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Perspective

Taking Advantage of Promiscuity of Cold-Active Enzymes

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Abstract: Cold-active enzymes increase their catalytic efficiency at low-temperature, introducing structural flexibility at or near the active sites. Inevitably, this feat seems to be accompanied by lower thermal stability. These characteristics have made cold-active enzymes into attractive targets for the industrial applications, since they could reduce the energy cost in the reaction, attenuate side-reactions, and simply be inactivated. In addition, the increased structural flexibility could result in broad substrate specificity for various non-native substrates, which is called substrate promiscuity. In this perspective, we deal with a less addressed aspect of cold-active enzymes, substrate promiscuity, which has enormous potential for semi-synthesis or enzymatic modification of fine chemicals and drugs. Further structural and directed-evolutional studies on substrate promiscuity of cold-active enzymes will provide a new workhorse in white biotechnology.

Keywords: cold-active enzyme; catalytic efficiency; broad substrate specificity; substrate promiscuity; psychrophile

Cold-active enzymes have drawn a lot of interest from academia and industry by virtue of their unique properties [1–5]. Since the description of thermolability of partially purified malic dehydrogenase from psychrophilic *Vibrio marinus* [6], a great number of cold-active enzymes from psychrophiles have been characterized [3]. Previous reviews have dealt with the structural and catalytic features of cold-active enzymes in depth along with their biotechnological and industrial applications [1–5]. Therefore, in this perspective, we will not simply cover the similar topics to the previous reviews, but address the different aspect of cold-active enzymes. In general, cold-active enzymes increase their catalytic rate (k_{cat}) by modulating their thermodynamic activation parameters: reduction of activation enthalpy ($\Delta H^{\ddagger}$) and increase of activation entropy ($\Delta S^{\ddagger}$) penalty [2,3,7]. To achieve the feat, the enzymes have evolved to have more conformational flexibility at low temperature, compared to mesophilic orthologs, mostly at the expense of thermal stability. The evolutionary changes are encrypted on the primary sequence of these enzymes, including, but not limited to, higher number of glycine residues, reduced frequency of proline and arginine residues, increase of lysine, reduction of hydrophobic core, increased surface hydrophobicity, and reduction of hydrogen bonds, etc. [2,8–10]. Higher catalytic activity at low and moderate temperature with low thermolability have made cold-active enzymes into attractive targets for industrial applications such as production of food, additives in detergents, and synthesis of fine chemicals and pharmaceuticals. They have a few advantages over mesophilic orthologs in that they could reduce the energy cost and chemical side-reactions, and they could be easily inactivated by heat, since they operate at low temperature

and perish easily at higher temperature [2,8–10]. Some cold-active enzymes currently being used in biotechnological and industrial applications are briefly listed in Table 1 [5].

Table 1. List of commercially available cold-active enzymes currently used in biotechnological and industrial applications.

Enzyme	Source Organisms	Function
Alkaline phosphatase (AP)	*Alteromonas undina* P2	Removal of 3′ and 5′ phosphate groups
	Antarctic bacterium TAB 5	
	Pandalus borealis	
Lipase	*Candida antarctica*	Resolution of chiral compounds and transesterification production of biodiesel
Uracil-DNA N-Glycosylase	*Gadus morhua*	Elimination of carryover polymerase chain reaction (PCR) products by hydrolyzing N-glycoside bond between uracil base and sugar skeleton, cutting uracil from dU-containing DNA.
	Marine bacterium BMTU 3346	
	Psychrophilic marine bacterium	
Nuclease	*Shewanella* sp. strain Ac10	Cleavage of phosphodiester bonds in DNA to yield oligonucleotides with 5′-phosphate and 3′-hydroxyl termini.
	Pandalus borealis	
Protease	Arctic marine microbial	Hydrolyzation of a variety of peptide bonds

