow the persistence of metabolic fluxes compatible with sustained growth at freezing temperatures. As shown in Figure 2, psychrophilic enzymes can be up to 10 times more active at low and moderate temperatures as compared with their mesophilic homologues. Furthermore, psychrophilic enzymes are heat-labile and are frequently inactivated

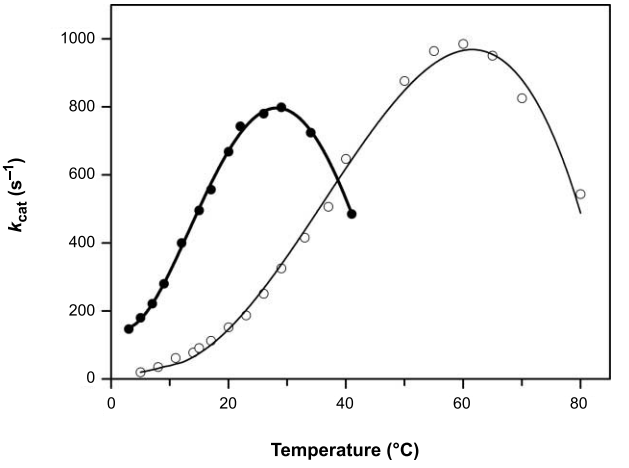


Figure 2. Temperature dependence of the activity of psychrophilic (filled symbols, heavy line) and mesophilic (open symbols) enzymes recorded at various temperatures illustrates the main properties of cold-adapted enzymes: cold-activity and heat-lability (data for α-amylases from the Antarctic *Pseudoalteromonas haloplanktis* and from the mesophile *Bacillus amyloliquefaciens*).

at temperatures that are not detrimental for their mesophilic counterparts. These specific traits are responsible for the three main advantages of cold-active enzymes in biotechnology: (*i*) as a result of their high activity, a lower concentration of the enzyme catalyst is required to reach a given activity, therefore reducing the amount of costly enzyme preparation in a process; (*ii*) as a result of their cold-activity, they remain efficient at tap water or ambient temperature, therefore avoiding heating during a process, either at domestic (e.g. washing machine) or industrial levels, and (*iii*) as a result of heat-lability, they can be efficiently and sometime selectively inactivated after a process by moderate heat input. Beside these traits specifically linked to temperature adaptation, an additional important aspect has to be mentioned: enzymes from organisms endemic to cold environments can be a valuable source of new catalysts possessing useful enzymological characteristics such as novel substrate specificities or product properties, as exemplified by lipases from the yeast *Candida antarctica* or by the xylanase from the bacterium *Pseudoalteromonas haloplanktis* (see below).

The conformation and 3D structures of psychrophilic proteins (about 30 have been solved by X-ray crystallography) are not markedly different from their mesophilic homologues and furthermore all amino acid side chains that are essential for the catalytic mechanism are strictly identical. It was found, however, that cold-active enzymes maintain the appropriate flexibility and dynamics of the active site at temperatures at which their mesophilic and thermophilic counterparts have severely restricted molecular motions [19–21]. This is achieved by the disappearance of discrete stabilizing interactions either in the whole molecule or at least in structures adjacent to the active site. Amongst these destabilizing factors, the most relevant are a reduced number of proline residues and of electrostatic interactions (ion pairs, H-bonds, aromatic interactions), a weakening of the hydrophobic effect, the strategic location of glycine residues, an improved interaction of surface side chains with the solvent or an improved charge-induced interaction with substrates and cofactors [22,23]. This adaptive destabilization of psychrophilic enzymes has been demonstrated to be responsible for both cold-activity and low thermal stability [24,25].

