



Discovery, Molecular Mechanisms, and Industrial Applications of Cold-Active Enzymes

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Cold-active enzymes constitute an attractive resource for biotechnological applications. Their high catalytic activity at temperatures below 25°C makes them excellent biocatalysts that eliminate the need of heating processes hampering the quality, sustainability, and cost-effectiveness of industrial production. Here we provide a review of the isolation and characterization of novel cold-active enzymes from microorganisms inhabiting different environments, including a revision of the latest techniques that have been used for accomplishing these paramount tasks. We address the progress made in the overexpression and purification of cold-adapted enzymes, the evolutionary and molecular basis of their high activity at low temperatures and the experimental and computational techniques used for their identification, along with protein engineering endeavors based on these observations to improve some of the properties of cold-adapted enzymes to better suit specific applications. We finally focus on examples of the evaluation of their potential use as biocatalysts under conditions that reproduce the challenges imposed by the use of solvents and additives in industrial processes and of the successful use of cold-adapted enzymes in biotechnological and industrial applications.

Keywords: cold-active enzymes, psychrophiles, biocatalysis, extremophiles, protein structure and function, protein engineering, biotechnological applications

INTRODUCTION

In the context of global needs for sustainability and clean manufacturing technologies, biocatalysts are an attractive alternative for the achievement of chemical transformations (Wohlgemuth, 2010; Bornscheuer et al., 2012). Enzymes are non-toxic, biodegradable, and efficient/selective biocatalysts with outstanding catalytic properties, offering high levels of safety, low energy consumption, and an overall environmentally friendly production procedure (Saha and Demirjian, 2001; Dunn, 2012; Wang M. et al., 2012). A high interest has been displayed in enzymes from organisms living on extreme ecosystems, because they work under harsh environments, which are conditions mostly found in industrial processes. Among extremophiles, microorganisms living in cold environments have become a very interesting source for the identification and isolation of novel cold-active enzymes (Russell, 2000; D'Amico et al., 2002a; Feller, 2003, 2010). The use of enzymes that remain active at low temperatures has a great potential for industrial biocatalysis in terms of energy savings

by lowering the required temperature of a reaction without sacrificing enzyme activity. Cold-active enzymes can also prevent undesirable chemical reactions occurring at higher temperatures, while simultaneously offering an amenable procedure for their rapid heat-inactivation due to their structural thermolability, which is of special interest in food industry for eliminating the use of chemical-based inactivation (Russell, 1998; Gerday et al., 2000; Georgette et al., 2004; Margesin and Feller, 2010). Although most cold-active enzymes have been isolated from psychrophiles and psychrotolerant microorganisms, some enzymes displaying high activity at low temperatures have also been obtained from mesophilic and even from thermophilic organisms.

MICROORGANISMS HAVE COLONIZED COLD PLACES ON EARTH

Despite the harsh conditions that cold environments present for human life, microorganisms have colonized cold places on Earth. Depending on their optimal growth temperature, these microorganisms can be psychrophilic or psychrotolerant. Psychrophilic organisms are able to grow at low temperatures, between -20 and 10°C , and unable to grow at temperatures higher than 15°C . Unlike psychrophiles, psychrotolerant organisms grow optimally at 20 – 25°C but also have a high metabolic activity and growth capacity at temperatures below 0°C (Pikuta et al., 2007). Typically, psychrotolerant microorganisms are found in terrestrial cold environments and psychrophiles in marine ecosystems. Microorganisms living on these cold places are mainly bacteria, yeasts, fungi and algae, and this biodiversity has been extensively reviewed (Cowan et al., 2007; Yumoto, 2013).

Constantly cold environments ($<5^{\circ}\text{C}$) cover $\sim 80\%$ of the Earth's biosphere and include mainly the Polar Regions, deep water and marine sediments of the oceans, and glaciers of high mountains (Pikuta et al., 2007; Huston, 2008). Polar regions account for 15% of the Earth's surface and include the Antarctic and the Arctic Circle with their polar ice sheets, glaciers, and permafrost (Cowan et al., 2007; Pikuta et al., 2007). To have an idea about the temperatures of this region, an example is the Antarctic air, which has annual temperatures below 0°C and during winter the temperature can reach -80°C (Cowan et al., 2007). Permafrost represents more than 20% of terrestrial soils (Deming, 2002) and it contains a large number of viable microorganisms which have retained their life over geological times (Rivkina et al., 2004). Organisms living in permafrost are mostly psychrotolerant and not psychrophiles (Morita, 1975). Deep water and marine sediments of the oceans cover 75% of the Earth's surface. They have an average temperature of 3°C , complete absence of light, high pressures and low nutrient availability, however numerous microorganisms have been identified and isolated from these ecosystems, the majority of them psychrophiles (Cowan et al., 2007). Glaciers, on other continents than the Polar Regions, cover an area of $15,861,766\text{ km}^2$. Here, microorganisms live in the liquid veins at ice grain inter-junctions and in the thin liquid film on the surfaces

of mineral grains, which contain substrates for their survival (Miteva, 2008).

COLD-ACTIVE ENZYME DISCOVERY

The most routine approach for discovering novel enzymes is the culture of microorganisms that express a protein of interest. This culture-dependent methodology has been successful for the isolation and characterization of many biocatalysts (Yang and Ding, 2014). Culture-independent techniques have emerged to increase the rate of enzyme discovery, since the microorganisms that can be cultured under laboratory conditions represent only a minor fraction (1–5%) of the microbial diversity, and therefore of their enzymes (Ekkers et al., 2012). For microorganisms from extreme environments a second problem arises when cultivation is attempted, as the harsh conditions that extremophiles need to grow increases the difficulty of obtaining enough biomass to have good DNA yields for cloning effectiveness (Ferrer et al., 2007). Some techniques to address this obstacle and improve the cultivation of cold-adapted microorganisms have recently been reviewed (Vester et al., 2015). Metagenomics is the main culture-independent approach and involves DNA extraction of an environmental sample followed by the construction of metagenome libraries for the isolation of target genes (Temperton and Giovannoni, 2012). Another approach, where no environmental sample is needed, is to use the vast information available in genome databases, which provides the possibility to identify novel enzymes by computational genomics (Gong et al., 2013). Considering that the access to extreme environments like constantly cold regions is not easy, genome mining emerges as a huge opportunity for the discovery of novel cold-adapted enzymes. However, to date it has not been used as the preferred alternative, maybe because only a few genomes of psychrophiles have been deposited in public databases.

Cold-active enzymes isolated by metagenomic approaches have been recently reviewed (Cavicchioli et al., 2011; Vester et al., 2015). Therefore, here we focus on cold-active enzymes derived from cultivated microorganisms and in some cases from synthetic genes.

Natural Hosts and Diversity of Cold-Active Enzymes

We have reviewed 92 cold-adapted enzymes that were successfully expressed in a heterologous host reported between 2010 and June 2016, which are detailed in **Table 1**. These enzymes were obtained mainly from psychrophilic or psychrotolerant organisms and bacteria or fungi (**Figures 1A,B**, respectively). These microorganisms were isolated from different and diverse environments, mainly from Polar Regions and marine environments. As explained later in this review, efficient catalysis at low temperatures requires an increase in protein flexibility, and therefore a reduction on enzyme stability. However, an interesting example of a cold-active enzyme isolated from a psychrophilic organism that had an unexpected high thermostability was reported for the superoxide dismutase DaSOD from *Deschampsia antarctica* (Rojas-Contreras et al.,

TABLE 1 | Source of cold-adapted enzymes microorganisms (published from 2010 to June 2016).

Class	Enzyme	Origin of sample	Organism source	Molecular technique	Heterologous expression host	Expression vector	T _{opt} (% residual activity at specific temperature)	pH _{opt}	Kinetics parameters (substrate)	References
Hydrolase	Xylanase	NS	<i>Flavobacterium johnsoniae</i>	Specific primers	<i>Flavobacterium johnsoniae</i>	Fj29	30 (50% at 4°)	8	K _m 8.41 mg/ml k _{cat} 17.95 s ⁻¹ (Birchwood)	Chen et al., 2013
Hydrolase	Esterase	Soil sample	<i>Pseudomonas</i> sp. S9	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> TOP10	pBAD/Myc-His A	35 (40% at 10°)	9	K _m 0.162 mM k _{cat} 3.31 s ⁻¹ (p-NP butyrate)	Wicka et al., 2016
Hydrolase	β-galactosidase	Antarctic soil	<i>Paracoccus</i> sp. 32d	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> LMG	pBAD/Myc-His A	40 (ND)	7.5	K _m 4.28 mM k _{cat} 140 s ⁻¹ (lactose)	Wierzbicka-Wos et al., 2011
Hydrolase	β-galactosidase	Antarctic soil	<i>Arthrobacter</i> sp. 32cB	Degenerated primers/genome walking	<i>E. coli</i> LMG194	pBAD/Myc-His A	28 (42% at 10°)	8	K _m 1.52 mM k _{cat} 30.55 s ⁻¹ (lactose)	Pawlak-Szukalska et al., 2014
Hydrolase	α-amylase	Antarctic	<i>Geomyces pannorum</i>	Degenerated primers/TAI-PCR	<i>Aspergillus oryzae</i>	pBC12F-NHA2	40 (20% at 0°)	5	K _m 3.22 mg/ml V _{max} 3.33 mg/min ml (soluble starch)	Mao et al., 2015
Hydrolase	β-glucosidase	Konjac field	<i>Paenibacillus xylanilyticus</i> KJ-03	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pCold I	20 (72% at 10°)	7	K _m 1.19 mM k _{cat} 16.87 s ⁻¹ (pNPRG)	Park et al., 2013
Hydrolase	Glucanase	NS	<i>Eisenia fetida</i>	Specific primers from a related sequenced genome	<i>E. coli</i> ArcticExpress RT (DE3)	pCold I	40 (38% at 10°)	5.5	ND	Ueda et al., 2014
Hydrolase	Esterase	Marine sediment	<i>Microbulbifer thermotolerans</i> DAU221	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pCold I	46 (10% at 1°)	8	K _m 0.099 mM V _{max} 550 μmol/min/mg (pNP-butyrate)	Lee, 2016
Hydrolase	β-galactosidase	Frozen soil	<i>Rahnella</i> sp. R3	Specific primers from a conserved region/TAI-PCR	<i>E. coli</i> BL21 (DE3)	pCold I	35 (27% at 4°)	6.5	K _m 1.5 mM k _{cat} 3 s ⁻¹ (lactose)	Fan et al., 2015
Hydrolase	Lipase	Antarctic	<i>Psychrobacter</i> sp.	Specific primers	<i>E. coli</i> BL21 (DE3)	pCold I + pG-KJE8	35 (30% at 5°)	8	ND	Shuo-shuo et al., 2011
Hydrolase	Nudix hydrolase MutT	Fish	<i>Aliivibrio salmonicida</i>	Specific primers	<i>E. coli</i> BL21 AI	pDest14	12 (ND)	7.5	K _m 0.0029 mM k _{cat} 0.713 s ⁻¹ (β-oxo-dGTP)	Lian et al., 2015
Hydrolase	Inulinase	Lead-zinc-rich soil	<i>Arthrobacter</i> sp. MN	Degenerated primers/TAI-PCR	<i>E. coli</i> BL21 (DE3)	pEASY-E1	35 (16% at 0 °C)	8	K _m 8.2 mM k _{cat} 5.75 s ⁻¹ (inulina)	Zhou et al., 2015
Hydrolase	Esterase	NS	<i>Streptomyces coelicolor</i> A3(2)	Specific primers	<i>E. coli</i> BL21 (DE3)	pET16b	35 (25% at 10°)	8.5	K _m 2.5 mg ml ⁻¹ k _{cat} 0.83 s ⁻¹ (succinylated casein)	Brault et al., 2012
Hydrolase	Esterase	Permafrost	<i>Psychrobacter cryohalobentis</i> K5T	Specific primers	<i>E. coli</i> BL21 (DE3)pLysS	pET20b	25 (70% at 5°)	ND	ND	Petrovskaya et al., 2015
Hydrolase	β-xylanase	Marine environment	<i>Saccharophagus degradans</i> 2-40	Specific primers	<i>E. coli</i> BL21 (DE3)	pET21a	30 (ND)	7	K _m 10.4 mg/mL k _{cat} ND (birchwood xylan)	Ko et al., 2016
Hydrolase	Esterase	Intestine of righteye flounder	<i>Acinetobacter venetianus</i> V28	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pET22a(+)	40 (70% at 5°)	9	ND	Kim, 2012

(Continued)

TABLE 1 | Continued

Class	Enzyme	Origin of sample	Organism source	Molecular technique	Heterologous expression host	Expression vector	T_{opt} (% residual activity at specific temperature)	pH _{opt}	Kinetics parameters (substrate)	References
Hydrolase	Esterase	Intestines/stomach of an Atlantic hagfish (Myxine glutinosa)	<i>Rhodococcus</i> sp. AW25M09	Specific primers	<i>E. coli</i> BL21 (DE3)	pET22b	30 (50% at 10 ^o)	11	K_m 0.753 mM K_{cat} 1.63 s ⁻¹ (pNP-butanolate)	De Santi et al., 2014
Hydrolase	Xylanase	Marine invertebrate <i>Halocynthia aurantium</i>	<i>Glaciecola mesophila</i> KMM241	Specific primers from a related sequenced genome	<i>E. coli</i> BL21(DE3)	pET22b	35 (8% at 0 ^o)	6	K_m 5.82 mg ml ⁻¹ K_{cat} 609 s ⁻¹ (Beech wood xylan)	Guo et al., 2013
Hydrolase	Esterase	Intestine of a blood clam	<i>Photobacterium</i> sp. MA1-3	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pET22b(+)	30 (45% at 5 ^o)	8	ND	Kim et al., 2013
Hydrolase	Esterase	Intestine of silver whiting	<i>Salinisphaera</i> sp. P7-4	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pET22b(+)	25 (ND)	9	ND	Kim et al., 2011
Hydrolase	Lipase	Soil	<i>Sorangium cellulosum</i>	Specific primers	<i>E. coli</i> BL21 (DE3)	pET22b(+)	30 (35% at 0 ^o)	8	K_m 0.174 mM K_{cat} 29s ⁻¹ (p-NP acetate)	Cheng et al., 2011
Hydrolase	Protease	ANTARCTIC	<i>Pseudoalteromonas</i> sp.	Degenerated primer/genome walking	<i>E. coli</i> BL21 (DE3)	pET22b(+)	25 (ND)	8	K_m 0.27 mM K_{cat} 199 s ⁻¹ (p-NP valerate)	Acevedo et al., 2013
Hydrolase	Xylanase	Soil sample	<i>Sorangium cellulosum</i> So9733-	Degenerate primers/TAIL PCR	<i>E. coli</i> BL21 (DE3)	pET22b(+)	30–35 ^o C (13.7% at 0 ^o C)	7	K_m 25.77 mg/ml K_{cat} 6.84 s ⁻¹ (Beechwood xylan)	Wang S. Y. et al., 2012
Hydrolase	α -glucosidase	Culture collection from Anhui University	<i>Pseudoalteromonas</i> sp. K8	Degenerated primers	<i>E. coli</i> BL21 (DE3)	pET22b(+)	30 (30% at 0 ^o)	8.5	K_m 0.27 mM K_{cat} 15 s ⁻¹ (pNPvG)	Li et al., 2016b
Hydrolase	Lipase	Antarctic seawater	<i>Shewanella frigidimarina</i> NCIMB 400	Degenerated primers	<i>E. coli</i> BL21 (DE3)	pET22b(+)	25 (35% at 10 ^o)	8	ND	Parra et al., 2015
Hydrolase	Protease	Antarctic seawater	<i>Pseudoalteromonas haloplanktis</i> TAC125	Protein sequence/specific primers	<i>E. coli</i> BL21 (DE3)	pET22b(+)	15 (20% at 5 ^o)	8	ND	de Pascale et al., 2010
Hydrolase	Xylanase	DNA of goat rumen fluid	-	Degenerate primer/TAIL PCR	<i>E. coli</i> BL21 (DE3)	pET22b(+)	30 (10% at 0 ^o)	6.5	K_m 1.8 mg ml ⁻¹ K_{cat} 584 s ⁻¹ (Beechwood xylan)	Wang et al., 2011
Hydrolase	β -galactosidase	NS	<i>Pyrococcus furiosus</i>	Specific primers	<i>E. coli</i> BL21 (DE3)	pET24a(+)	90 (8% at 0 ^o)	7	ND	Dong et al., 2014
Hydrolase	Esterase	Sea floor	<i>Thalassospira</i> sp. GB04J01	Specific primers	<i>E. coli</i> BL21 (DE3)	pET26b	45 (20% at 10)	8.5	K_m 0.94 mM K_{cat} 47.7 s ⁻¹ (pNP-acetate)	De Santi et al., 2016
Hydrolase	Pullulanase	Soil sample	<i>Exiguobacterium</i> sp. SH3	Specific primers	<i>E. coli</i> BL21 (DE3)/ <i>B. subtilis</i> WB600	pET26b(+) pHY300PLK	45 (30% at 10)	8.5	K_m 2.8 mg/ml K_{cat} 37s ⁻¹ (pullulan)	Rajaei et al., 2015
Hydrolase	Esterase	NS	<i>Pseudomonas mandelii</i>	Specific primers	<i>E. coli</i> BL21 (DE3)	pET28a	40 (ND)	8.5	K_m 0.21 mM K_{cat} 3.4 s ⁻¹ (p-NP acetate)	Lee et al., 2013