In the context of industrial applications of cold-active enzymes, the features mentioned above have been well exploited. However, less investigated has been the broadening of the substrate specificity of cold-active enzymes due to their intrinsic characteristics, which may lead to increased enzyme promiscuity, compared to mesophilic orthologs [2,3,11–15]. Notably, enzymes with broad substrate specificity frequently have large active site to embrace substrates of varying sizes and shapes [16–18]. Especially, it is likely that the hydrophobic substrate binding sites could promote binding of the non-native substrates by hydrophobic effects. The increased exposure of hydrophobic residues in cold-active enzymes may stimulate substrate promiscuity [19–21]. The broader substrate specificity of cold-active enzymes, another intriguing property, could expand their application to the molecular design or semi-synthesis for fine chemicals and drugs. In this article, we focus on promiscuity of cold-active enzymes with a few recent reports for the possible application in organic synthesis. Note that we mainly refer to the cold-active enzymes as a cold-adapted enzymes from psychrophilic microorganisms.

Mesophilic and/or thermophilic enzymes showing broad substrate specificity have been well reviewed [18,22–24]. A partial list of mesophilic/thermophilic promiscuous enzymes along with psychrophilic orthologs are listed in Table 2. The critical features of enzymes to show promiscuity are the presence of an highly mobile active site loop [25], highly reactive active sites [23], active site embedded with cofactors [23], and highly hydrophobic substrate [26]. Some of these are similar to natural characteristics of cold-active enzymes. Hence, cold-actives enzymes could be promiscuous furthermore or fortuitously, compared to their mesophilic orthologs. In addition, it is probable that the substrate promiscuity of cold-active enzymes could be escalated or arisen by thermal fluctuation compared to mesophilic orthologs, without losing their advantages mentioned above. This may occur by chance due to the experimental conditions, which are different from natural environment. In the condition of in vivo and low temperature, cold-active enzymes should have strict substrate specificity. However, cold-active enzymes examined at room temperature may undergo an increased thermal fluctuation at the flexible loop regions near the active site and it may result in an unregulated substrate binding of xenobiotic compounds. This unexpected property of cold-active enzymes could be useful for enzyme-mediated semi-synthesis of fine chemicals and drugs. However, we should consider that the structural flexibility of the active sites is very similar between cold-active and mesophilic enzymes, meaning that substrate promiscuity applies to a very limited number of cold-active enzymes, since

the active site residues are highly conserved [27–29]. Through comparative computational studies of cold- and warm-active enzymes, Åqvist and his coworkers supposed that structural flexibility of cold-active enzymes generally located at surface loops of the protein may confer higher reaction rates at low temperature [7,27–29]. Except for *Candida antarctica* Lipase B [24,30], to date, only a handful of reports on enzyme promiscuity and possible application of cold-active enzymes have been investigated [14,15,31,32].

Table 2. Partial list of mesophilic/thermophilic and cold-active enzymes showing substrate promiscuity.