## Bioprospecting the psychrophilic genetic resources

Two reports of the United Nation University-Institute of Advanced Studies (UNU-IAS) have described the bioprospecting activities in the Antarctic [10] and in the Arctic [9]. The former report stressed that the absence of clear rules governing the use and ownership of genetic resources from Antarctica, resulting from the peculiar international status of the continent, obviously inhibits commercially oriented research and information exchanges. Such concerns have been frequently debated [26]. On the other hand, the latter report devoted to the Arctic provides an extensive survey of companies active in this field and of the patents and products derived from Arctic organisms. This survey clearly demonstrates the potential of psychrophiles in an unsuspected wide range of applications and the intense commercial activity in the field. More recently, UNU-IAS has launched Bioprospector, an online database (http://www.bioprospector.org/bioprospector/) surveying patents, commercial products and companies involved in applied research using genetic resources from both the Antarctic and the Arctic (sections for marine and Pacific resources are also available). This excellent initiative, accompanied by relevant publications, is currently the most updated survey of biotechnological applications based on psychrophiles.

The main areas of interest in terms of investigation, patenting and commercial products are summarized below [9,10] and some relevant examples are given in the next sections:

(1) Enzymes: their use in a wide range of industrial processes including food technology, as well as laboratory reagents in molecular biology or medical research.
(2) Biomolecules: generally as food additives such as dietary supplements for use in aquaculture, livestock and human diets, with special focus on polyunsaturated fatty acids and on antifreeze proteins.
(3) Pharmaceutical and medical uses: mainly focused on screening for new antibiotics or anti-cancer drugs but also on cosmetics and nutraceuticals.
(4) Bioremediation by biostimulation or bioaugmentation to degrade pollutants by cold-adapted microorganisms following accidental spills or past waste disposal practices.

A special mention should be made of the very large number of products derived from the Arctic shrimp *Pandalus borealis* and from the Antarctic krill *Euphausia superba*, mainly by Scandinavian and Japanese companies, in research, medical and pharmaceutical applications (alkaline phosphatase, hydrolase for prevention of immune rejection reactions, hypotensor peptide, anti-inflammatory agent), in food, feed and beverage processes, cosmetics or nutraceuticals (krill oils rich in omega-3 fatty acids).

## Bioremediation with psychrophiles

The capacity of a broad spectrum of microorganisms to utilize hydrocarbons as the sole source of carbon and

energy (biodegradation) has been recognized already by ZoBell in 1946 [27] and was the basis for the development of biological remediation methods. Bioremediation attempts to accelerate the natural biodegradation rates through the optimization of limiting environmental conditions and is an ecologically and economically effective method. Low-temperature biodegradation of organic contaminants in cold ecosystems is a result of the degradation capacity of the indigenous psychrophilic microbial population. They transform or mineralize organic pollutants into less harmful, non-hazardous substances, which are then integrated into natural biogeochemical cycles. Most studies on hydrocarbon bioremediation in cold regions have focused on the treatment of petroleum hydrocarbons, since cold environments are increasingly exposed to petroleum exploration, production and transport, and these activities increase the risk of accidental oil release.

Evidence for the biodegradation activity of indigenous bacteria and fungi [28–30] in contaminated terrestrial and aquatic cold environments is provided by high numbers and activities of hydrocarbon degraders, the prevalence of genotypes with catabolic pathways for the degradation of a wide range of hydrocarbons [31], and high mineralization potentials. Such environments include polar and alpine soils, permafrost, sediments, sea, and also subsoils and groundwaters of temperate climates where temperatures rarely exceed 10°C. Hydrocarbon degraders in cold regions are confronted with special challenges, which – besides reduced enzymatic reaction rates – include increased viscosity of liquid hydrocarbons, reduced volatility of toxic compounds, low levels of nutrients, limited bioavailability of contaminants, and sometimes extremes of pH and salinity [30,32,33]. Until recently, frozen soils have been considered to be a practically impermeable barrier to pollutants. Meanwhile, studies have confirmed that hydrocarbons can penetrate even into ice-saturated soils [34]. Microbial activities have been measured at temperatures close to the freezing point of water and in marine ice at temperatures lower than –10°C, indicating that slow hydrocarbon biodegradation occurs in oil-contaminated ice [29].

Several remediation schemes have been implemented successfully at petroleum-contaminated sites in the Arctic during the past decade (for reviews, see the works given at [32,35,36] below). Successful on-site treatments include biopiles [37] and landfarming, which is now well-developed for cold-regions and offers low-cost treatment of petroleum-contaminated soils [38,39]. Engineered bioremediation implies the use of mechanized systems (e.g. forced aeration, heating and insulation systems) and allows the remediation of large volumes of petroleum-contaminated soils to clean-up standards within 2–3 treatment seasons in Alaska [36,40] and the lengthening of the usual short Arctic bioremediation season (June–September) by 3 months from May to November.