(Continued)

TABLE 1 | Continued

Class	Enzyme	Origin of sample	Organism source	Molecular technique	Heterologous expression host	Expression vector	T_{opt} (% residual activity at specific temperature)	pH _{opt}	Kinetics parameters (substrate)	References
Hydrolase	Xylanase	Sediment sample from a soda lake	<i>Bacillus</i> sp. SN5	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pET28a	40 (29% at 5°)	7	K_m 0.6 mg/ml k_{cat} ND (beechwood xylan)	Bai et al., 2012
Hydrolase	β -glucosidase	Antarctic soil	<i>Exiguobacterium antarcticum</i> B7	Specific primers	<i>E. coli</i> Rosetta	pET28a	30 (25% at 5°)	7	K_m 1.07 mM k_{cat} 32.96s ⁻¹ (pNPpG)	Crespim et al., 2016
Hydrolase	Pullulanase	Soil of fruit market garbage dump	<i>Paenibacillus polymyxa</i> Nws-pp2	Degenerated primers	<i>E. coli</i> BL21 (DE3)	pET28a	35 (40% at 10°)	6	K_m 15.25 mg/ml V_{max} 20.1 U/mg (pullulan)	Wei et al., 2015
Hydrolase	Glycogen branching enzyme	CGMCC	<i>Rhizomucor miehei</i>	Degenerate primers/RACE PCR	<i>E. coli</i> BL21 (DE3)	pET28a (+)	25 (ND)	7.5	ND	Wu et al., 2014
Hydrolase	Lipase	Deep-sea sediments	<i>Psychrobacter</i> sp. C18	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pET28a(+)	30 (18% at 0°)	8	ND	Chen et al., 2010
Hydrolase	β -mannanase	Slag of a phosphate rock-stacking site	<i>Sphingomonas</i> sp. JB13	Degenerate primer/TAI-PCR	<i>E. coli</i> BL21 (DE3)	pET28a(+)	40 (20% at 10°C)	6.5	K_m 5 mg ml ⁻¹ k_{cat} 211.9 s ⁻¹ (locust bean gum)	Zhou et al., 2012
Hydrolase	Endoglucanase	Lake sediment	<i>Paenibacillus</i> sp. /HB B 3084	Specific primers	<i>E. coli</i> BL21 (DE3)	pET28a(+)	40 (70% at 5°)	5	K_m 40.5 mg/ml V_{max} 0.692 IU/ml (CMC)	Dhar et al., 2015
Hydrolase	Esterase	Sediment of soda lake	<i>Alkalibacterium</i> sp. SL3	TAIL-PCR	<i>E. coli</i> BL21 (DE3)	pET28a(+)	30 (70% at 0°)	9	K_m 0.15 mM k_{cat} 307.69s ⁻¹ (pNP-acetate)	Wang et al., 2016
Hydrolase	β -glucanase	Deep-sea sediment	<i>Pseudomonas</i> sp. MM15	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pET28a+	30 (70% at 10°)	4.5	ND	Yang and Dang, 2011
Hydrolase	β -amylase	NS	<i>Arabidopsis thaliana</i>	Specific primers	<i>E. coli</i> BL21 (DE3)	pET29a	30 (20% at 0°)	6	ND	Monroe et al., 2014
Hydrolase	Lipase	Glacier soil	<i>Acinetobacter</i> sp. XMZ-26	Degenerated/genome walking	<i>E. coli</i> BL21 (DE3)	pET30a(+)	15 (39% at 0°)	10	K_m 0.075 mM k_{cat} 561 s ⁻¹ (p -NP octanoate)	Zheng et al., 2011
Hydrolase	β -glucosidase	Gut of longhorned beetle (<i>Batocera horsfieldi</i>) larvae	<i>Serratia</i> sp. TN49	Degenerate primer/TAI-PCR	<i>E. coli</i> BL21 (DE3)	pET30a(+)	35 (25% at 10°)	7.5	K_m 7.79 mM k_{cat} 22.6 s ⁻¹ (pNPG)	Zhou et al., 2011
Hydrolase	Lipase	CGMCC	<i>Stenotrophomonas maltophilia</i> GS11	Specific primers	<i>E. coli</i> BL21 (DE3)	pET30a(+)	35 (55% at 5)	8	ND	Li et al., 2016a
Hydrolase	Lipase	Siberian cryopeg	<i>Psychrobacter cryohalobentis</i> K5	Specific primers	<i>E. coli</i> BL21 (DE3)	pET32a	25 (60% at 5°)	8.5	ND	Novototskaya-Vlasova et al., 2013b
Hydrolase	β -mannanase	Soil	<i>Bacillus subtilis</i> Bs5	Specific primers from a related sequenced genome	<i>E. coli</i> Rosetta_gami (DE3)	pET32a	35 (ND)	5	ND	Huang et al., 2012

(Continued)

TABLE 1 | Continued

Class	Enzyme	Origin of sample	Organism source	Molecular technique	Heterologous expression host	Expression vector	T_{opt} (% residual activity at specific temperature)	pH _{opt}	Kinetics parameters (substrate)	References
Hydrolase	Esterase	Siberian permafrost	<i>Psychrobacter cryohalobantis</i> K5 ^T	Specific primers	<i>E. coli</i> BL21 (DE3)	pET32a(+)	35 (82% at 0°C)	8.5	ND	Novototskaya-Vlasova et al., 2012
Hydrolase	Lipase	Siberian cryopeg	<i>Psychrobacter cryohalobantis</i> K5	Specific primers	<i>E. coli</i> BL21 (DE3)	pET32a(+)	25 (80% at 5°C)	9	ND	Novototskaya-Vlasova et al., 2013a
Hydrolase	Esterase	Seawater	<i>Photobacterium</i> sp. strain J15	Degenerated primers	<i>E. coli</i> Rosetta-garni (DE3) pLysS	pET32b(+)	20 (50% at 4°C)	8	ND	Shakiba et al., 2016
Hydrolase	Alkaline phosphatase	Mantle tissue of the marine mussel	<i>Cobelia marina</i>	Specific primers	<i>E. coli</i> Rosetta (DE3)	pET40b (+)	40 (ND)	9.5	K_m 0.3 mM K_{cat} 24,000 s ⁻¹ (pN-phosphate)	Golotin et al., 2015
Hydrolase	α -galactosidase	Marine environment	<i>Pseudoalteromonas</i> sp. KMM 701	Specific primers	<i>E. coli</i> Rosetta(DE3)	pET40b(+)	20 (ND)	7	K_m 0.412 mM K_{cat} 0.588 s ⁻¹ (pNP- α Gal)	Bakunina et al., 2014; Balabanova et al., 2010
Hydrolase	Lipase	Dirty and cool stream water	<i>Pseudomonas</i> sp. TK-3	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pET47b	20 (30% at 5°C)	8	ND	Tanaka et al., 2012
Hydrolase	Protease	NS	<i>Shewanella arctica</i>	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> Tuner (DE3) pLacI	pETBlue1	60 (20% at 0°C)	8	K_m 0.175% (w/v) K_{cat} 5.186 s ⁻¹ (casein)	Ooura et al., 2015
Hydrolase	Lipase	NS	<i>Candida albicans</i>	Specific primers	<i>P. pastoris</i>	pGAPZaA	15 (50% at 5°C)	5	K_m 0.27 mM K_{cat} 551 s ⁻¹ (p-NP caprylate)	Lan et al., 2011
Hydrolase	β -mannosidase	NS	<i>Aspergillus niger</i> CBS 513.88	Synthesized from known sequence	<i>P. pastoris</i> X33	pGAPZaA	45 (22% at 0°C)	5	K_m 2.87 mg/ml K_{cat} 492.29 s ⁻¹ (guar gum)	Zhao W. et al., 2011
Hydrolase	Lipase	NS	<i>Malassezia globosa</i>	Synthesized from known sequence	<i>P. pastoris</i> X-33	pGAPZaA	15 (50% at 5°C)	6	ND	Xu et al., 2015
Hydrolase	Lipase	NS	<i>Bacillus</i> sp.	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> JM109	pGEM-T	35 (55% at 10°C)	8	K_m 3.3 mM K_{cat} 2.4 x 10 ⁻⁵ s ⁻¹ (pNP laurate)	Khurana et al., 2015
Hydrolase	β -galactosidase	NS	<i>Lactococcus lactis</i>	Specific primers	<i>E. coli</i> NovaBlue (DE3)	pGEMT-Easy	15-55 (60% at 5°C)	6-7.5	K_m 0.82 mM K_{cat} 102 s ⁻¹ (lactose)	Vincent et al., 2013
Hydrolase	Phytase	NS	<i>Bacillus licheniformis</i>	Specific primers	<i>E. coli</i> BL21 (DE3)	pGEMT-Easy Vector	75 (40% at 4°C)	7	K_m 178 μ M K_{cat} 1163.5 s ⁻¹ (phytic acid)	Borgi et al., 2014
Hydrolase	α -amylase	Surface seawater	<i>Zunongwangia profunda</i>	Specific primers	<i>E. coli</i> BL21 (DE3)	pGEX-6P-1	35 (39% at 0°C)	7	K_m 2.3 mM K_{cat} 329.58 s ⁻¹ (soluble starch)	Qin et al., 2014
Hydrolase	Esterase	Deep seawater	<i>Psychrobacter pacificensis</i>	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pGEX-6p-1	25 (70% at 10°C)	7.5	K_m 0.034 mM K_{cat} 5.75 s ⁻¹ (p-NP butyrate)	Wu et al., 2015

(Continued)

TABLE 1 | Continued

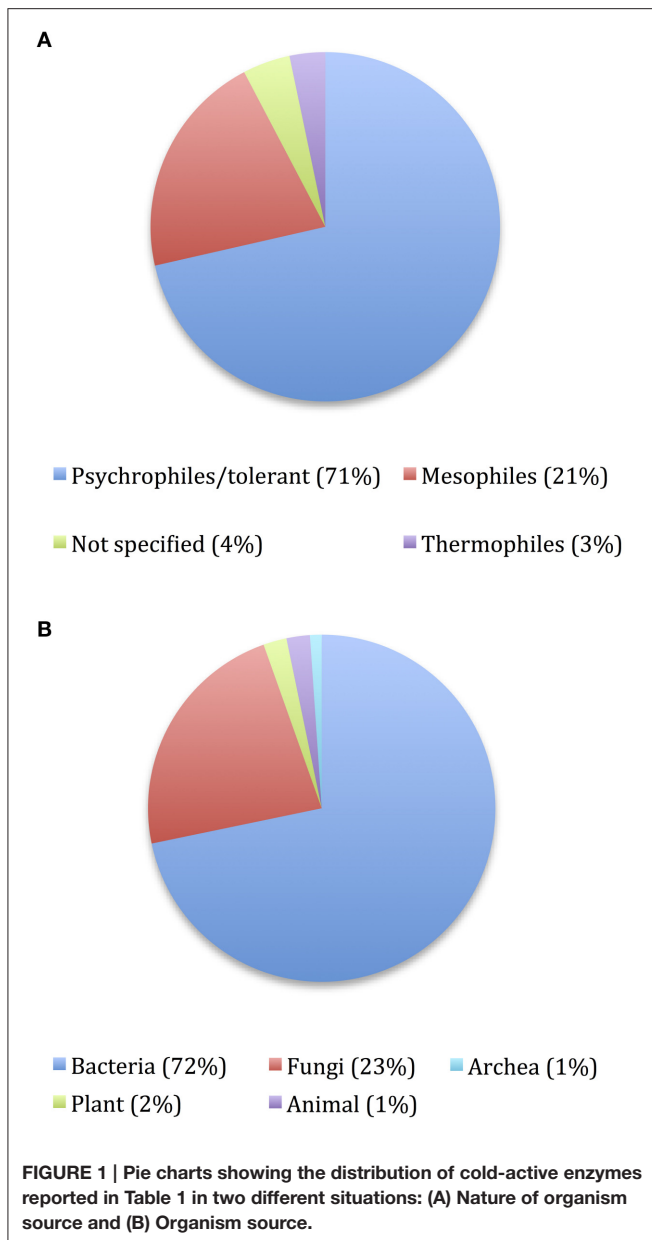
Class	Enzyme	Origin of sample	Organism source	Molecular technique	Heterologous expression host	Expression vector	T _{opt} (% residual activity at specific temperature)	pH _{opt}	Kinetics parameters (substrate)	References
Hydrolase	Esterase	Deep-sea sediments	<i>Psychrobacter celer</i> 3Pb1	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pGEX-6p-1	35 (41% at 0 °C)	7.5	K _m 0.033 mM K _{cat} 9.21 s ⁻¹ (p-NP butyrate)	Wu et al., 2013b
Hydrolase	Esterase	Sediments in the Gulf of Mexico	<i>Psychrobacter pacificensis</i>	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> BL21 (DE3)	pGEX-6p-1	25 (55% at 0°C)	7.5	K _m 0.7667 mM K _{cat} 3.92 s ⁻¹ (p-NP butyrate)	Wu et al., 2013a
Hydrolase	Xylanase	Seawater	<i>Zunongwangia profunda</i>	Specific primers	<i>E. coli</i> BL21 (DE3)	pGEX-6p-1	30 (23% at 0°)	6.5	K _m 1.15 mg/ml K _{cat} 80.33 s ⁻¹ (beechwood xylan)	Liu et al., 2014
Hydrolase	Esterase	Marine environment	<i>Serratia</i> sp.	Specific primers from a related sequenced genome	<i>E. coli</i> BL21 (DE3)	pGEX-6P-1	10 (92% at 0)	8.5	K _m 0.074 mM K _{cat} 2339 s ⁻¹ (pNP-acetate)	Jiang et al., 2016
Hydrolase	Esterase	Surface seawater	<i>Zunongwangia profunda</i>	Specific primers	<i>E. coli</i> BL21 (DE3)	pGEX-6P-1	30 (75% at 0)	8	K _m 0.121 mM K _{cat} 110 s ⁻¹ (pNP-butyrate)	Rahman et al., 2016
Hydrolase	β-galactosidase	Antarctica deep lake	<i>Halorubrum lacusprofundi</i>	Specific primers	<i>Halobacterium</i> sp. NRC-1	pKJ408	50 (10% at 0°)	6.5	ND	Karan et al., 2013
Hydrolase	Trypsin	Antarctic	<i>Euphausia superba</i>	Peptide sequence/degenerated and specific primers/RACE PCR	<i>E. coli</i> TB1	pMAL-c2E	50 (ND)	9	K _m ND K _{cat} 6 s ⁻¹ (BAPNA)	Oliviera-Nappa et al., 2013
Hydrolase	Lipase	Antarctic	<i>Penicillium expansum</i>	Degenerated primers/genome walking	<i>E. coli</i> <i>Origami B</i> (DE3)	pMAL-c5E	10 (ND)	8	ND	Mohammed et al., 2013
Hydrolase	Chitosanase	Fresh water lake	<i>Janthinobacterium</i> sp. strain 4239	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> DH10B	pMGJ1042	45 (30% at 10°)	5	ND	Johnsen et al., 2010
Hydrolase	Xylanase	Beech stump	<i>Bispora antennata</i>	Degenerated primers/TAIL-PCR	<i>P. pastoris</i> (GS115)	pPIC9	35 (21% at 0°)	5.5	K _m 1.65 mg/ml V _{max} 236 mmol/min/mg (birchwood xylan)	Liu et al., 2015
Hydrolase	Pectin methyltransferase	Wastewater of food processing	<i>Penicillium chrysogenum</i> F46	Specific primers from a related sequenced genome	<i>P. pastoris</i> GS115	pPIC9	40 (52% at 10°)	5	K _m 0.55 mg/ml V _{max} 15.78 mmol/min/mg (pectin)	Pan et al., 2014
Hydrolase	Polygalacturonase	Desert sand	<i>Achaetomium</i> sp. Xz8	Degenerate primers/TAIL-PCR	<i>P. pastoris</i> GS115	pPIC9	45 (10% at 0°)	6	K _m 0.32 g/l V _{max} 97.951 mmol/min/mg (polygalacturonic acid)	Tu et al., 2013
Hydrolase	Lipase	CGMCC	<i>Rhizomucor endophyticus</i>	Degenerated primers/RACE	<i>P. pastoris</i> GS115	pPIC9 K	40 (75% at 0)	6	K _m 2.3 mM K _{cat} 0.891 s ⁻¹ (pNP-caprylate)	Yan et al., 2016
Hydrolase	Lipase	NS	<i>Candida Parapsilosis</i>	Specific primers	<i>P. pastoris</i> GS115	pPIC9K	35 (45% at 5°)	6.5	ND	Neang et al., 2014
Hydrolase	Lipase	NS	<i>Candida tropicalis</i>	Specific primers	<i>P. pastoris</i> GS115	pPIC9K	45 (36% at 5°)	6.5	ND	Neang et al., 2014
Hydrolase	Pullulanase	Sea water	<i>Shewanella arctica</i>	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> M15	pQE-30	35 (25% at 10)	7	K _m 0.1% K _{cat} 86.9 s ⁻¹ (pullulan)	Elleuche et al., 2015