Mesophilic or Thermophiles					Psychrophiles			
Enzyme Class	**Abbreviation**	**Source Organisms**	**Substrate**	**Product**	**Abbreviation**	**Source Organisms**	**Substrate**	**Product**
Lipase	PPL	Porcine pancreas [33]	Aldehyde [b] Nitromethane [b]	β-nitroalcohols [b] (ee%–85%)	SPL	*Dasyatis pastinaca* [34]	Triacylglycerols [a]	Glycerol [a] Acetic acid [a]
	ANL	*Aspergillus Niger* [35]	Anilines [b] 1,3-Diketones [b]	Acetanilide [b]	ZC12	*Psychrobacter* sp. *ZY124* [36]	Fatty acid esters [a] p-nitrophenyl esters [b]	(S)-1-phenylethanol (ee%–92%) p-nitrophenol [b] Carboxylic acid [b]
					KM12	*Bacillus Licheniformis* [37]	Fatty acid esters [a] Long chain p-nitrophenyl Esters [b]	Glycerol [a] Fatty acid [a] p-nitrophenol [b] Carboxylic acid [b]
	r-CALB	*Aspergillus oryzae* [38]	Fatty esters [a]	Glycerol [a] Fatty acid [a]	CALB	*Candida Antarctica* [35]	Aldehyde [b] Nitromethane [b] Anilines [b] 1,3-Diketones [b]	β-nitroalcohols [b] (ee%–90%) Acetanilide [b]
	SGNH	*Sinorhizobium meliloti* [39]	p-nitrophenyl acetate [b] Butyrate [b] Valerate [b] α- and β-naphthyl acetate [b] (R)- and (S)-methyl-3-hydroxy-2 -methylpropionate [b]	p-nitrophenol [b] Acetic acid [b] Butyric acid [b] Valeric acid [b] β-naphthanol [b] (R)- and (S)-3-hydroxy-2-methylpropionate [b] Methanol [b]	SGNH	*Halocynthiibacter arcticus* [31]	Fatty acid esters [a] tert-butyl acetate [b] Glucose pentaacetate [b] p-nitrophenyl esters [b]	Glycerol [a] Fatty acid [a] tert-butyl alcohol [b] Acetic acid [b] Glucose [b] p-nitrophenol [b] Carboxylic acid [b]
	HSL	*Bacillus halodurans* [40]	p-nitrophenyl palmitate [a] Unsaturated fatty acyl esters [a]	p-nitrophenol [a] Palmitic acid [a] Unsaturated fatty acid [a] Glycerol [a]	SHL	*Salinisphaera* sp. P7-4 [41]	Fatty acid esters [a] p-nitrophenyl esters [b] Glyceryl tributyrate [b] 4-methylumbelliferyl (4 MU)-acetate [b] 7-aminocephalosporanic acid [b] Glucose pentaacetate [b] (R,S)-naproxol acetate	Glycerol [a] Fatty acid [a] p-nitrophenol [b] Carboxylic acid [b] Glycerol [a] tert-butyric acid [b] 4-Methylumbelliferone [b] Deacetylated aminocephalosporanic acid [b] Acetic acid [b] Glucose [b] (R)-naproxol (ee%–8.3%)

Table 2. *Cont.*

Enzyme Class	Mesophilic or Thermophiles				Psychrophiles			
	Abbreviation	Source Organisms	Substrate	Product	Abbreviation	Source Organisms	Substrate	Product
Dehydrogenase	Dm7α-HSDH	*Deinococcus marmoris* [25]	Steroids [a] α-ketoesters [b]	Keto-steroids [a] α-hydroxyester [b]				
	Ngi1_7αHSDH	*Metagenome* [25]						
	Ec7α-HSDH	*Escherichia coli* [25]						
	Ca7α-HSDH	*Clostridium absonum* [25]			KstDs	*Rhodococcus ruber* [42]	4-androstene-3, 17-dione [a] 9α-hydroxy-4-androstene-3, 17-dione [a]	1,4-androstadiene-3,17-dione [a] 9-α-hydroxy-1,4-androstadiene-3, 17-dione [a]
	Hh7α-HSDH	*Halomonas halodenitrificans* [25]						
	Ca7β-HSDH	*Clostridium absonum* [25]						
	Cae7β-HSDH	*Collinsella aerofaciens* [25]						
	Hh7β-HSDH	*Halomonas halodenitrificans* [25]			KshAB	*Mycobacterium tuberculosis* [43]	1,4-androstadiene-3,17-dione [a]	3-hydroxy-9,10-seconandrost-1,3, 5(10)-trien-9,17-dione [a]
	Bsp7β-HSDH	*Brucella* sp. [25]						
	Rs7β-HSDH	*Rhodobacter sphaeroides*						
	Csp12α-HSDH	*Clostridium* sp. [25]						
	Sc7β-HSDH	*Stanieria cyanosphaera* [25]						
	CaADH	*Clostridium acetobutylicum* [44]			*Mo*ADH	*Moraxella* sp. TAE123 [45]		Carboxylic aicd
	MtADH	*Mycobacterium tuberculosis* [46]			N/A			