The most widely used bioremediation procedure in cold soils is biostimulation of the indigenous microorganisms by supplementation of appropriate nutrients (and optimization of other limiting factors, such as oxygen content, pH and temperature control); however, care has to be taken to avoid inhibition of biodegradation due to overfertilization [41]. Bioaugmentation by inoculating allochthonous hydrocarbon degraders has been used as a bioremediation option to treat petroleum-contaminated sites in Alaska, Canada, Greenland, and Norway. This strategy generally underperformed or gave no better results than fertilization (for reviews, see the works given at [32,36] below). In addition, such inocula are more expensive than commercially available fertilizers. Bioaugmentation with non-indigenous or genetically modified/engineered microorganisms is banned in Antarctica, Norway, Iceland, and Sweden [36]. The construction of psychrophiles with specific degradative capabilities has already been reported 20 years ago [42] and was based on the transfer of the TOL plasmid from the mesophile *Pseudomonas putida* by conjugation to a psychrophile of the same species; the transconjugant degraded toluene at temperatures as low as 0°C. Recently, the gene coding for a monooxygenase involved in the degradation of aromatic hydrocarbons from the mesophile *Pseudomonas stutzeri* was recombinantly expressed in the Antarctic *Pseudoalteromonas haloplanktis* [43]. However, the performance of such strains has still to be proven. Preconditions of a successful application of bioaugmentation are the expression of the biodegrading activities in the polluted environment and the survival of the inoculated strains at least for the time necessary for bioremediation.

Bacteria and fungi able to degrade high amounts of organic compounds within a short time at low temperatures represent a promising source as inocula for accelerated wastewater treatment. For example, a cold-adapted *Arthrobacter psychrolactophilus* strain displayed all the features necessary for its use as microbial starter, both from the viewpoint of biosafety and production. At 10°C, the strain induced a complete clarification of a synthetic wastewater turbid medium, it hydrolyzed proteins, starch and lipids, and improved the biodegradability of organic compounds in the wastewater [44]. Another example is low-temperature degradation of phenol, which is the most common representative of aromatic toxic pollutants in a wide variety of wastewaters. Psychrophilic bacteria (*Rhodococcus* spp.) and yeasts (e.g. *Rhodotorula psychrophenolica*) fully degraded up to 12.5–15 mM phenol at 10°C under fed-batch cultivation; with some strains phenol degradation occurred even at temperatures as

low as 1°C [45]. Immobilization may improve phenol degradation by psychrophilic yeast strains [46]. Fixed-biofilm reactors inoculated with bacterial consortia were used for the degradation of chlorophenols and high removal efficiencies were obtained at temperatures down to 4°C [47]. Interestingly, a temperature decrease resulted in a relative increase of the *Gamma-Proteobacteria* within the consortia. A further compound currently of interest is methyl tert-butyl ether (MTBE); its extensive use as both an octane enhancer and as an oxygenate in unleaded gasoline over the past decades has led to widespread pollution of surface water and groundwater. An aerobic mixed bacterial culture capable of utilizing MTBE and growing from 3 to 30°C could be suitable for the cleanup of MTBE-contaminated aquifers. When inoculated into groundwater samples, it degraded MTBE simultaneously with other VOC (volatile organic compounds) pollutants [48].



## Psychrophiles as cell factories

To facilitate biotechnological applications of psychrophiles and of their products, recombinant protein secretion systems efficiently working at low temperature are indispensable. The production level of cold-active (heat-labile) proteins by wild-type strains is usually too low for the production on an industrial-scale. The first recombinant production of a cold-active enzyme (alpha-amylase from Antarctic *Pseudoalteromonas haloplanktis*) in an Antarctic host bacterium of the same species was described in 2001 [49]. The cold gene-expression-system was further developed and optimized for the recombinant extracellular secretion of heterologous proteins in *P. haloplanktis*, with enzymes originating from various Antarctic *P. haloplanktis* strains and a mesophilic yeast [50,51]. The simultaneous secretion of proteolytic enzymes that degraded the recombinant products could be considerably reduced by inactivating the secretion system with the use of a gene insertion strategy; the mutant strain still secreted the cold-active enzyme (alpha-amylase) as efficiently as the wildtype and in a stable form [52].