(Continued)

TABLE 1 | Continued

Class	Enzyme	Origin of sample	Organism source	Molecular technique	Heterologous expression host	Expression vector	T _{opt} (% residual activity at specific temperature)	pH _{opt}	Kinetics parameters (substrate)	References
Hydrolase	Lipase	Soil at a car service area	<i>Staphylococcus epidermidis</i> AT2	Specific primers	<i>E. coli</i> (DE3) pLacI	pTrcHis2-TOPO	25 (ND)	8	ND	Kamarudin et al., 2014
Hydrolase	β-galactosidase	Arctic	<i>Alkalicoccus bacillus ikense</i>	Genomic DNA library/phenotype screening/specific primers	<i>E. coli</i> TOP10	pUC18	20 (60% at 0°)	8	ND	Schmidt and Stougaard, 2010
Hydrolase	Esterase	Human saliva	<i>Lactobacillus plantarum</i> WCFS1	Specific primers	<i>E. coli</i> BL21 (DE3)	pUR3-TEV + pGRO7	5 (ND)	6	ND	Esteban-Torres et al., 2014b
Hydrolase	Esterase	Human saliva	<i>Lactobacillus plantarum</i> WCFS1	Specific primers	<i>E. coli</i> BL21 (DE3)	pUR3-TEV + pGRO7	20 (90% at 5°)	6.5	ND	Esteban-Torres et al., 2014a
Hydrolase	Protease	Compost	<i>Bacillus</i> sp. B001	Degenerate primers/genomic DNA digestion and self-ligation/reverse PCR	<i>B. subtilis</i> WB600	pWB980	60 (ND)	10	K _m 0.44 mM K _{cat} 4181 s ⁻¹ (casein)	Deng et al., 2011
Hydrolase	Lipase	Antarctic soil	<i>Geomyces</i> sp. P7	Inverse PCR	<i>S. cerevisiae</i> (BJ5465)	pYES 2.1	35 (15% at 0°)	8	K _m 8.5 mM K _{cat} 118 s ⁻¹ (p-NP acetate)	Florczak et al., 2013
Hydrolase	β-glucosidase	Root surface of the salt marsh grass <i>Spartina anglica</i>	<i>Marinomonas</i> MWYL1	Synthesized from known sequence	<i>E. coli</i> DH5a	pYPX251	40 (20% at 5°C)	7	K _m 0.9 mg ml ⁻¹ K _{cat} 475.4 s ⁻¹ (oNPGic)	Zhao W. et al., 2012
Isomerase	Arabinose isomerase	Provided by other laboratory	<i>Shewanella</i> sp. ANA-3	Specific primers	<i>E. coli</i> BL21 (DE3)	pET15b	15 (90% at 4°)	5.5-6.5	K _m 33.7 mM V _{max} 164 mmole/s/mg (L-arabinose)	Rhimi et al., 2011
Ligase	Glutathione synthetase	Antarctic sea	<i>Pseudoalteromonas haloplanktis</i>	Specific primers	<i>E. coli</i> BL21 (DE3)	pET28a(+)	15 (ND)	7.8	K _m 0.25 mM K _{cat} 1.93 s ⁻¹ (γ-glutamylcysteine)	Albino et al., 2012
Oxidoreductase	Superoxide dismutase	Antarctic	<i>Deschampsia antarctica</i>	Specific primers	<i>E. coli</i> BL21-SI	NS	20 (80% at 0°)	7	ND	Rojas-Contreras et al., 2015
Oxidoreductase	Nitroreductase	Urinary tract	<i>Staphylococcus saprophyticus</i>	Specific primers	<i>E. coli</i> BL21 (DE3)	pET14b	20 (80% at 3°)	7.5	K _m 0.0498 mM K _{cat} 2.2 s ⁻¹ (NFZ)	Çelik and Yetis, 2012
Oxidoreductase	Glutaredoxin	Antarctic sea ice	<i>Pseudoalteromonas</i> sp. ANI78	Specific primers for Gix from the genera	<i>E. coli</i> BL21 (DE3)	pET28a (+)	30 (25.5% at 0°C)	8	K _m 0.46 mM V _{max} 14.3 nmol/mL/min (HED)	Wang Q. et al., 2014
Oxidoreductase	Glycine oxidase	Marine sediment sand	<i>Bacillus licheniformis</i>	Specific primers	<i>E. coli</i> BL21 (DE3)	pGEX-6p-1	40 (60% at 0°)	8.5	K _m 11.22 mM K _{cat} 0.08 s ⁻¹ (glyphosate)	Zhang et al., 2016
Transferase	Serine hydroxymethyl transferase	Arctic polar sea ice	<i>Psychromonas ingrahamii</i>	Synthesized from known sequence	<i>E. coli</i> HMS174 (DE3)	pET28a	30 (ND)	7.2	K _m 1.6 mM K _{cat} 1.78 s ⁻¹ (L-allo-threonine)	Angelaccio et al., 2012
Transferase	Glutathione S-transferase	Antarctic sea ice	<i>Pseudoalteromonas</i> sp. ANT506	Degenerated primers	<i>E. coli</i> BL21 (DE3)	pET28a (+)	40 (14.2% at 0°)	7	K _m 1.01 mM K _{cat} ND (glutathione)	Shi et al., 2014

BAPNA, Nu-benzoyl-L-arginine 4-nitroamide; CGMCC, China General Microbiological Culture Collection Center; CMC, carboxymethyl cellulose; dGTP, deoxyguanosine triphosphate; HED, hydroxyethyl disulfide; ND, not determined; NS, not specified; pNP-αGal, p-nitrophenyl-α-D-galactopyranoside; oNPGic, 2-Nitrophenyl-β-D-galactopyranoside; pNP-αG, 4-Nitrophenyl-α-D-glucopyranoside; pNPAG, 4-Nitrophenyl-β-D-glucopyranoside; NFZ, nitrofurazone; RACE, Rapid amplification of cDNA ends; TAIL-PCR, Thermal Asymmetric Interfacial PCR.



2015). The optimal temperature of this enzyme is 20°C, it retains 80% of activity at 0°C and has detectable activity at -20°C, but also DaSOD possess high thermostability, its activity was not affected at 80°C, and the half-life time was 35 min at 100°C.

There are good examples of cold-active enzymes isolated from mesophilic organisms. Most of the time, a high activity at low temperatures is unexpected during the characterization of the catalytic properties of a mesophilic enzyme. This was the case of a *Candida albicans* lipase (Lan et al., 2011), which shows a low sequence identity with those of known lipases from psychrophilic organisms, but has an optimal temperature of 15°C. Other example is the lipase from *Staphylococcus epidermidis*, isolated from a car service area, with an optimal temperature of 25°C (Kamarudin et al., 2014). Other interesting case was reported by Monroe et al. (2014), where the cold-active properties from

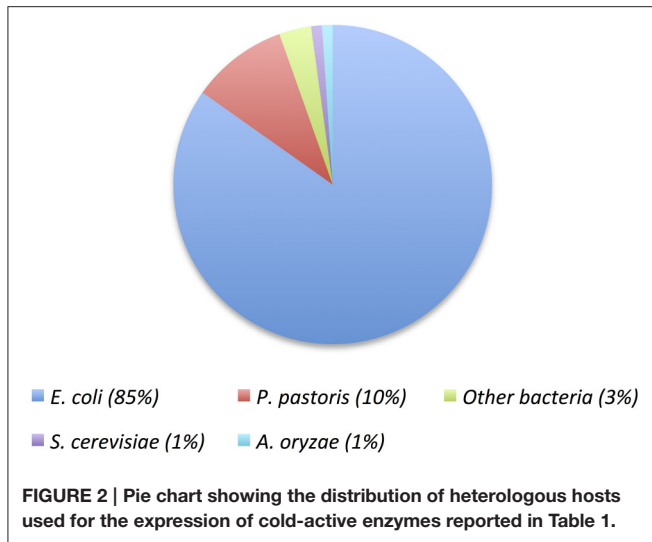
β -amylase 3 from *Arabidopsis* were inferred from the fact that this enzyme was more active during nighttime, compared to β -amylase 1 that had the opposite behavior. Both enzymes were overexpressed and purified from *Escherichia coli* confirming that they were differentially thermal adapted. β -amylase 3 had a lower optimal temperature, greater residual activity at low temperatures and less thermal stability than β -amylase 1.

More surprising is to discover a thermophilic enzyme with high activity at low temperatures. This was the case of a β -galactosidase isolated from *Pyrococcus furiosus* (Dong et al., 2014) with optimal activity at 90°C (130 U/mg). The enzyme was still active at 0°C, retaining 8% of its activity. Despite the decrease in activity compare to its optimal temperature, the lactase activity of *P. furiosus* at 0°C was still 40% of the optimal activity from the main β -galactosidase use in the food industry (28 U/mg at 50°C and pH 7.0) from *K. marxianus*. In addition, the lactase activity of *P. furiosus* at 0°C was 31% of the optimal activity of a cold-active β -galactosidase from *Arthrobacter psychrolactophilus* strain F2 (33 U/mg at 10°C and pH 8.0).

GENE CLONING AND RECOMBINANT EXPRESSION SYSTEMS FOR COLD-ACTIVE ENZYMES

The usual approach to obtain sufficient enzyme yield for purification, characterization, and final use consists of the recombinant expression of enzymes in a heterologous host. Mesophilic hosts are the most commonly used systems for heterologous expression of genes encoding cold-active enzymes (Table 1). However, the optimal growth temperature of these microorganisms is not compatible with the temperature that cold-active enzymes need to properly fold in order to retain their structure and functional activity (Bjerga et al., 2016). One alternative to circumvent these folding issues in *E. coli* is to lower the incubation temperatures of the cell culture to 18°C after induction (Feller et al., 1998), although this also decreases the host growth rate and thus the synthesis rate of heterologous enzyme is also reduced. Here, we briefly summarize the standard strategies for the expression of cold-active enzymes, which have been largely used for most of the enzymes reviewed in Table 1, followed by a more extensive revision of novel strategies for improving the expression of cold-active enzymes aiming to enhance their solubility, protein yield, and proper folding.

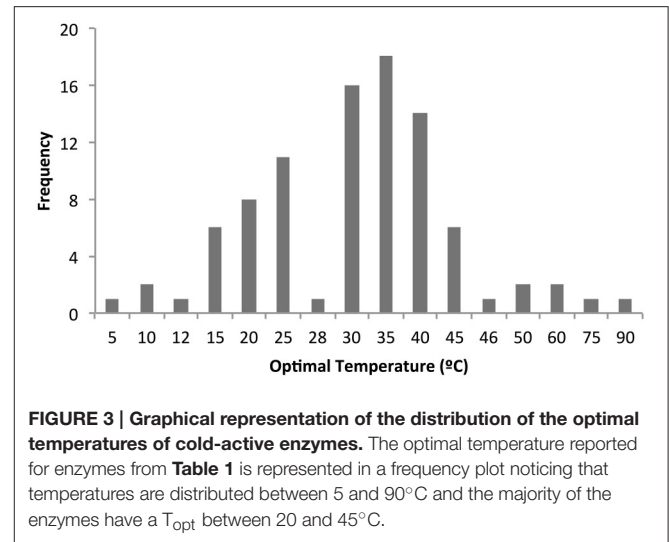
The starting point of most of the reviewed enzymes was the isolation of a cold-adapted organism with an interesting enzymatic activity. The main cloning strategy was the design of specific primers for gene amplification using the genomic DNA of the strain as template, (~48% of enzymes in Table 1). This is only possible if the genome of the species (or a very close relative) has been sequenced or the gene has been deposited in Gene Bank, and also if the microorganism can be properly cultured in order to obtain its genomic material. If the organism is not available or impossible to grow, the alternative is to synthesize the gene with an optimal codon usage for the host; this was the case of four cold-adapted enzymes described in Table 1 (Zhao W. et al., 2011, 2012; Angelaccio et al., 2012; Xu et al., 2015).



When the gene sequences were not available, the preferred cloning strategy was the creation of a genomic library, with subsequent clone screening, followed by sequencing the candidate clone to finally obtain a sequence that can be inserted into an expression vector (~21% of enzymes in **Table 1**). Degenerated primers for partial gene amplification, complemented with TAIL PCR, genome walking, RACE or inverse PCR, were used to a lesser extent.

The selected expression host was by far *E. coli* (**Figure 2**). Different genotypes were used, but in most cases BL21 (DE3) was the preferred strain. As we will see below, only one of these enzymes was expressed in an optimized strain for cold-active enzymes, ArcticExpress. Nevertheless, other expression hosts have been used, such as *Halobacterium* sp. for the expression of a cold-adapted hydrolase, and *Pichia pastoris*, used as the expression host for 9 proteins including various fungal enzymes. Other expression hosts that were rarely used are shown in **Table 1**.