Table 2. *Cont.*

Enzyme Class	Mesophilic or Thermophiles				Psychrophiles			
	Abbreviation	Source Organisms	Substrate	Product	Abbreviation	Source Organisms	Substrate	Product
Isomerase	PriA	*Actinobacteria* [47]	N′-[(5′-phosphoribosyl) formimino] -5-aminoimidazole-4-carboxamide Ribonucleotide [a] Phosphoribosyl anthranilate [b]	N′-[(5′-phosphoribulosyl) formimino]-5-aminoimidazole-4-carboxamide ribonucleotide [a] 1-[(2-carboxyphenyl) amino] -1-deoxyribulose 5-phosphate [b]	N/A			
	TIM	*Pyrococcus furiosus* [48]	Cellobioside–resorufin [a]	Resorufin [a]	TIM	*Pseudomonas* sp. [49]	glyceraldehyde-3-phosphate [a] Triose phosphate [a]	Dihydroxyacetone phosphate [a]
Transferase	PpATaseCH	*Pseudomonas protegens* [50]	2,4-diacetylphloroglucinol [a] Aniline derivatives [b]	1,3,5-trihydroxy -Phloroglucinol [a] N-acetanilide derivative [b]	N/A			
	HCT	Switchgrass *Arabidopsis* [51]	Lignin [b]	p-Coumaroyl conjugates	N/A			
	hGSTT1-1	Human [52]	Glutathione [a] 7-amino-4-chloromethyl coumarin[b]		rHsGST	*Halomonas* sp. ANT108 [53]	Glutathione [a] Chlorodinitrobenzene [a]	Glutathione-Chlorodinitrobenzene [a] conjugate
Reductase	CNR	*Haemophilus influenzae* [54]	Chloramphenicol [a] 4-nitrobenzene derivatives [b]	Reduced chloramphenicol [a] Aniline derivatives [b]	N/A			
	FabI	*Plasmodim falciparum* [55]	Crotonyl Coenzyme A [a], *trans*-2-pentenal [b] 3-pentene-2-one [b]	Saturated Crotonyl Coenzyme A [a] 2-pentanal [b] pentane-2-one [b]	Fab A, Fab B, Fab D, Fab F, Fab G, Fab H and Fab Z	*Shewanella putrefaciens* WS13 [56]	Fatty acid metabolism	
	DHDPR	MRSA [57]	Dihydrodipicolinate [a] NADPH [a]	Tetrahydrodipicolinate [a]	*Pa*DHDPR	*Paenisporosarcina* sp. TG-14 [58]	Dihydrodipicolinate [a] NADPH [a]	Tetrahydrodipicolinate [a]

Table 2. *Cont.*

Enzyme Class	Abbreviation	Mesophilic or Thermophiles: Source Organisms	Mesophilic or Thermophiles: Substrate	Mesophilic or Thermophiles: Product	Psychrophiles: Abbreviation	Psychrophiles: Source Organisms	Psychrophiles: Substrate	Psychrophiles: Product
Synthases	DTSs	*Kitasatospora griseola*, *Streptomyces cyslabdanicus*, *Salinispora arenicola*, *Bradyrhizobium japonicum*, *Erwinia tracheiphila* [59]	Terpentedienyl diphosphate [a] copalyl diphosphate (CPP) [a] *ent*-CPP [a] (E,E,E)-geranylgeranyl diphosphate [b] (Z,Z,Z)-nerylneryl diphosphate [b] (E,E)-farnesyl diphosphate [b] (E,E,E,E)-geranylfarnesyl diphosphate [b] syn-CPP [b] 7-*endo*-CPP [b], ent-7-endo-CPP [b] 8α-hydroxy-CPP [b] 8β-hydroxy-*ent*-CPP [b] 9α-hydroxy-CPP [b] kolavenyl diphosphate (KPP) [b] ent-KPP [b] halimadienyl diphosphate (HPP) [b] syn-HPP [b] *syn*-halima-5(10) [b] 13*E*-dienyl diphosphate [b] mutildienyl diphosphate [b]		N/A			
	NaI	*Escherichia coli* K12 [60]	*N*-acetylglucosamine (GlcNAc) [a] Pyruvate [a]		MvNeuB	*Moritella viscosa* [61]	*N*-acetylneuraminic acid [a] Phosphoenolpyruvate [b]	
Hydroxylase	EctD	*Pseudomonas stutzeri* [62]	Ectoine [a] Homoectoine [b]		EctD	*Sphingopyxis alaskensis* [63]	L-aspartate-β-semialdehyde [a]	
	TfdB-JLU	*Rhodococcus opacus* 1G *Rhodococcus erythropolis* *Pseudomonas* sp. NCIB934 [64,65]	2,4-dichlorophenoxyacetic acid [a] Chlorophenol [b] Dichlorophenol [b] Trichlorophenol derivatives [b]		N/A			