Another recombinant protein expression system working at low temperature was developed by using an Antarctic *Shewanella* sp. strain [53] and was based on the selection of a suitable promoter and a broad-host-range plasmid. High yields of ß-lactamase were produced in the *Shewanella* sp. strain at 4°C; the enzyme yield produced at 4°C was 64% of that obtained at 18°C. The efficiency of the system was demonstrated by the production of foreign proteins (putative peptidases and a glucosidase) from the psychrophile *Desulfotalea psychrophila*.

Site-specific mutants of psychrophiles are a useful tool to study cold-adaptation and expression of cold-active enzymes at low temperature [54]. For example, the role of a substrate-binding subunit of a specific transporter in a Siberian psychrophile *Psychrobacter arcticus* strain in the transport of several substrates at low temperatures could be elucidated.

Contrary to the above-mentioned works, which focused on the construction of cold expression systems for *Proteobacteria*, an expression system was developed for high G+C Gram-positive bacteria that are known to occur frequently in cold environments [55]. Seven psychrophilic isolates from Greenland ice cores belonging to various genera of the class *Actinobacteria* (*Arthrobacter*, *Microbacterium*, *Curtobacterium* and *Rhodoglobus*) were transformed with a shuttle vector that was constructed by using a small cryptic plasmid from a pychrophilic *Arthrobacter agilis* strain and conferred antibiotic resistance. In some isolates, plasmid stability was higher at 5°C than at 25°C, which points to the efficiency of the expression system within a restricted low temperature range.

## High added value psychrophilic enzymes and biomolecules

In a pioneer work, Kobori *et al.* [56] have purified and characterized a heat-labile alkaline phosphatase from an Antarctic bacterium isolated in seawater samples from McMurdo Sound. Alkaline phosphatases are mainly used in molecular biology for the dephosphorylation of DNA vectors prior to cloning to prevent recircularization, for the dephosphorylation of 5'-nucleic acid termini before 5'-end labelling by polynucleotide kinase or for removal of dNTPs and pyrophosphate from PCR reactions. However, the phosphatase has to be carefully removed after dephosphorylation to avoid interferences with the subsequent steps. Furthermore, *E. coli* and calf intestinal alkaline phosphatase (that was the preferred enzyme for these applications) are heat-stable and require detergent addition for inactivation. It follows that heat-labile alkaline phosphatases are excellent alternatives as they are inactivated by moderate heat treatment allowing one to perform the subsequent steps in the same test tube and minimizing nucleic acid losses. While the scientific report of Kobori *et al.* [56] specifically stressed the usefulness of their heat-labile alkaline phosphatase as a new tool in molecular biology, this interesting finding was apparently not turned into a marketed product, possibly because gene cloning and heterologous expression were not well established at that time. Fifteen years later, the group of V. Bouriotis isolated an alkaline phosphatase from another Antarctic bacterium and cloned its gene in *E. coli* [57], solved its crystal structure [58] and also showed that its properties can be further improved by directed evolution in terms of

high activity and heat-lability [59]. This heat-labile alkaline phosphatase, sold as Antarctic phosphatase, is now proposed on the market by New England Biolabs (USA). In the same context, the heat-labile alkaline phosphatase from the Arctic shrimp *Pandalus borealis* is also available for instance from Biotec Pharmacon ASA (Norway) or GE Healthcare Life Sciences (UK).