Half of the cold-adapted genes were cloned in plasmids from the pET system for their expression. Only five of the genes were cloned in pCold vectors, whose advantages are described later in this review. Fusion constructs were also used for cloning 10 genes, eight in pGEX-6P-1, which allow the fusion expression of proteins to GST, and two in pMAL-c, which express proteins fusion to MBP. Other vectors are detailed in **Table 1**. Concerning enzyme purification, for more than half of the enzymes from **Table 1** the purification process was aided by fusion to a His tag. The majority of the enzymes were overproduced in the cytoplasm in a soluble form (72%). Only 15% were secreted and 8% were insoluble. Only two enzymes were purified from the periplasm and one was expressed in the outer membrane through fusion with an autotransporter domain (Petrovskaya et al., 2015; **Table 1**). Almost all enzymes were characterized, providing data from their optimal temperature (T_{opt}), optimal pH (pH_{opt}) and kinetic parameters like k_{cat} and K_m . The distribution of the optimal temperatures of the enzymes is displayed in **Figure 3**, and shows that T_{opt} are distributed between 5 and



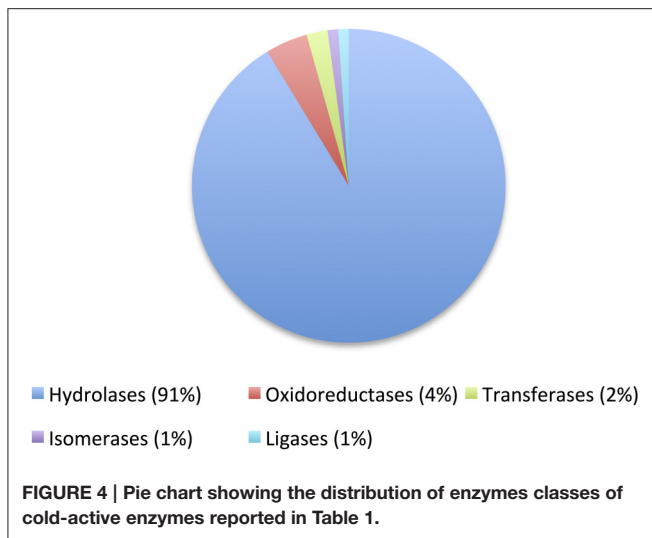
90°C, with 80% of the enzymes having a T_{opt} between 20 and 45°C.

It is important to underline that for *in vitro* characterization of enzymes, T_{opt} is obtained by measuring the enzyme activity at fixed temperatures and conditions, so it is likely that these numbers provide an approximate value for T_{opt} . Nevertheless, the distribution of T_{opt} displayed in **Figure 3** has a fundamental meaning, as this parameter often reflects the temperature of the environmental niches inhabited by their source organisms: albeit their source organisms are either psychrophilic or psychrotolerant (**Figure 1A**), the ability of these enzymes to remain active in the cold is the result of either complete or incomplete evolutionary adaptations of their structure and sequence for functioning at low temperatures, with enzymes from psychrotolerant being often identified as examples of incomplete evolution (Georlette et al., 2004). Regardless of the degree of completeness of their cold-adaptations, these enzymes are evidently cold-active, as demonstrated by the retention of an important percentage of their activity between 0 and 10°C for almost all of the enzymes in **Table 1**.

By far hydrolases were the preferred class for cold-enzyme discovery (**Figure 4**). Unsurprisingly, cold-adapted hydrolases are the most frequent proteins for which their three-dimensional structures have been solved (**Table 2**). Among them, lipases and esterases were the favorites (18 and 20% of enzymes in **Table 1**, respectively), which is the same case reported recently for cold-active enzymes obtained by metagenomic approaches where all the proteins were hydrolases (30% lipases and 30% esterases) except one (Vester et al., 2015).

Novel Strategies for Improving the Expression of Cold-Active Enzymes

Several strategies have been suggested to promote proper expression and folding of cold-active enzymes expressed in heterologous host, increasing their solubility, activity, and yield. These strategies are summarized in **Figure 5** and include the use of: (i) molecular chaperones; (ii) cold-active promoters; (iii)



fusion partners; (iv) psychrophilic hosts, and (v) a combination of these strategies.

i) Molecular Chaperones

Molecular chaperones are ubiquitous proteins that help newly synthesized polypeptides and denatured proteins to reach their native conformation. They are widely distributed in bacteria, yeast, plants, and animals (Evstigneeva et al., 2001). Originally, they were discovered because their encoding genes were expressed under heat shock conditions, therefore this family of molecules was named heat shock proteins (HSP). However, genes encoding molecular chaperones are also induced under other stressful conditions including UV irradiation, hypoxia, and chemical challenges, among others (Whitley et al., 1999). There are various chaperones families, which are named by their molecular size ranging from HSP40 to HSP100 and the small HSPs (Hartl et al., 2011). In *E. coli*, chaperones with quaternary structure are also known as chaperonins and include the GroEL/GroEL systems, whereas monomeric chaperones include the DnaK/DnaJ systems (Bukau et al., 2000; de Marco, 2007).

In 2004, Ferrer and coworkers reported that expressing chaperones Cpn60 and Cpn10 from the psychrophilic bacterium *Oleispira antarctica* RB8 in *E. coli*, lowered its minimal growth temperature below 15°C (Ferrer et al., 2003, 2004a). They used this chaperone-*E. coli* system to express a heat-labile esterase, reporting for the first time a successfully expression system for heat-sensitive proteins. They demonstrated that the low temperature improved proper folding of the enzyme, enhancing its specific activity 180-fold in comparison to the enzyme purified from the usual *E. coli* strain grown at 37°C (Ferrer et al., 2004b). Today, a competent *E. coli* strain that co-express cold-active chaperones Cpn60 and Cpn10 is commercialized by Agilent Technologies under the name of ArcticExpress.

Kim et al. (2015) co-expressed a cold active esterase together with PsyGroELS, a chaperonin from the psychrophilic bacterium *Psychrobacter* sp. PAMC21119, in an *E. coli* strain. The expression was performed at 10°C and they compared the enzyme activity

using the previously reported chaperones Cpn60 and Cpn10, finding better results with PsyGroELS for this particular enzyme. They conclude that PsyGroELS not only confers cold-tolerance to *E. coli*, but also is effective for co-expression of stable psychrophilic proteins.

Another example of chaperone co-expression was recently described by Esteban-Torres et al. (2014a) using GroES/GroEL chaperones. First, they cloned the cold-active esterase Lp_2631 into the pURI3-TEV expression vector for protein production, but the recombinant protein was expressed as inclusion bodies when *E. coli* BL21 (DE3) was used as host. To solve this, they used the plasmid pGro7 that produces GroES/GroEL chaperones. When Lp_2631 was co-expressed with the molecular chaperones in the *E. coli* host, the protein was expressed in the soluble fraction of the cells.

ii) Cold-active promoters

Quing and coworkers developed cold-shock expression vectors (pColdI-IV) harboring the *cspA* promoter from *CspA*, the major cold shock protein of *E. coli*, allowing high expression of several genes upon induction by cold-shock (Qing et al., 2004). They reported that pCold vectors are highly complementary to the widely used pET vectors for the expression of 38 genes. pCold vectors have been used to functionally express various proteins in *E. coli* at low temperature, most of them from mesophilic organisms including human proteins that were difficult to obtain with other systems (Hayashi and Kojima, 2008). Surprisingly only a few examples have been described for the expression of enzymes from psychrophilic organisms. One of them corresponds to the pCold I vector, used to functionally express a cold-active β -galactosidase (rBglAp) that was found to be extremely heat-labile in *E. coli* (Nakagawa et al., 2007).

Shuo-shuo and coworkers cloned the cold-active lipase gene Lip-948, from the Antarctic psychrotrophic bacterium *Psychrobacter* sp. G into the plasmid pColdI and transformed it into *E. coli* BL21, obtaining substantive expression of lipase LIP-948 with a yield of 39% of total protein, most of which was present as inclusion bodies (Shuo-shuo et al., 2011). Co-expression of pColdI-Lip-948 with chaperone pTf16 and pGro7 decreased the amount of insoluble LIP-948, while the soluble expression was enhanced when pColdI-Lip-948 was co-expressed with “chaperone team” plasmids (pKJE7, pG-Tf2, pG-KJE8), respectively. LIP-948 was most efficiently expressed in soluble form when it was co-expressed with pG-KJE8, which was up to 19.8% of intracellular soluble proteins. Also, pCold vectors have been used for the expression of proteins with fusion partners, as it is described below.

iii) Fusion Partners

Fusion partners are solubility-enhancing tags used to increase both the solubility and expression level of recombinant enzyme expression (Hayashi and Kojima, 2010). They are located at the N- or C-terminus of the target protein and in some cases a specific cleavage site is placed between the tag and the target protein to allow their excision after purification. The most common fusion partners correspond to glutathione-S-transferase (GST), maltose-binding protein (MBP), thioredoxin (Trx), G β -1

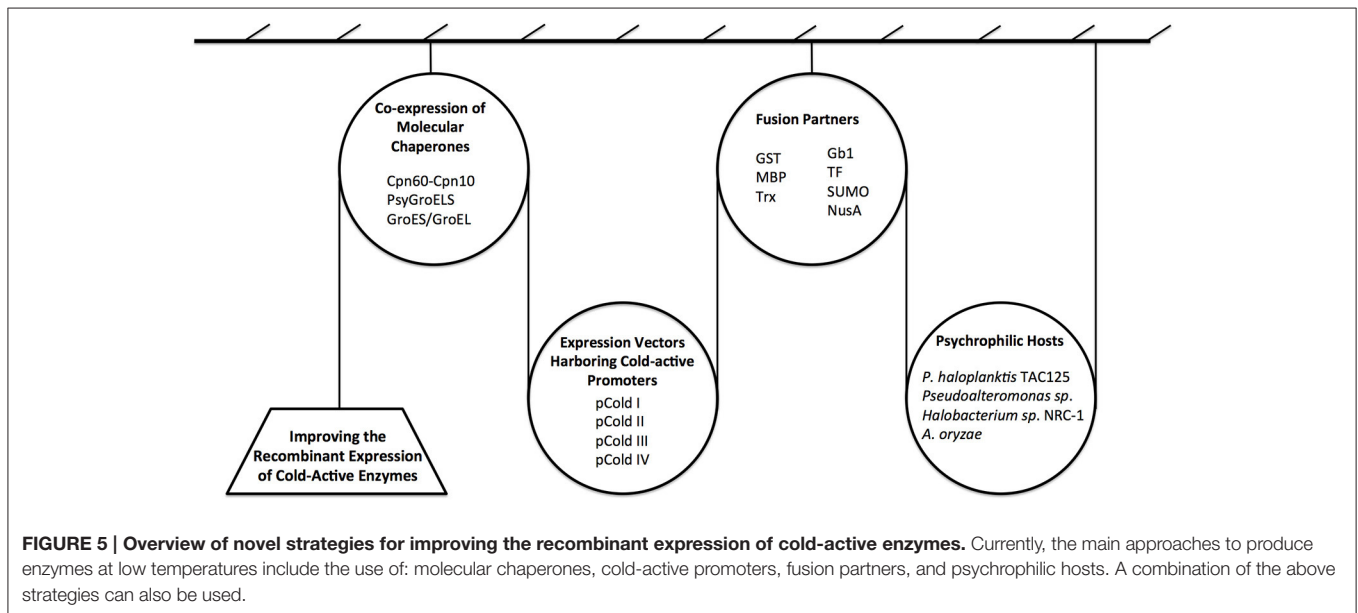
TABLE 2 | Solved structures of cold adapted enzymes deposited in the Protein Data Bank.

Enzyme	Classification	Source organism	PDB ID	References
Alkaline phosphatase	Hydrolase	<i>Shewanella</i> sp.	3A52	Tsuruta et al., 2010
Alkaline phosphatase	Hydrolase	<i>Vibrio</i> sp.	3E2D	Helland et al., 2009
Alkaline phosphatase	Hydrolase	Antarctic bacterium TAB5	2IUC	Wang et al., 2007
Alkaline phosphatase	Hydrolase	<i>Pandalus borealis</i>	1K7H	de Backer et al., 2002
Amidase	Hydrolase	<i>Nesterenkonia</i> sp.	3HXK	Nel et al., 2011
Aminopeptidase	Hydrolase	<i>Colwellia psychrerythraea</i>	3CIA	Bauvois et al., 2008
Cellulase	Hydrolase	<i>Pseudoalteromonas haloplanktis</i>	1TVN, 1TVP	Violot et al., 2005
Chitinase	Hydrolase	<i>Moritella marina</i>	4MB3, 4MB4, 4MB5	Malecki et al., 2013
Elastase	Hydrolase	<i>Salmo salar</i>	1ELT	Berglund et al., 1995
Endonuclease I	Hydrolase	<i>Aliivibrio salmonicida</i>	2PU3	Altermark et al., 2008
Esterase	Hydrolase	<i>Thalassospira</i> sp.	4V2I	De Santi et al., 2016
Esterase	Hydrolase	Arctic metagenomic library	4AO6	Fu et al., 2013
Esterase	Hydrolase	<i>Oleispira antarctica</i>	3I6Y, 3S8Y	Lemak et al., 2012
Esterase	Hydrolase	<i>Pseudoalteromonas</i> sp.	3HP4	Brzuszkiewicz et al., 2009
Lipase	Hydrolase	<i>Proteus mirabilis</i>	4GW3, 4GXN	Korman and Bowie, 2012
Lipase	Hydrolase	<i>Photobacterium lipolyticum</i>	2ORY	Jung et al., 2008
Lysozyme	Hydrolase	<i>Bombyx mori</i>	1GD6	Matsuura et al., 2002
Pepsin	Hydrolase	<i>Gadus morhua</i>	1AM5	Karlsen et al., 1998
Peptidase	Hydrolase	<i>Serratia</i> sp.	2B6N	Helland et al., 2006
Protease	Hydrolase	<i>Flavobacterium</i> sp.	3U1R	Zhang et al., 2011
Protease	Hydrolase	<i>Pseudomonas</i> sp.	1G9K, 1H71	Aghajari et al., 2003
Protein tyrosine phosphatase	Hydrolase	<i>Shewanella</i> sp.	1V73	Tsuruta et al., 2005
Pyrophosphatase	Hydrolase	<i>Oleispira antarctica</i>	3I4Q	Kube et al., 2013
S-formylglutathione hydrolase	Hydrolase	<i>Pseudoalteromonas haloplanktis</i>	3LS2	Alterio et al., 2010
Serine protease	Hydrolase	<i>Bacillus subtilis</i>	2GKO	Almog et al., 2009
Serine protease	Hydrolase	<i>Vibrio</i> sp.	1S2N, 1SH7	Arnórsdóttir et al., 2005
Trypsin	Hydrolase	<i>Oncorhynchus keta</i>	1MBQ	Toyota et al., 2002
Trypsin	Hydrolase	<i>Salmo salar</i>	2TBS	Smalås et al., 1994
Uracil-DNA N-glycosylase	Hydrolase	<i>Gadus morhua</i>	1OKB	Leiros et al., 2003
Xylanase	Hydrolase	<i>Aegilops speltoides</i>	5AY7, 5D4Y	Zheng et al., 2016
Xylanase	Hydrolase	<i>Pseudoalteromonas haloplanktis</i>	1H12, 1H13, 1H14	Van Petegem et al., 2003
α -amylase	Hydrolase	<i>Pseudoalteromonas haloplanktis</i>	1B0I	Aghajari et al., 1998
β -galactosidase	Hydrolase	<i>Arthrobacter</i> sp.	1YQ2	Skalova et al., 2005
β -glucanase	Hydrolase	<i>Eisenia fetida</i>	3WC3	Arimori et al., 2013
β -glucosidase	Hydrolase	<i>Exiguobacterium antarcticum</i>	5DT5, 5DT7	Zanphorlin et al., 2016
β -glucosidase	Hydrolase	<i>Micrococcus antarcticus</i>	3W53	Miao et al., 2016
β -lactamase	Hydrolase	<i>Pseudomonas fluorescens</i>	2QZ6	Michaux et al., 2008
Prolyl isomerase	Isomerase	<i>Cenarchaeum symbiosum</i>	2RQS	Jaremko et al., 2011
Sedoheptulose 7-phosphate isomerase	Isomerase	<i>Colwellia psychrerythraea</i>	5BY2	Do et al., 2015b
Triose phosphate isomerase	Isomerase	<i>Moritella marina</i>	1AW1, 1AW2	Alvarez et al., 1998
3-octaprenyl-4-hydroxybenzoate carboxylase	Lyase	<i>Colwellia psychrerythraea</i>	4RHE, 4RHF	Do et al., 2015a
Citrate synthase	Lyase	<i>Arthrobacter</i> sp.	1A59	Russell et al., 1998
Ectoine synthase	Lyase	<i>Sphingopyxis alaskensis</i>	5BY5, 5BXX	Widderich et al., 2016
Tryptophan synthase	Lyase	<i>Shewanella frigidimarina</i>	3VND	Mitsuya et al., 2014
Catalase	Oxidoreductase	<i>Aliivibrio salmonicida</i>	2ISA	Riise et al., 2007
Ectoine hydroxylase	Oxidoreductase	<i>Sphingopyxis alaskensis</i>	4Q5O, 4MHR, 4MHU	Höppner et al., 2014
Isocitrate dehydrogenase	Oxidoreductase	<i>Desulfotalea psychrophila</i>	2UXQ, 2UXR	Fedøy et al., 2007
L-leucine dehydrogenase	Oxidoreductase	<i>Flavobacterium frigidimaris</i>	2YY7	Yoneda et al., 2010
Lactate dehydrogenase	Oxidoreductase	<i>Champscephalus gunnari</i>	2V65	Coquelle et al., 2007
Leucine dehydrogenase	Oxidoreductase	<i>Sporosarcina psychrophila</i>	3VPX	Zhao Y. et al., 2012