Table 2. *Cont.*

Enzyme Class	Abbreviation	Mesophilic or Thermophiles: Source Organisms	Substrate	Product	Abbreviation	Psychrophiles: Source Organisms	Substrate	Product
	cPAH	*Chromobacterium violaceum* [66]	Phenylalanine [a]		PAH	*Colwellia psychrerythraea* [67]	Phenylalanine [a]	
Hydrolases	Mhg	*Pseudomonas fluorescens* [68]	γ-lactamase [a] Perhydrolase [a] Esters [b]		HsGST	*Glaciozyma antarctica* [69]	Dienelactone [a] p-nitrophenyl esters [b]	
Dehydratase	DaDHT	*Herbaspirillum huttiense* [70]	D- altronate [a] L-fuconate [b] D-Arabinonate [b] L-xylonate [b] D-idonate [b] L-gluconate [b]	Pyruvate [b] L-Lactate [b] Glycolate [b] Glycerate [b]	LsDHT	*Pseudomonas aeruginosa* [71]	L-serine [a]	
Esterase	EaGPE	*Enterobacter aerogenes* [72]	Diesters glycerol-3-Phosphoethanolamine [a] 4-nitrophenyl phosphate (NPP) [b] Bis(4-nitrophenyl) phosphate (BNPP) [b] Diethyl-nitrophenylphosphate (paraoxon) [b]	Glycerol [a] Ethanolamine Phosphoric acid [a] Carboxylic acid [a] Diethyl phosphate [b] p-nitrophenol [b]	N/A			
	PLL	*Saccharolobus solfataricus* [73]	Paraoxon [a] chemical warfare nerve agents [b]	Diethyl phosphate [a] p-nitrophenol [a]	N/A			
	PLL	*Deinococcus radiodurans* [74]	Phosphotriesters [a] Organophosphates [b]	Phosphoric acid [a] Alcohols [a]	N/A			
	AXE	*Bacillus subtilis* [75]	Glycerol triacetate [b] Ethyl acetate [b]	Glycerol [b] Acetic acid [b] Ethanol [b]	PbAcE	*Paenibacillus* sp. R4 [32]	Acetyl xylan [a] p-nitrophenyl esters [b] α-β-naphthyl esters [b] carbohydrate esters [b] tertiary alcohol esters [b] lipids [b], and antibiotics [b]	Xylanol [a] Acetic acid [a] p-nitrophenol [b] Carboxylic acid [b] α-β-naphthanol [b] Carbohydrate [b] tert- alcohol [b] Fatty acid [b] Glycerol [b]
	N/A				*Sf*SFGH	*Shewanella frigidimarina* [76]	p-nitrophenyl esters [b] glucose pentaacetate [b]	p-nitrophenol [b] Carboxylic acid [b] Glucose [b] Acetic acid [b]