Two other psychrophilic enzymes are also marketed for molecular biology applications taking advantage of the heat-labile property. Shrimp nuclease selectively degrades double stranded DNA: for instance, it is used for the removal of carry-over contaminants in PCR mixtures, and then it is heat-inactivated prior to addition of the template. This enzyme is produced in recombinant form in *Pichia pastoris* and is available from Biotec Pharmacon ASA (Norway), USB Corporation (USA) or Thermo Scientific (UK). Heat-labile uracil-DNA N-glycosylase from Atlantic cod (*Gadus morhua*), that presents typical cold adaptation features [60], is also used to remove DNA contaminants in sequential PCR reactions. When PCR is performed with dUTP instead of dTTP, PCR products become distinguishable from target DNA, and can be selectively degraded by uracil-DNA N-glycosylase. Following degradation of contaminants, the enzyme is completely and irreversibly inactivated after heat treatment. Heat-labile uracil-DNA N-glycosylase, produced in recombinant form in *E. coli*, is available from Biotec Pharmacon ASA (Norway).

Cold-active chaperones have also found very useful application in the production of recombinant proteins. High-level expression of heterologous proteins in *E. coli* can result in the production of large amounts of incorrectly folded proteins, generating aggregates of inactive protein generally in the form of inclusion bodies. To circumvent this insolubility problem, low-temperature cultivation of *E. coli* represents a classical strategy and co-expression of chaperones also frequently improves the recovery of soluble proteins. Chaperones are a ubiquitous class of proteins that assist the folding of nascent polypeptides, preventing misfolding or even repairing misfolding. In this context, the chaperonins Cpn10 and Cpn60 (homologous to GroES and GroEL in *E. coli*) from the Antarctic bacterium *Oleispira antarctica* were shown to improve the growth of *E. coli* at low temperatures and to remain optimally active as folding catalysts at these low temperatures [61]. Taking advantage of these properties, the ArcticExpress *E. coli* cells from Stratagene (USA) have been engineered to co-express the cold-active chaperonins with the recombinant protein of interest, therefore improving protein processing at low temperatures and increasing the yield of active, soluble recombinant protein.

To conclude this section with beauty, two cosmetic additives are worth mentioning. Antarticine-NF3 is a glycoprotein with antifreeze properties produced by the bacterium *Pseudoalteromonas antarctica*, that has been patented by Spanish researchers (EP20020738185, US7022668). It was found that Antarticine is effective for scar treatments and re-epithelialization of wounds. This glycoprotein is now included in some cosmetic regeneration creams (sometimes under the name Antarctilyne). It is also proposed in association with edelweiss extract: this is of course reminiscent of the peculiar resistance to harsh conditions of both the Antarctic bacterium and the Alp flower. Finally, extracts of the Antarctic algae *Durvillea antarctica* are included in cosmetics to improve skin vitality such as in the Extra Firming Day Cream, a top seller of Clarins (France).

## Psychrophilic enzymes and proteins in large scale processes

At the industrial level, the best-known representative of polar microorganisms is certainly the yeast *Candida antarctica*, as its species name unambiguously refers to the sampling origin. This yeast produces two lipases, A and B, the latter being sold for instance as Novozym 435 by Novozymes (Denmark). Although the moderate heat-stability of this lipase in aqueous solutions can be of concern, this enzyme is stabilized in its immobilized form. As a result of its substrate and stereospecificity, lipase B is involved in a very large number of organo-synthesis applications related to food/feed processing, pharmaceuticals or cosmetics [62]. In a survey of patents related to Antarctica [10] it was shown that lipases from *C. antarctica* by far dominate the number of process- or product-based patents. This is a significant example of the potential for novel catalysts from genetic resources in cold environments.

The market for enzymes used in detergents represents 30–40% of all enzymes produced worldwide. Amongst these enzymatic cleaning agents, subtilisin (an alkaline serine protease predominantly produced by *Bacillus* species) largely dominates this market. At the domestic level, the current trend is however to use detergents at lower washing temperatures because of the associated reductions in energy consumption and costs as well as to protect texture and colours of the fabrics. Accordingly, cold-active subtilisins are required for optimal washing results at tap water temperatures and the current advertisements for cold-active detergents indicate that this goal has been reached. The first psychrophilic subtilisins isolated from Antarctic *Bacillus* species have been extensively characterized to comply with such requirement [63,64]. However, they suffered from a low heat-stability that can compromise

their storage but also from a low chemical stability towards the detergent components. Therefore, subtilisins currently incorporated in cold-active detergents are engineered enzymes that combine storage stability, alkaline stability and activity and cold-activity. Although psychrophilic subtilisins are not components *per se* of cold-active detergents, they have largely contributed to the advancement of this economically attractive concept.