(Continued)

TABLE 2 | Continued

Enzyme	Classification	Source organism	PDB ID	References
Malate dehydrogenase	Oxidoreductase	<i>Aquaspirillum arcticum</i>	1B8P, 1B8U, 1B8V	Kim et al., 1999
Phenylalanine hydroxylase	Oxidoreductase	<i>Colwellia psychrerythraea</i>	2V27, 2V28	Leiros et al., 2007
Superoxide dismutase	Oxidoreductase	<i>Pseudoalteromonas haloplanktis</i>	3LJF, 3LJ9, 3LIO	Merlino et al., 2010
Superoxide dismutase	Oxidoreductase	<i>Allivibrio salmonicida</i>	2W7W	Pedersen et al., 2009
Adenylate kinase	Transferase	<i>Marinibacillus marinus</i>	3FB4	Davlieva and Shamoo, 2009
Adenylate kinase	Transferase	<i>Sporosarcina globispora</i>	1S3G	Bae and Phillips, 2004
Aminotransferase	Transferase	<i>Psychrobacter</i> sp.	4RKC, 4RKD	Bujacz et al., 2015
Aspartate carbamoyltransferase	Transferase	<i>Moritella profunda</i>	2BE7	De Vos et al., 2007
Serine hydroxymethyltransferase	Transferase	<i>Psychromonas ingrahamii</i>	4P3M	Angelaccio et al., 2014



domain of protein G (Gb1), nascent chain chaperone trigger factor (TF), small ubiquitin-like modifier (SUMO), and N-utilizing substance A (NusA).

Regarding expression of cold-adapted enzymes using fusion partners, Trx fusion tag has been used to obtain high yield of soluble psychrophilic yeast proteins in *E. coli* host (Illias et al., 2014). Moreover, the effects of seven different N-terminal fusion partners were studied to improve the solubility of proteins from the psychrophilic fish *Vibrio salmonicida* in *E. coli*. Among the fusion partners, MBP and NusA showed to be the best for expression yield and protein solubility (Niiranen et al., 2007). In addition, two different expression host strains and three cell culture incubation temperatures were used. Concerning the host strain, *E. coli* BL21-AI was shown to be superior to BL21(DE3)RIL CodonPlus for protein expression, but the product solubility was not affected by the choice of host. In terms of the incubation temperature for protein expression, the protein yield increased with temperature, although the effect on solubility was the contrary in most cases. They also concluded that small proteins were easier to express.

Another example for the soluble expression of a cold-active enzyme using MBP as the fusion partner was reported for a lipase from marine Antarctic origin (Parra et al., 2008). First, the expression system *E. coli* BL21(D3E)/pET22b(+) was used but the protein was obtained as inclusion bodies. After using the expression system *E. coli* TB1/pMAL-c2E, which expressed a fusion MBP-lipase protein, the enzyme was obtained in a soluble and active form. Hayashi and Kojima (2008) used the pCold I vector and modified it in order to express proteins fused to a GST tag. They were able to successfully express 9 proteins which they could not obtain using a conventional *E. coli* expression system. Later, the same authors used the pCold-GST system to successfully express 78 proteins from mesophilic organisms, showing that the primary sequence length of these proteins was not correlated with the expression level in the soluble fraction. They also developed three other cold-shock vectors using the fusion partners GB1, Trx, and MBP, showing that all systems were successful in obtaining soluble fusion proteins, with the pCold-GST system being the preferred and the pCold-MBP system the second choice. Furthermore, they reported that the use of a C-terminal 6 proline tag was

successful in inhibiting the degradation of the protein during protein expression and purification, therefore being useful for enzyme stabilization (Hayashi and Kojima, 2010).

iv) Psychrophilic hosts

To overcome the decrease in protein yield and overall process productivity when *E. coli* strain is cultured at low temperatures, psychrophilic bacteria have been used as expression hosts (Parrilli et al., 2008b).

The most studied psychrophilic host is *Pseudoalteromonas haloplanktis* TAC125, which uses a modified *E. coli* cloning vector with psychrophilic molecular signals. This host was reported for the expression of a cold-adapted α -amylase as secretion carrier for extra-cellular protein targeting (Cusano et al., 2006a,b). Later, authors developed a *P. haloplanktis* TAC125 mutant strain that secreted a reduced number of exo-proteases, therefore reducing the extra-cellular proteolytic activity (Parrilli et al., 2008a).

In other study, an expression vector derived from psychrophilic bacterium *Pseudoalteromonas* sp. BSi20429 was constructed and *Pseudoalteromonas* sp. SM20429 was used as the psychrophilic bacterial strain. The system was first reported using a mesophilic promoter from *E. coli* and used for the active expression of a cold-adapted cellulase at 25–30°C (Zhao D. et al., 2011). Later, the mesophilic promoter was replaced by another from *Pseudoalteromonas* sp. BSi20429 that acted as a strong promoter at low temperatures and was also inducible by xylan, thus enabling the recombinant expression at lower temperatures. Multiple cloning sites and a His tag were also added to the expression vector, making these system useful for expressing *Pseudoalteromonas* enzymes that could not be maturely expressed in *E. coli* (Yu et al., 2015).

Another interesting study is an example to overcome the barrier of studying polyextremophilic enzymes. For halophilic enzymes, a high salt concentration is a requirement to obtain an active protein during overexpression in heterologous hosts. Karan et al. (2013) purified and characterized a halophilic and cold-active β -galactosidase from the cold-adapted haloarchaeon, *H. lacusprofundi*. They used the haloarchaeon, *Halobacterium* sp. NRC-1 strain as host in combination with a cold-shock protein gene promoter, *cspD2*, also from the host. They produced the recombinant β -galactosidase at 20-fold higher levels compared to *H. lacusprofundi*.

Finally, an example of a eukaryotic expression system for genes codifying cold-active enzymes comes from the work performed by Mao et al. (2015), who developed a novel uracil-deficient *Aspergillus oryzae* host for heterologous expression. This system was used to express an α -amylase from the psychrophilic fungus *Geomyces pannorum*.

v) Combination of the above strategies

Combination of these strategies has also been successfully used for the expression and purification of cold-adapted enzymes. A cold-adapted endo-1,4- β -glucanase from the earthworm *Eisenia fetida* was cloned in the pColdI vector and successfully expressed using the host strain ArcticExpress RT (DE3) (Ueda et al., 2014). Bjerga and Williamson (2015) optimized an expression

system for cold-adapted proteins based on the pCold-II vector. They expressed five genes derived from metagenomic DNA from marine Arctic sediments and used three hosts strains including BL21 CodonPlus(DE3)RIL, ArcticExpress(DE3)RIL, and Rosetta2(DE3)pLysS, obtaining the best results using the latter. The yields of soluble protein were increased using fusion partners like MBP, TF, TRX, and SUMO, reporting the best results using large fusion partners like MBP and TF (Bjerga and Williamson, 2015).

EVOLUTIONARY AND MOLECULAR MECHANISMS OF THE COLD-ADAPTATION OF ENZYMES

The ability of unicellular organisms to thrive in cold environments requires a vast array of adaptations in all levels, which enables to compensate for the perturbations stressed by these extreme environments. These adaptations cover from changes in the lipid composition of the cell membrane (Russell and Fukunaga, 1990) to sequence and structure changes in enzymes ensuring the efficiency of all biochemical reactions (Gerday et al., 2000).

The critical role of thermal adaptations on an enzyme's ability to remain highly active in the cold is easily understood if we consider that the metabolic and growth rates of psychrophilic and psychrotolerant species near the freezing point of water are higher than those of mesophilic organisms at the same temperature (Mohr and Krawiec, 1980; Knoblauch et al., 1999). To achieve this, enzyme function must be tuned in order to cope with the inherent temperature-dependent reduction of chemical rates and enable life in cold environments. Structurally, enzymes also require modification of their thermal stability and the dynamics of their three-dimensional structure in order to compensate for the freezing effects of low temperatures (Feller and Gerday, 2003), while at the same time avoiding catastrophic cold-induced unfolding events that impede proper function (Ramírez-Sarmiento et al., 2013). As thermal adaptations in enzymes are achieved by amino acid substitutions, insertions and deletions, the evolution and molecular basis of these adaptations in cold-adapted enzymes can be extracted mainly based on the comparison of their structural and functional features against mesophilic and thermophilic homologs. Here, we cover the main catalytic features of cold-adapted enzymes and the evolutionary and molecular mechanisms that allow these adaptations.

Functional Adaptations for High Catalytic Activity at Low Temperatures

The main mechanistic goal of the evolutionary adaptations in cold-active enzymes is to maintain a high catalytic activity at low temperatures. These activities are required to sustain metabolic activity in extremely cold environments, in some cases even near -20°C (Rivkina et al., 2000).

At very low temperatures the kinetic energy is insufficient to allow overcoming the kinetic barriers associated with an enzymatic reaction (Siddiqui and Cavicchioli, 2006). Nevertheless, cold adapted enzymes generally have optimum

temperatures of activity and higher reaction rates at lower temperatures than their mesophilic homologs. We can rationalize this behavior if we examine the temperature dependence of the rate of chemical reactions as envisioned by the Arrhenius equation (Laidler, 1984):

$$k_{\text{cat}} = Ae^{-E_a/(RT)} \quad (1)$$

Where k_{cat} is the catalytic rate, E_a is the activation energy of the reaction, R is the gas constant, T is temperature and A is a collision frequency factor. This equation illustrates how the catalytic rate depends on the temperature, such that it decreases upon decreasing temperature. For example, the catalytic rate of a mesophilic enzyme with E_a values ranging 50–75 kJ·mol⁻¹ decreases 2–3-fold upon lowering the temperature every 10°C (Tattersall et al., 2012).

As suggested by the Arrhenius equation, the detrimental effect of lowering the temperature on the catalytic turnover can be countered by decreasing the activation energy, such that the thermal dependence of the catalytic reaction is reduced. Extensive reviews have been made about the changes in activation energies of chemical reactions catalyzed by psychrophilic enzymes and their mesophilic and thermophilic homologs, consistently showing that the activation energies are in fact decreased in cold-adapted enzymes (Lonhienne et al., 2001; Matsuura et al., 2002; D'Amico et al., 2002b, 2003a,b; Mavromatis et al., 2003; Garsoux et al., 2004; Liang et al., 2004; Fedøy et al., 2007; Leiros et al., 2007; Lian et al., 2015).

The temperature-dependence of reaction rates given by the Arrhenius equation explains the increase in k_{cat} needed for catalyzing reactions at low temperature under saturating substrate concentrations, whereas substrate binding represented by the Michaelis-Menten constant (K_m) could also play an important role in increasing the catalytic efficiency (defined as k_{cat}/K_m) of cold-adapted enzymes at low temperatures (Feller and Gerday, 1997; D'Amico et al., 2002a). We can rationalize how these changes in the kinetic parameters are tuned within a cold-adapted enzyme through the transition state theory, in which equilibrium between the ground (ES) and a stable activated (ES^\ddagger) enzyme-substrate complex is assumed:



In this scenario, the temperature dependence of the catalytic rate can be related to the free energy of activation (ΔG^\ddagger) between the ground and activated that has to be overcome during an enzymatic reaction using the Eyring equation (Eyring, 1935):

$$k_{\text{cat}} = \frac{k_B T}{h} e^{-\frac{\Delta G^\ddagger}{RT}} = \frac{k_B T}{h} e^{\left(\frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{RT}\right)} \quad (3)$$

Where k_B is the Boltzmann constant, h is the Planck constant and ΔH^\ddagger and ΔS^\ddagger are the enthalpic and entropic contributions to the free energy of activation, respectively.

The comparative analysis of the kinetic parameters of a large number of psychrophilic enzymes has shown that their k_{cat} at low temperatures is similar to those observed for mesophilic enzymes

at warm temperatures (D'Amico et al., 2002a; Siddiqui and Cavicchioli, 2006). In that scenario, either ΔH^\ddagger must decrease or ΔS^\ddagger must increase, as it is clear from Equation (3) (Lonhienne et al., 2000).

The contribution of ΔH^\ddagger can be understood in terms of the interactions that are broken while transitioning from the ground enzyme-substrate complex to the transition state of the reaction (Figure 6). Thus, a decrease of the enthalpic contribution translates into a reduction of the number of interactions that must be broken during this process (Siddiqui and Cavicchioli, 2006). This enthalpy decrease for psychrophilic enzymes is consistent with the decrease of the activation energy of the reactions catalyzed by these enzymes, as $\Delta H^\ddagger = E_a - RT$ (Lonhienne et al., 2000).