Table 2. *Cont.*

Mesophilic or Thermophiles					Psychrophiles			
Enzyme Class	**Abbreviation**	**Source Organisms**	**Substrate**	**Product**	**Abbreviation**	**Source Organisms**	**Substrate**	**Product**
	Est	*Bacillus subtilis* DR8806 [77]	p-nitrophenyl acetate [a]	p-nitrophenol [a] Acetic acid [a]	Est12	*Butyrivibrio proteoclasticus* [78]	Carbohydrate esters [a] short- and middle-chain p-nitrophenol esters [b]	Carbohydrate [a] Carboxylic acid [a] p-nitrophenol [b]
Lactonase	SsoPox	*Sulfolobus solfataricus* [79]	Acyl-homoserine lactones [a] Paraoxon [b]	Acyl-homoserine [a] Lactones [a] Diethyl phosphate [b] p-nitrophenol [b]	AidP	*Planococcus* sp.[80]	Acyl-homoserine lactones[a]	Acyl-homoserine [a] Lactones [a]
Peptidase	ST1737	*Sulfolobus tokodaii* [33]	Aldehyde [b] Nitromethane [b]	β-nitroalcohols (ee%–85%)	ColAP	*Colwellia psychrerythraea* [81]	l-alanine-4-nitroanilide hydrochloride [a]	Alanine [a] p-nitroaniline [a]
	ST0779	*Sulfolobus tokodaii* [33]	Aldehyde [b] Nitromethane [b]	β-nitroalcohols (ee%–94%)	N/A			
	SAt	*Aspergillus terreus* [82]	Azocasein [a]	Leucine [a] Glycine [a] p-nitroaniline [a] Benzyl alcohol [a]	LsPc	*Lysobacter* sp. [83]	Azocasein [a] Gelatin and feather [b]	Leucine [a] Glycine [a] p-nitroaniline [a] Benzyl alcohol [a] Alanine [b] Arginine [b] Aspartic acid [b] Proline [b] Hydroxy Proline [b]
	ApAAP	*Aeropyrum pernix* [84]	Abz-GFEPF(NO2)RA [b] Abz-GFRPF(NO2)RA [b] Abz-SAVLQSGF(NO2)A [b] Abz-EALFQGPF(NO2)A [b] Ac-Phe-Nap [b] Gly-Phe-Nap [b] Ac-Phe-Nan [b]		SpAAP	*Sporosarcina psychrophila* [85]	N-acetyl-L-leucine-p-nitroanilide [a] Butyl-p-nitrophenyl esters [b]	N-acetyl-L-leucine [a] p-nitroaniline [a] Butyric acid [b] p-nitrophenol
Glucanase	ENG16A	*Coprinopsis cinerea* [86]	β-glucan [a] Laminarin [a]	Short chain carbohydrates	GaEbG	*Glaciozyma antarctica* PI12 [87]	Laminarin [a] Lichenin [b] glucan polysaccharides [b]	Short chain carbohydrates

a—Natural substrate. b—Promiscuous substrate.

The cold-active lipase B (CALB) from psychrophilic *Candida Antarctica* is one of the most studied cold-active enzymes with broad substrate specificity [4]. CALB has a structure similar to α/β hydrolases containing both α-helices and β-strand. The catalytic triad of Ser, His, and Asp/Glu is embedded near the surface within a catalytic pocket [88]. CALB has open, large, and medium substrate-binding pockets, which imparts structural flexibility and broad substrate specificity [89]. The flexible structure of CALB allows the efficient catalysis of various industrially important reactions. CALB catalyzes the epoxidation of α,β-unsaturated aldehydes with hydrogen peroxide to give α,β-epoxy aldehydes (Figure 1A) [90]. Aldol addition of ethanethiol to α,β-unsaturated hexanal gives diastereomeric addition products 2-methyl-3-(ethylthio)pentanal by CALB mutant (Ser105Ala) (Figure 1B) [91]. CALB also efficiently catalyzes aldol addition reaction between tricyclic diketone to give bis-product within 4 h (Figure 1C) [92]. Under polar and nonpolar solvents, CALB catalyzes different reactions [93]. For example, CALB catalyzes the amidation reaction between α,β-unsaturated ester and amine to give α,β-unsaturated amide under a polar solvent, whereas Michael addition is favored under a non-polar solvent to give β-amino ester (Figure 1D) [93]. The Baeyer–Villiger oxidation reaction of α-alkyl cyclic ketone to give ethyl 5-acetoxypentanoate can also be catalyzed by CALB (Figure 1E) [94]. These results imply that CALB could accommodate various substrates, which could be useful for the catalysis of industrially important reactions.