The xylanase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* is a nice example of the successful biotechnological transfer from academic research to industry. Xylanases are glycoside hydrolases that degrade the polysaccharide beta-1,4-xylan, thus breaking down hemicellulose, one of the major components of plant cell walls. Xylanases are also a key ingredient of industrial dough conditioners used to improve bread quality. It was found that the Antarctic enzyme belonged to a new class of xylanases as both its amino acid sequence and fold were distinct from previously characterized xylanases. The psychrophilic enzyme was therefore subjected to intensive investigations aimed at elucidating the structural origins of its high cold activity and weak stability as well as at understanding its enzymological mode of action [65–69]. Furthermore, baking trials have revealed that the psychrophilic xylanase was very effective in improving the dough properties and final bread quality with, for instance, a positive effect on loaf volume [70]. This efficiency appears to be related to the high activity of the psychrophilic xylanase at cool temperatures required for dough resting and to its specific mode of xylan hydrolysis. Following careful production optimization of this peculiar xylanase, the product is now sold by Puratos (Belgium). This is apparently the psychrophilic enzyme produced at the highest amounts to date.

Beta-galactosidase, or lactase, is also a glycoside hydrolase that specifically hydrolyzes the milk sugar lactose into galactose and glucose. It should be stressed that 75% of the world population suffers from lactose intolerance arising from deficient synthesis of intestinal lactase in adults and resulting in digestive disorders due to fermentation of lactose by enteric bacteria. In this context, a cold-active lactase from an Antarctic bacterium has been patented (WO 01/04276A1) for its capacity to hydrolyze lactose during milk storage at low temperatures [71]. It is worth mentioning that commercially available lactases require milk heating to become active. This heating step has, however, detrimental effects on milk quality as it alters the aspect, the taste and texture (Maillard reactions, activation of proteases, coagulation, and so on). Although the psychrophilic lactase is apparently not used for this specific application, it is expected that it will be produced soon in large quantities by Nutrilab NV (Belgium) to hydrolyze lactose (a by-product of the dairy industry) in the process of the high value sweetener D-tagatose, a natural monosaccharide with low caloric value and glycaemic index.

A last example will give a sweet taste for psychrophilic proteins. Antifreeze proteins are, for most of them, small glycoproteins first discovered in polar fish [72] that allow them to thrive at sub-zero temperatures without freezing. As a result of their peculiar surface properties, antifreeze proteins bind to ice crystal seeds and inhibit growth of ice in body fluids that would otherwise be fatal. The precise mechanism of ice adsorption-inhibition by antifreeze proteins remains poorly understood because of the complex water–ice interface and of the structural diversity of these proteins. Besides this interesting finding, the gene of an antifreeze protein from the ocean pout, an eel-like fish found in northern and Arctic oceans, has been cloned and expressed in the baker's yeast *Saccharomyces cerevisiae*. This recombinant protein is now included in several edible ice cream brands from Unilever (The Netherlands–England) under the name of ice-structuring protein. It controls ice re-crystallization following thawing-freezing cycles that otherwise drastically reduces taste and texture quality. Furthermore, the antifreeze protein enables the production of healthier ice creams that are lower in fat, added sugar and with fewer additives. Approval for the use of this technology has been granted by regulatory administrations in many countries.

## Conclusions

Amongst the extremophiles thriving at extreme biological temperatures, thermophiles have been generally considered as the most promising source of biotechnological innovations. However, recent developments based on cold-adapted organisms and on their biomolecules, such as those mentioned here, have clearly demonstrated the huge potential of psychrophiles. This potential appears to be even larger than for thermophiles when considering both the broader psychrophilic biodiversity, that encompasses microorganisms, plants and animals [73], and the broader fields of application. Last but not least, most biotechnological applications of psychrophiles are environmentally friendly and contribute to energy saving, both aspects being of increasing significance.

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