Compensation of the change of enthalpic contributions for the formation of the transition state is achieved by an antagonist change in ΔS^\ddagger , which explains why k_{cat} does not increase exponentially upon changing ΔH^\ddagger as a product of the cold adaptation of psychrophilic enzymes (Lonhienne et al., 2000). This compensation is such that the activation entropy difference between a mesophilic and a psychrophilic enzyme is always negative and the absolute value of their entropy difference, $T\Delta(\Delta S^\ddagger)$, is always large (Lonhienne et al., 2000). This entropic compensation can be conceptualized in the context of the protein structure as an increase in flexibility of regions of the protein covering the enzyme's active site or other extensive changes in flexibility throughout the protein (Gerday et al., 1997). Extensive

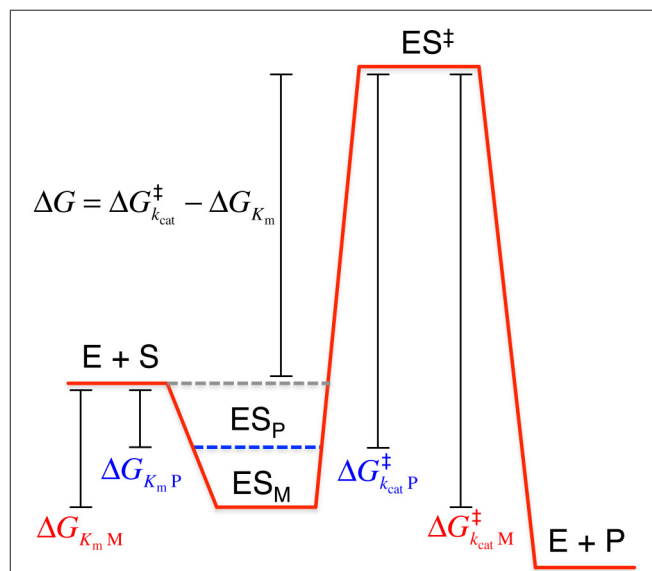


FIGURE 6 | Free energy changes between psychrophilic and mesophilic enzymes along the enzyme reaction coordinate from substrates (S) to products (P), according to the transition state theory.

The energy of the enzyme-substrate complex for the psychrophilic enzyme (ES_P) is higher than for the mesophilic homolog (ES_M), due to changes on the free energy of activation caused by decreasing the number of interactions broken to reach the transition state (enthalpic contribution) and increasing the protein flexibility (entropic compensation). These free energy changes lead to an increase in k_{cat} and a concomitant increase in K_m . ‡, transition state.

evidence from enzyme kinetics has shown that this is true for all cold-adapted enzymes studied so far (Siddiqui and Cavicchioli, 2006). Moreover, as a result of these changes in entropy and enthalpy, a small reduction of the free energy of activation and an increase of the conformational distribution of the ground state of the enzyme-substrate complex occurs (Figure 6). This trade-off between activity and stability is what leads to a small reduction of the free energy of activation in cold-adapted enzymes.

The enthalpic-entropic changes experienced by psychrophilic enzymes and represented in Figure 6 have two different consequences. First, increasing the flexibility of an enzyme through changes in plasticity of the active site leads to increased substrate promiscuity (Nobeli et al., 2009) because substrates with small variations in size and conformation can now fit into the more accessible binding site (Struvay and Feller, 2012; Feller, 2013) as it has been demonstrated for cold-adapted *Sporosarcina psychrophila* acylaminoacyl peptidase (Brunialti et al., 2011), *Shewanella gelidimarina* nitrate reductase, *Psychromonas ingrahamii* serine hydroxymethyltransferase (Angelaccio et al., 2012) and *Psychrobacter* sp. aminotransferase (Bujacz et al., 2015). This broader substrate utilization can be advantageous for protein engineering strategies focused in enhancing the specificity toward chemical reactions of biotechnological interest (Zhang et al., 2016). Second, these free energy changes, particularly the enthalpic changes, cause a decrease in substrate binding affinity. In this context, cold-active enzymes increase their k_{cat} at the expense of an increase in K_m (Feller and Gerday, 2003). In fact, stepwise single and multiple mutations engineered on a psychrophilic α -amylase to reconstruct the amino acid substitutions found in a mesophilic homolog exhibit a striking correlation of k_{cat} and K_m , such that both decrease concomitantly upon increasing the number of mesophilic residues in the cold-adapted enzyme (Cipolla et al., 2011). Nevertheless, some enzymes from psychrophilic organisms that operate under subsaturating substrate concentrations within the cytoplasm exhibit a decrease in this kinetic parameter as an evolutionary strategy for cold adaptation (Bentahir et al., 2000; Hoyoux et al., 2001; Lonhienne et al., 2001).

In summary, cold-adapted enzymes generally exhibit an increase of their catalytic rate (k_{cat}) allowed by a decrease in enthalpy due to a reduced number of protein-ligand interactions and an increase in entropy due to changes in their stability and flexibility, which can also lead to advantageous properties such as substrate promiscuity. In the following section we rationalize how these changes in stability and flexibility are embodied in the primary, secondary, tertiary (and sometimes quaternary) structure of these enzymes.

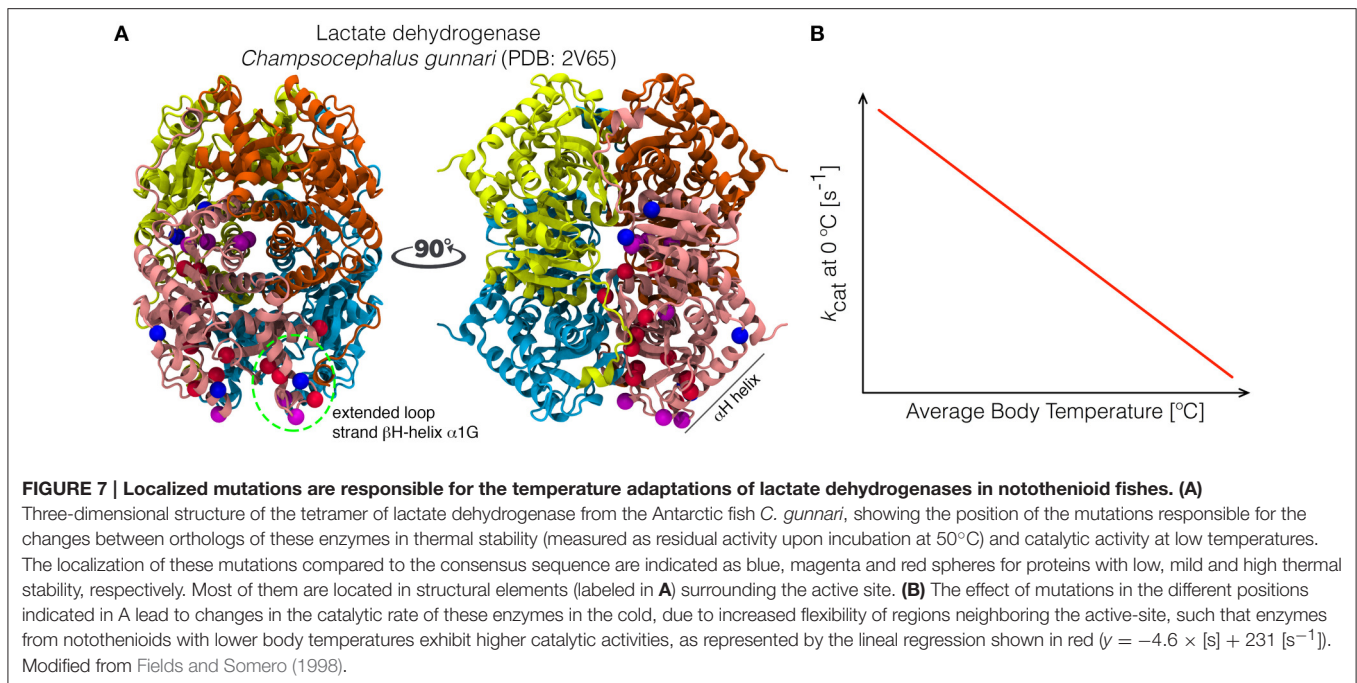
Sequence and Structure Changes Enabling High Enzymatic Activities at Low Temperatures

Thermophilic enzymes are known for having a higher thermostability than mesophilic enzymes and for being poor biocatalysts at room temperature (Gerday et al., 2000). Such thermostability, which is required to withstand heat denaturation at high temperatures, leads to increased conformational rigidity

at temperatures where mesophilic enzymes usually catalyze their reactions (Závodszy et al., 1998). Interestingly, the conformational fluctuations are similar when comparing mesophilic and thermophilic enzymes at their respective optimal activity temperatures in which both K_m and k_{cat} are also optimal, the so-called “corresponding state” hypothesis (Závodszy et al., 1998). This evidence led to conclude that evolutionary adaptations, in the form of sequence and structure changes, allow a balance between protein stability and conformational flexibility that are responsible of proper function in the environmental niche’s temperature of the source organism. In consistency with this idea, it has been argued that the plasticity or flexibility of cold-adapted enzymes is what enables their high specific activity at low temperatures and with a low energy cost (Gerday et al., 2000). It is now broadly accepted that the trade-off between thermostability and activity, and in particular the balance between stability and flexibility, is what evolves in enzymes in order to suit different environmental niches: for enzyme catalysis to be efficient at low temperatures protein flexibility must be increased, otherwise the reduced thermal fluctuations will diminish the conformational mobility and consequently compromise catalytic efficiency (Arnold et al., 2001). Moreover, it has been suggested that the encounter of cold-active enzymes with optimal activities at temperatures higher than their physiological conditions is evidence of an incomplete evolutionary adaptation to low temperatures (Georlette et al., 2004).

Some of the first and most detailed evidences of this apparent increase in conformational flexibility came from the study of A4 lactate dehydrogenases (A4-LDH) from nine Antarctic and three South American notothenioid teleosts, which inhabited niches with temperatures ranging from -1.8 to 10°C (Fields and Somero, 1998). Enzyme activity assays revealed that the catalytic rate of A4-LDH from teleosts inhabiting the coldest environments were higher at 0°C than their homologs, with k_{cat} decreasing linearly as a function of average body temperature. More importantly, deduction of their amino acid sequences from RT-PCR and DNA sequencing showed that most of the minimal residue substitutions between A4-LDH that led to these catalytic differences were not distributed randomly, but located in two regions in the vicinity of the active site (helix αH and an extended loop connecting an helix with catalytic residues) whose conformational changes are rate-limiting steps for catalysis (Figure 7). Their results suggested that the observed substitutions increased the flexibility of these regions, leading to more rapid conformational changes and thus increasing k_{cat} (Fields and Somero, 1998).

Similar suggestions of the increased flexibility of cold-adapted enzymes were made based on gene cloning, protein purification and sequence analysis, combined with homology modeling of several enzymes generated using already crystallized mesophilic and thermophilic enzymes as templates (Russell, 2000). Such analysis led to the identification of interesting amino acid substitutions consistently found to occur in several cold-adapted enzymes when compared to their mesophilic and thermophilic homologs, such as the reduction of the number of surface salt bridges due to replacement of basic residues by glutamine or



asparagine, changes in the distribution of surface charges, a reduced hydrophobicity of the protein core due to substitutions of bulky aromatic residues by more flexible nonpolar residues, a decrease in the number of hydrogen bonds in the protein structure, an increase in length of loop regions, among others (Davail et al., 1994; Feller et al., 1994; Smalås et al., 1994; Feller and Gerday, 1997; Russell, 2000). However, confirmation of these changes through the resolution of crystal structures of cold-adapted enzymes was lacking, mostly due to the difficulty of crystallizing these proteins (Russell, 2000).

The first crystal structures of cold-adapted enzymes were obtained for elastase (Berglund et al., 1995) and trypsin (Smalås et al., 1994) of Atlantic salmon, whereas the first solved crystal structures of bacterial psychrophilic enzymes corresponded to α -amylase (Aghajari et al., 1998), triose phosphate isomerase (Alvarez et al., 1998), and citrate synthase (Russell et al., 1998). Currently there are more than 50 different cold-adapted enzymes deposited in the Protein Data Bank (Berman et al., 2000), most of them coming from psychrophilic bacteria. The accession codes of most of these solved structures (excluding repeated structures of the same enzymes with ligands or mutations) are provided in **Table 2**, including a few remarkable examples of the use of metagenomic libraries to collect novel cold-adapted enzymes (Fu et al., 2013). It is worth noting that most of these enzymes correspond to hydrolases (**Table 2**), which comes as no surprise given that these are the most identified and studied cold-adapted enzymes for biotechnological and industrial applications, as we will see later on in this review.

Comparison of these deposited structures against mesophilic and thermophilic homologs confirmed that only minor structural modifications are needed to adapt warm-adapted enzymes to cold temperatures and that active-site residues involved in the

reaction mechanisms are strictly conserved between homologous enzymes adapted to different temperatures (D'Amico et al., 2002a). Such comparisons allow determining the preferred amino acid exchanges and the localization of these changes within the protein structure, the variety of evolutionary strategies toward cold adaptation and also enable the reconstruction of the evolutionary steps that mediate temperature adaptations in the laboratory via rational design (Tsigos et al., 2001; Mavromatis et al., 2003).

In terms of changes in protein sequence, a systematic comparative analysis of multiple sequence and structure alignments containing 21 psychrophilic enzymes belonging to different structural families and 427 homologous mesophilic and thermophilic allowed to create a distance matrix of residue substitutions often found to allow adaptation to low temperatures: charged residues Arg and Glu tend to be replaced at exposed sites on α -helices by Lys and Ala, respectively; Val is replaced by Ala at buried regions in α -helices; and the content of Ala and Asn increases whereas Arg decreases in exposed sites (Gianese et al., 2001). Very similar results were recently obtained using archaeal genome analysis combined with high-throughput homology modeling (Saunders et al., 2003) and also using proteome-wide approaches on six completely sequenced species of psychrophilic and mesophilic bacteria (Metpally and Reddy, 2009). However, the location of these and other substitutions and the number of substitutions vary on a great extent depending on the enzyme under examination, meaning that each protein family adopts different structural strategies to adapt to low temperatures (Gianese et al., 2002).

For example, in the case of *M. marina* triose phosphate isomerase, a single substitution of an alanine located within a loop that contacts the phosphate moiety of its substrate by a

serine that is conserved in mesophilic enzymes is sufficient to increase the thermal stability and decrease the catalytic activity at low temperatures (Alvarez et al., 1998). The same is applicable in some cases for tuning mesophilic enzymes in order to sustain catalytic activities in the cold, as exemplified by the rationally designed single-point mutation I137M of *Bacillus subtilis* LipJ (Goomber et al., 2016b). Most frequently, evolutionary changes are related to multiple changes that lead to a more accessible and/or a more flexible active site due to substitution of bulky residues, insertions and deletions (Russell et al., 1998; Kim et al., 1999; Schröder Leiros et al., 2000; Toyota et al., 2002; Aghajari et al., 2003; Van Petegem et al., 2003; Tsuruta et al., 2005, 2008; Leiros et al., 2007; Riise et al., 2007; Jung et al., 2008; Merlino et al., 2010; Jaremko et al., 2011; Malecki et al., 2013; Zheng et al., 2016), which in some cases are accompanied by the introduction of discrete amino acid substitutions in the active site that thermodynamically favor protein-ligand interactions at low temperatures, thus decreasing K_m (Lonhienne et al., 2001). Finally, the most extensive changes involve large portions throughout the protein structure and are related to optimization of the surface electrostatic potential to allow better interactions with the solvent and changes in ion-pair interactions (Bell et al., 2002; de Backer et al., 2002; Leiros et al., 2003; Bae and Phillips, 2004; Kumar and Nussinov, 2004; Arnórsdóttir et al., 2005; Helland et al., 2006; De Vos et al., 2007; Fedøy et al., 2007; Wang et al., 2007; Michaux et al., 2008; Pedersen et al., 2009; Alterio et al., 2010; Arimori et al., 2013; Bujacz et al., 2015), reduction of the number of hydrogen bonds (Matsuura et al., 2002; Bae and Phillips, 2004; Altermark et al., 2008; Michaux et al., 2008; De Santi et al., 2016), changes in loop extension, amino acid content, and flexibility (Bauvois et al., 2008; Helland et al., 2009; Zhang et al., 2011; Fu et al., 2013; Miao et al., 2016; Zheng et al., 2016), introduction or loss of disulfide bonds to modulate local stability (Violot et al., 2005; Helland et al., 2006; Wang et al., 2007), differential flexibility of domains in multidomain enzymes (Watanabe et al., 2005; Bauvois et al., 2008; Angelaccio et al., 2014), and enhanced protein solvation due to increased exposure of hydrophobic residues to the solvent (Aghajari et al., 1998; Russell et al., 1998; Maes et al., 1999; Bell et al., 2002; Van Petegem et al., 2003; Zhao Y. et al., 2012; Zheng et al., 2016). A summary of the most usual modifications responsible for cold-adaptation are shown in **Figure 8**. It is worth noting that not all of these mechanisms are required to explain the cold-adaptation of a given enzyme (De Maayer et al., 2014), although several proteins exhibit more than one of these mechanisms occurring in parallel (Coquelle et al., 2007), which suggest that comparative analysis within protein families might be better suited to solve the sequence-structure factors that explain the evolutionary adaptations of an enzyme of interest. Although it is rare to find proteins showing other mechanisms of cold adaptation, more extensive changes in protein topology (Tsuruta et al., 2005) or modifications of the oligomerization state that allows to increase the flexibility of solvent-exposed hydrophobic regions while simultaneously stabilizing the native fold of the enzyme (Skalova et al., 2005; Zanphorlin et al., 2016) have been also observed. However, these should be considered as evolutionary alternatives rather

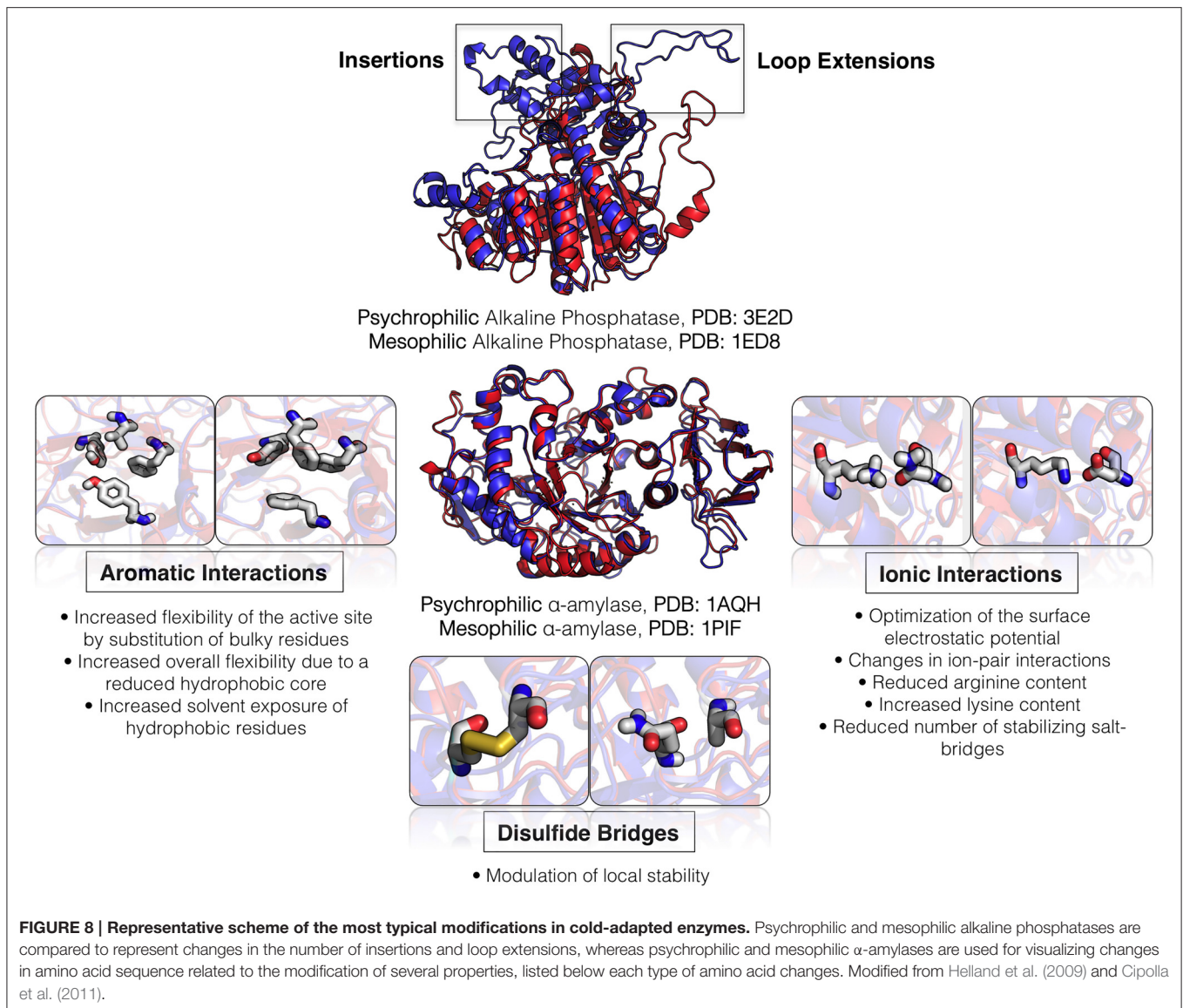
than as general mechanisms for enhanced flexibility in cold environments.

Experimental and Computational Approaches to Study the Global and Localized Conformational Flexibility of Cold Adapted Enzymes

While solving the structures of these enzymes helped to deepen our understanding of the molecular mechanisms behind adaptation to cold temperatures, they only provide a static view of the position of these sequence changes within the three-dimensional space, thus lacking of an exploration of protein dynamics, with the only exception of those structures solved by NMR (Jaremko et al., 2011). Therefore, the combination of this structural information with experiments that assess the conformational flexibility of cold-adapted enzymes and the direct use of these structures as inputs for molecular dynamics is crucial to provide a solid framework for further experimental and computational protein engineering approaches.

Among the experiments performed to demonstrate the increased flexibility of cold-adapted enzymes, dynamic quenching of tryptophan fluorescence by increasing concentrations of acrylamide (Eftink and Ghiron, 1975) is commonly used. Acrylamide ascertains the accessibility of tryptophan residues within a protein as a decrease in fluorescence by means of physical contact (Eftink and Ghiron, 1976), thus reflecting the ability of the quencher to penetrate the protein structure and providing information of its permeability (D'Amico et al., 2003b). Typically, the fluorescence quenching constants (as reported by the Stern-Volmer constant) of psychrophilic enzymes are higher than for mesophilic proteins at both low and warm temperatures, thus indicating a more permeable structure (Huston et al., 2008; Tang et al., 2012), and the variation of fluorescence quenching (i.e., the change in the Stern-Volmer constant) within a temperature range where the native state prevails decreases in the order psychrophilic > mesophilic > thermophilic (D'Amico et al., 2003a; Georlette et al., 2003, 2004; Cipolla et al., 2012), thus indicating that cold-adapted enzymes possess higher flexibility. These experiments can also be combined with mutational analysis to explore the interplay between sequence variation, protein flexibility, and catalytic activity (Cipolla et al., 2011; Sigtryggsdóttir et al., 2014; Truongvan et al., 2016).

Further identification of the spots responsible for increased flexibility within a psychrophilic protein requires assessment of local regions of the protein. A successful approach for such task consists of the use of chimeric enzymes, in which a gene encoding for a given psychrophilic protein is divided into several regions that are then replaced by similar regions from a mesophilic homolog (Yoneta et al., 2004; Watanabe et al., 2005). This strategy allows not only to confirm that in some cases the entire protein does not necessarily need to be flexible to achieve high catalytic activity in at low temperatures, but also to identify which protein regions are responsible for the increased flexibility (Yoneta et al., 2004), which can



be further combined with mutational analysis to identify the key residues responsible for cold-adaptation (Hayashi et al., 2014). These experiments allowed the identification of the C-terminal region of the cold-adapted isocitrate dehydrogenase from *Colwellia maris* as responsible for its psychrophilic characteristics (Yoneta et al., 2004). Another powerful strategy corresponds to amide hydrogen/deuterium exchange mass spectrometry (Balasubramaniam and Komives, 2013), in which the exchange between backbone amide protons and the deuterium from the surrounding solvent is used as a mass probe for the solvent accessibility of a protein, whereas quenching and pepsin digestion of the protein followed by mass spectrometry analysis of the resulting peptides allows to localize the sites of exchange within the protein. In these experiments, highly flexible regions become fully deuterated in a few minutes, whereas well-packed regions such as the hydrophobic core exhibit a low extent of exchange. The advantages of this strategy is that it can be applied

to proteins of any size (Balasubramaniam and Komives, 2013), under varying temperature (Ramírez-Sarmiento et al., 2013), and solvent conditions (Medina et al., 2016) and in the absence and presence of ligands (Chalmers et al., 2011). Comparative analysis of deuterium incorporations of local regions of a psychrophilic and a thermophilic alcohol dehydrogenase led to strengthen the notion that only those functional regions related to substrate binding exhibit greater flexibility in the cold-active enzyme than in the warm-adapted homolog, suggesting that local flexibility can be uncoupled from thermal stability (Liang et al., 2004).

The use of solved or homology modeled psychrophilic protein structures in molecular dynamics allows the assessment of dynamical features relevant for cold adaptation with atomistic resolution. Constant temperature simulations of psychrophilic enzymes at several temperatures within the range 10–45°C enabled the identification of loops near active sites that exhibit

higher flexibility in comparison with their mesophilic homologs, as in the case of Uracil-DNA glycosylases (Olufsen et al., 2005), elastases (Papaleo et al., 2006), and β -glucosidases (Zanphorlin et al., 2016), and the optimization of ion-pair networks near the active sites of elastases (Papaleo et al., 2007) and serine proteases (Tiberti and Papaleo, 2011). In some cases, these simulations show good correlation between the optimal temperature of catalytic activity and the increased flexibility of functional regions of the protein (Aurilia et al., 2009) and also highlight other loops distant from the active site that exhibit preservation of similar flexibilities between psychro-, meso-, and thermophilic enzymes at their optimal temperature for catalysis (Kovacic et al., 2016). Other quasi-harmonic entropy approximations have been used for comparative analysis of simulations of psychrophilic enzymes in their free and substrate-bound forms, allowing the identification of key determinants of structural flexibility at the residue-level (Kosugi and Hayashi, 2011). More recently, complex molecular simulation strategies have allowed bridging the reduction of the activation energies of enzymatic reactions with the increased flexibility of cold-adapted enzymes. Hybrid quantum-mechanics/molecular-mechanics simulations on a psychrophilic α -amylase from *Pseudoalteromonas haloplanctis* revealed that formation of the transition state of the enzymatic reaction is accompanied by a rearrangement of a loop neighboring the active site, such that it interacts with the substrate via water-mediated and direct interactions, and is crucial for the reduction of the free energy barrier of the hydrolysis reaction (Kosugi and Hayashi, 2012). Moreover, energetic estimations of peptide hydrolysis by psychrophilic trypsins calculated using free energy perturbation simulations in which the flexibility of the protein surface is systematically reduced through position restraints of different strengths, showed that this protein rigidity is sufficient to increase the activation energy as in mesophilic enzymes, thus strongly suggesting that softness of the protein-water surface is what tunes the temperature adaptation of catalytic rates (Isaksen et al., 2016).

The molecular mechanisms of cold-adaptation and the hotspots of conformational flexibility captured through the application of these experimental and computational approaches are not only compelling evolutionary and theoretical challenges to pursue, but also provide paramount information to integrate in protein engineering and design endeavors. As we will see below, a vast number of rational design and directed evolution approaches used to improve catalysis at low temperatures are proposed based on localized conformational flexibility spots revealed by these types of analysis.

PROTEIN ENGINEERING OF COLD-ACTIVE ENZYMES

Protein engineering has emerged as a strategy to optimize a specific property of an enzyme *in vitro*, such as their thermal stability, substrate specificity and activity at extreme temperatures. This is performed through the introduction of mutations into a protein sequence in order to allow

“evolution” toward a target feature. Rational design and directed evolution are the two most general approaches to attempt protein engineering. Rational design is based on site-specific mutagenesis, therefore the structure, function, and catalytic mechanisms of the protein must be known (Arnold, 2001; Tang and Zhao, 2009; Bornscheuer et al., 2012; Reetz, 2013). When no detailed structural information of the enzyme is available, the typically applied strategies are error-prone PCR (epPCR) (Leung et al., 1989) and DNA shuffling (Stemmer, 1994). An actual trend of directed evolution is the creation of “smarter,” high-quality libraries, with a reduced library size and fast in reaching beneficial mutations (Kazlauskas and Lutz, 2009; Bornscheuer et al., 2012; Kille et al., 2013; Parra et al., 2013; Wijma et al., 2013).

As we have largely stressed in this review, the temperature adaptability of the catalytic properties exhibited by enzymes obtained from organisms adapted to extreme environments, makes them interesting biocatalysts for biotechnological and commercial applications. However, further improvements to the activity, substrate specificity, or stability of cold-adapted enzymes are often needed to better suit specific industrial applications. In this regard, the elucidation of the molecular mechanisms and the trade-off between thermostability and activity underpinning the cold-adaptation of enzymes have been crucial for the application of protein engineering strategies that either enhance some of the properties of cold-adapted enzymes or modify meso- and thermophilic enzymes to be able to catalyze reactions at low temperatures. Here, we discuss some examples of successful applications of several protein-engineering approaches for achieving these goals.

Rational design has been used to improve the thermal stability and activity of cold-adapted citrate synthases by introduction of residue substitutions and loop insertions that reduce the accessibility of the active site in hyperthermophilic homologs, leading to an enzyme with increased thermal stability and lower optimal temperatures of activity (Gerike et al., 2001). Other rational designs focused on increasing the flexibility due to the introduction of more flexible residues, such as the single-point mutation I137M in the mesophilic *Bacillus subtilis* lipase [Lip], which led to a 17°C downshift of the optimal temperature of activity and to cold adaptation (Goomber et al., 2016b). A triple mutant of a psychrophilic alkaline phosphatase in which the bulky aromatic residues on the substrate binding sites were replaced by more flexible amino acids, led to an enzyme with increased stability that retains the psychrophilic character of the wild-type enzyme (Tsigos et al., 2001). Other attempts of increasing the rigidity of the active site of these enzymes through engineering of disulfide bonds have led to more stable proteins but accompanied by a large reduction of their catalytic rates (Ásgeirsson et al., 2007). Site-directed mutagenesis of cold-adapted endo-1,5- α -L-arabinanase has also allowed to shift the optimum pH of activity toward acidic conditions for their use in pectin extraction and juice clarification (Wang S. et al., 2014). Finally, computational analysis of residue packing and atomic displacement parameters in structures of cold-active lipases allowed identification of highly flexible regions within a protein, whose residues were experimentally manipulated via

rational design and site saturation mutagenesis to obtain variants with seven-fold increased thermal stability without loss of their cold-adapted properties (Cesarini et al., 2012).

Site saturation mutagenesis has been used to identify substitutions that affect enzyme activity and are not easily predicted by rational approaches. Site saturation mutagenesis on a cold-active β -galactosidase, which was unexpectedly inactivated by a rationally designed mutation, led to the identification of a double mutation within the active site that increased the catalytic activity in 2.5-fold and showed faster hydrolysis of skim milk's lactose at low temperature than the wild-type enzyme (Coker and Brenchley, 2006). The same strategy of saturation was exhaustively applied onto all the 88 loop residues of a mesophilic lipase from *Bacillus subtilis*, finding 5 substitutions within loops around the enzyme's active site that increased their conformational flexibility and, when combined into a 5-residue mutant, led to a lipase with a seven-fold catalytic activity enhancement at 10°C and increased catalytic activity within the range 5–60°C when compared to the wild-type enzyme (Kumar et al., 2014).

The most successful strategy for engineering novel cold-adapted enzymes and optimizing the properties of enzymes extracted from organisms inhabiting cold environments has been the use of directed evolution. Results from this strategy usually illustrate that cold adaptation of enzymes can be achieved through multiple routes. One of the first examples corresponds to random chemically-induced mutagenesis and low-temperature activity screening assays on mesophilic alkaline serine protease subtilisin, which led to obtain two different triple mutants, whose substitutions were located in different regions of the protein, with each one leading to improved catalytic activity at 10°C due to either a decrease in K_m (Taguchi et al., 1998) or an increase in k_{cat} (Taguchi et al., 1999). A similar strategy based on error-prone PCR was applied on psychrophilic lipases from *C. antarctica* (Zhang et al., 2003) and *Pseudomonas fragi* (Gatti-Lafranconi et al., 2008), leading to enzyme with increased half-life times against thermal inactivation, and on a metagenomically isolated mesophilic *Bacillus* lipase, generating a single mutation that conferred optimal activity at 10°C due to increased localized flexibility and reduced thermal stability (Goomber et al., 2016a). Combination of directed evolution with subsequent rounds of rationally designed site-directed mutagenesis, led to 6 substitutions within structured and unstructured regions near the active site of a thermophilic subtilase, which were not related to the substitutions found in naturally occurring cold-adapted homologs but enhanced casein hydrolysis at low temperatures, due to a downshift both in the thermal stability and the optimal catalytic temperature (Zhong et al., 2009). The same strategy was applied onto a xylanase from *Paenibacillus campinasensis* to improve its resistance to high alkaline and temperature conditions for their potential use in pulp and paper industry (Zheng et al., 2014).

A more coarse approximation consists of DNA shuffling for either generating chimeric enzymes of psychrophilic and warm-adapted homologs or allowing combinatorial extension of variants generated by mutagenesis. Transferring a highly flexible 12-residue region of a psychrophilic subtilisin into a mesophilic

homolog from *Bacillus lentus* generated a chimeric enzyme with cold adaptation characteristics (Tindbaek et al., 2004). Also, the combination of directed evolution with DNA shuffling on a glycine oxidase from *Bacillus licheniformis* led to engineering a cold-adapted enzyme with increased catalytic activity against the herbicide glyphosate, which can be potentially used to confer resistance on genetically modified crops (Zhang et al., 2016).

It is worth noting that, although most of the attempts for unleashing the full biotechnological potential of cold-adapted enzymes as biocatalysts rely on protein engineering strategies, the application of chemical modification strategies, such as protein immobilization, have been also successful in improving the stability of cold-adapted enzymes for industrial processes and also to enable the removal and recovery of these enzymes for continuous use. One of these few examples corresponds to the immobilization of a cold-adapted pullulanase “extremozyme” from *Exiguobacterium* sp. on epoxy-functionalized silica particles, which significantly improved the thermal stability after hour-long incubations at 60 and 70°C in comparison to the fast inactivation of the free enzyme after 5 min incubation at the same temperatures, thus constituting a potential candidate for starch hydrolysis at low temperatures (Rajaei et al., 2015).

POTENTIAL BIOTECHNOLOGICAL APPLICATIONS OF THE REVIEWED ENZYMES

The use of cold-adapted enzymes in chemical processes not only allows energy saving, but also performing chemical reactions at low temperatures in order to avoid chemical side-reactions that can occur at higher temperatures (Siddiqui, 2015). Moreover, the rapid inactivation of cold-active enzymes at moderate temperature because of their heat-lability is a good option for food industry, fine-chemical synthesis, and molecular biology applications (Cavicchioli et al., 2011). The biotechnological potential of cold adapted enzymes is very broad and have been extensively reviewed (Gerday et al., 2000; Cavicchioli et al., 2002, 2011; Gomes and Steiner, 2004; Marx et al., 2004, 2007; Margesin et al., 2007; Huston, 2008; Margesin and Feller, 2010; Nevalainen et al., 2012; Feller, 2013; Elleuche et al., 2014; Sarmiento et al., 2015; Siddiqui, 2015). Specific examples of biocatalysis using cold-active enzymes isolated from bacteria (Morita et al., 1997; Russell, 1998), yeast (Buzzini et al., 2012; Alcaíno et al., 2015), and fungi (Nevalainen et al., 2012) have also been documented, as well as specific reviews of the biotechnological potential of pectinases for food industry (Adapa et al., 2014), lipases (Joseph et al., 2008; López-López et al., 2014; Maiangwa et al., 2015), and xylanases (Collins et al., 2005; Dornez et al., 2011). Here, we summarize the biotechnological potential of some of the enzymes from **Table 1**.

Testing Cold-Active Enzymes under Additives or Industrial-Like Conditions

Enzymes used in chemical processes need to be active in the presence of other additives required for these reactions. Organic

solvents are widely used, either pure or mixed with aqueous solvents. In general, to obtain some information about cold-active enzymes after purification, residual enzymatic activity was studied in the presence of different additives, such as metal ions, EDTA, DTT, β -mercaptoethanol, and protease inhibitors. In addition, enzyme stability upon addition of organic solvents and salt was also studied for some cold-active enzymes. The effect of different additives was assessed for 76% of the cold-active enzymes examined in **Table 1**.

In reaction mixtures, organic solvents are used to increase the solubility of hydrophobic substrates, as in biodiesel production through transesterification reactions using lipases and esterases. However, enzyme activity is reduced in organic solvents because water molecules are lost (Doukyu and Ogino, 2010). At low temperatures, cold-active enzymes are able to grasp more tightly to available water molecules because they have a low inherent surface hydrophobicity (Karan et al., 2012). For this reason, cold-active enzymes preserve their catalytic activity in organic solvents because they are able to maintain a tight hydration shell. One example of a cold-active esterase that is active under high concentrations of different solvents and additives is the esterase LipA from *Sorangium cellulosum* (Cheng et al., 2011). The enzyme retained high level of activity in the presence of 0.1–1% of the commercially available detergents (Tween 20, Tween 80, Triton X-100). Also, the activity was tested after incubation with 16 different solvents. Diethylether, chloroform, benzene, toluene, *p*-xylene, cyclohexane, *n*-hexane, *n*-heptane, and isooctane increase LipA activity and the others have minor negatives effects, conserving always more than 50% activity. Another study of a cold-active enzyme stable in organic solvent was reported for lipase AT2 from *S. epidermidis* (Kamarudin et al., 2014). This cold-active enzyme was found stable in both hydrophilic and hydrophobic organic solvents. The enzyme displayed stability not only in methanol, ethanol and acetone but the lipolytic activity was also enhanced in the presence of DMSO and diethyl ether. In addition, the enzyme was catalytically active in toluene and *n*-hexane mixture, which is the preferred solvent in most of the transesterification reactions. One last example of lipases/esterases active in non-aqueous solvent systems is the lipase ReLipA from *R. endophyticus* (Yan et al., 2016) which exhibited excellent ability to catalyze the synthesis of methyl oleate, ethyl oleate, and butyl oleate in isooctane solvent system with a maximum yield of 82.2%. In addition, the enzyme is stable in different organic solvents.

Since an important number of cold-active enzymes are isolated from marine environments, some of them are also halophiles. One example is a salt-tolerant esterase, Est12 from *Psychrobacter celer*, which catalyze reactions and degrade organic matters under high salt concentrations (Wu et al., 2013a,b). Est12 was isolated from deep-sea sediments and showed enhanced activity and stability in 4.5 M NaCl, with K_m decreasing from 0.069 to 0.033 mM *p*-NB and k_{cat} doubled to around 9.21 s^{-1} compared to the enzyme without salt. Moreover 0.5 and 1% (v/v) non-ionic detergents (Tween 20, Tween 80, Triton 100 and CHAPS) significantly enhanced the activity, in some cases up to 200%. After incubation with 5–30% (v/v) ethanediol, methanol, DMSO as well as 5–20% isopropanol

and ethanol for 1 h, Est12 retained more than half of its activity.

More in depth, based on the remarkable activity of Pul-SH3 in the presence of SDS, two commercial detergents, Rika (7.5% v/v) and Fadisheh (2.5% w/v), were used to assess the potential application of the enzyme for washing purposes. The results showed that the enzyme was highly active in the presence of these detergents by 80.4 and 93.7%, respectively. In addition, the stability of the enzyme against the commercial detergents was interestingly high, so that the remaining activity after a 10-day holding at room temperature with Rika (7.5% v/v) and Fadisheh (2.5% w/v) was about 54.5 and 85%, respectively (Rajaei et al., 2015).

From the cold-active enzymes reviewed in **Table 1**, most of them claim potential uses in industrial processes. However, only a few went a step further and made at least a small trial in a real application, commonly for the food industry. These examples include three β -galactosidases for the hydrolysis of lactose in milk, one methylesterase for fruit firming, one polygalacturonase for juice industry, and a glycogen branching enzyme with biotechnological potential in bread production.

The first example was previously mentioned in this review and reported by Dong and coworkers (Dong et al., 2014) and corresponds to a cold-active but thermostable β -galactosidase. The enzyme was expressed as both a soluble protein and in the form of inclusion bodies. The active inclusion bodies of β -galactosidase were easily isolated by nonionic detergent treatment and directly used for lactose conversion in a repetitive batch mode. The enzyme lost \sim 5% (90°C) or 1% (10°C) activity after each reaction cycle. More than 54% (90°C) or 88% (10°C) of the original enzyme activity was retained after 10 conversion cycles under optimum conditions. These results suggest that the recombinant thermostable β -galactosidase may be suitable for the hydrolysis of lactose in milk processing, with the advantages of being active at low temperatures and cost-convenient. The second example is the attractive activity of *Lactococcus lactis* β -galactosidase at low temperatures, for which its efficiency as biocatalyst to bioconvert lactose within milk during storage was explored. For this purpose, they performed lactose hydrolysis in milk at 4 and 10°C. Using the free enzyme or immobilized cells, bioconversion rates of nearly 98% were achieved after 7 and 6 h of incubation, respectively. The immobilized cells were recycled and used several times, followed by enzyme activity measurements. Using immobilized *E. coli* NovaBlue cells expressing the β -galactosidase, more of the 96% of the initial activity was retained after 10 cycles of use at 4°C (Vincent et al., 2013). One last example of a β -galactosidase with potential applications in milk and dairy product industry is the enzyme from *Antarctic Arthrobacter* sp. 32cB, which has the capacity to hydrolyzed 90% of the lactose in 1 mL of milk at 10°C in 24 h (Pawlak-Szukalska et al., 2014).

Another example of enzymes with potential applications in food industry is an acidic and cold-active pectin methylesterase PE8F46 that was identified from *P. chrysogenum* and successfully expressed in *P. pastoris* (Pan et al., 2014). This enzyme was shown to significantly improve the firmness of pineapple

dices in combination with calcium lactate, compared with a commercial pectinase complex. Thus, it represents an excellent candidate for food processing in the fruit and vegetable industry, considering the requirement of low-temperature to keep fruit quality.

An example concerning the juice industry is polygalacturonase, Endo-PG I, which was shown to reduce the viscosity of papaya juice by 17.6%, and increased its transmittance by 59.1% (Tu et al., 2013). When combined with a commercial pectin methylesterase, it showed higher efficiency with a synergy degree of more than 1.25. Currently, the widely used polygalacturonase has a pH optimum of 3.5, which is lower than the papaya juice pH (5.7). Endo-PG I have a slightly acid pH optimum (6.0), is cold active and stable in a large range of temperatures, properties required for potential applications in the juice industry.

The last example of application in food industry is a glycogen branching enzyme (RmGBE) from the thermophilic fungus *Rhizomucor miehei* that showed interesting cold-adapted characteristics (Wu et al., 2014). Addition of RmGBE to wheat bread resulted in a 26% increase in specific volume and a 38% decrease in crumb firmness in comparison with the control. Besides, the retrogradation, determined by measuring the crumb firmness and chewiness of bread, was significantly retarded along with the enzyme reaction. These properties make RmGBE highly useful in the food and starch industries.

Two nice examples of cold-active enzymes with potential uses in biomedicine were also reported for a α -galactosidase and a nitroreductase. The possibility to generate a universal blood type from B-type blood for application in transfusion therapy has been studied using enzymes. Some α -galactosidases are capable of removing the antigenic component from surface carbohydrates of group B red blood cells. One example is the cold-active α -galactosidase from *Pseudoalteromonas* sp. strain KMM 701 that showed to convert B red blood cells into blood type O cells at neutral pH (Balabanova et al., 2010). The activity of the enzyme was first observed when it was purified from its natural host producer. In view of its application, this cold-active enzyme was then overproduced in a heterologous host (Bakunina et al., 2014). An example of a potential cold-active enzyme for prodrug therapy was described using a cold-active nitroreductase, Ssap-NtrB (Çelik and Yetis, 2012). Despite Ssap-NtrB derived from a mesophilic bacterium, it showed optimal activity at 20°C against cancer prodrugs. Authors comment that the cold-activity of this novel enzyme will be useful for therapies in combination with cryotherapy, exposing the target tissue to low temperatures in order to trigger the enzyme activity to activate the drug only where is required. Moreover, the enzyme could also be used for bioremediation of compounds of explosive and volatile nature in regions where high activity at low temperatures is needed.

CONCLUSIONS AND PERSPECTIVES

In this article, we have reviewed cold-active enzymes discovered between 2010 and June 2016 from culture-dependent

bioprospecting and also some few enzymes discovered by genome mining of psychrophilic microorganisms, aspects that have not been reviewed elsewhere. Interestingly, these cold-active enzymes were isolated not only from microorganisms living in cold environments, but also from mesophilic and even thermophilic microbes. By far, hydrolases were the most frequent class of enzymes isolated, probably because of the vast potential applications that this type of cold-active enzymes might have, due to their significant activities in diverse reactions and their potential catalysis of novel hydrolytic transformations (López-Iglesias and Gotor-Fernández, 2015). Lipases and esterases covered together 42% of the hydrolases from **Table 1**, which is consistent with the worldwide use of lipases due to their features, as they are easy to handle, active in non-aqueous medium and are able to catalyze chemo-, regio-, and enantio-selective transformations (Kumar et al., 2016). The second largest class was oxidoreductases, but only with four representatives compared to the 84 hydrolases. A similar trend was observed for the representation of different types of cold-adapted enzymes for which their structures have been solved, as shown in **Table 2**. Therefore, there is a big opportunity for the isolation of novel cold-active enzymes from members of other classes, which have been less explored. The majority of the enzymes were isolated from microorganisms living in diverse places of the Polar Regions and oceans, and most of these microorganisms were bacteria. For enzyme production, the most used host was *E. coli* (85%) followed by *P. pastoris* (10%). Concerning expression vectors, the common pET vectors were the choice. It is surprising that despite the existence of special designed hosts and expression vectors for the recombinant production of cold-active enzymes, still classic *E. coli*/pET systems are preferable. We argue that more studies comparing different expression systems for cold-active enzymes are needed, in order to give more evidences of the advantages of using other hosts and expression plasmids. Indeed, we have also addressed the progress made in the overexpression and purification of cold-adapted enzymes, giving examples of enzymes that were only obtained soluble when using special expression systems and fusion partners.

We have also covered the evolutionary and molecular origins of the temperature adaptations exhibited by these enzymes, as well as diverse computational and experimental techniques to ascertain these adaptations. The value of understanding the molecular mechanism of these adaptations comes from their potential use in protein engineering strategies, some of which we also covered in this review. While the most used technique for protein engineering corresponds to directed evolution and the most straightforward example of the use of these insights were rational design strategies, the identification of rigid and flexible regions within proteins allows establishment of potential hotspots for the modification of the structural properties of these localized regions by site saturation mutagenesis.

Given the extensive literature on the applications of cold-active enzymes in biocatalysis, we only cover specific examples of potential applications given for enzymes reviewed in **Table 1**.

However, only a few of the characterized enzymes were studied for a real industrial application and most of them in the food industry. It will be interesting to see more original articles covering other examples of a concrete use of these remarkable enzymes in the future, which are known to be very relevant for various industrial processes and whose applications will be potentially widespread in the following years.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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