Similar to other acylaminoacyl peptidases (AAPs), the cold-active AAP (*Sp*AAP) from psychrophilic bacterium *Sporosacrina psychrophila* possesses promiscuous ester hydrolysis activity [85,95]. The *Sp*AAP exhibited hydrolytic activity on native *N*-acylated amino acids *p*-nitroanilide substrates, and on several promiscuous *p*-nitrophenyl esters of fatty acids of different chain length (C4-C18). Similar characteristics in the primary sequence were found in the primary sequence *Sp*AAP with a lack of disulfide bonds, which probably confers structural flexibility for broader substrate specificity [95]. Contrary to hyperthermophilic AAP from *Aeropyrum pernix* (*Ap*AAP) [84,96,97], *Sp*AAP had a lower K_m and k_{cat} for *N*-acyl amino acids, but, interestingly, higher K_m and k_{cat} for short-chain fatty acids esters. This discrepancy was supported by finding that the diameter of a tunnel in the β-propeller domain to the active site is wider in *Sp*AAP than in mesophilic and thermophilic orthologs [98].

The cold-active acetyl xylan esterase (*Pb*AcE) from psychrophilic soil microbe *Paenibacillus* sp. had a broad substrate specificity (Figure 2A) [32]. Structural analysis of *Pb*AcE suggested that flexible subunit movements and different active site loop conformations might allow *Pb*AcE to have these characteristics different from other mesophilic and thermophilic homologs. *Pb*AcE forms hexamer in solution and each monomer has α/β hydrolase fold with conserved catalytic triad residues of Ser185, Asp274, and His303. Activity assay data showed that *Pb*AcE has typical properties of cold-active enzymes with strong low-temperature activity and broad substrate specificity. *Pb*AcE has moderate and strong deacetylation activity for glucose penta-acetate and xylan acetate as well as reversible acetylation activity for xylan. In addition, *Pb*AcE exhibited strong deacetylation activity for lipids and tertiary alcohol esters. These results proposed that *Pb*AcE can accommodate various substrates and it could be useful for deacetylation biocatalyst of other xenobiotic ligands. For example, acetyl group of beta-lactam antibiotics such as cefotaxime, 7-Aminocephalosporic acid (7-ACA), and cephalosporin C were successfully removed by *Pb*AcE (Figure 2B). The enzymatic modification of antibiotics is of significant importance to overcome the antibiotic resistance issue by increasing the chemical diversity. Furthermore, the possibility of commercial application of *Pb*AcE was assessed by ensuring that the activity was maintained more than 80% even after immobilization followed by repetitive use of 18 times.

Le et al. reported that the recombinant *Ha*SGNH1-type lipase from *Halocynthiibacter arcticus* catalyzed the synthesis of biodiesel and flavor compounds [31]. *Ha*SGNH1 had a sandwich structure containing five central parallel β-sheets between two layers of four α-helices per layer. The catalytic triad of S18, D171, and H174 was embedded near the surface within a catalytic pocket. The solvent-exposed substrate-binding pocket offered the substrate flexibility to *Ha*SGNH1. The substrate-binding pocket encircled with five amino acids, Asn83, Met86, Arg87, Phe131, and Ile173, regulated the entry of substrates via noncovalent interactions. *Ha*SGNH1 was applied for the hydrolysis of tert-butyl esters,

carbohydrate esters, and p-nitrophenyl esters. *Ha*SGNH1 specifically hydrolyzed butyl esters such as *p*-nitrophenyl butyrate, 1-naphthyl acetate, *tert*-butyl acetate, and glyceryl tributyrate. To improve the catalytic activity of *Ha*SGNH1 six mutants, Asn83Leu (1), Met86Glu (2), Met86Arg (3), Arg87Leu (4), Phe131Ala (5), and Ile173Phe (6) were generated using site-directed mutagenesis. Only four showed 120% enhanced hydrolysis of *p*-nitrophenyl butyrate than wild-type *Ha*SGNH1. Two showed higher activity towards carbohydrate acetates, while three showed for larger substrates like p-nitrophenyl hexanoate. The bulky phenyl side chain of six preferred bindings to short-chain fatty esters like p-nitrophenyl acetate. The immobilized *Ha*SGNH1-CELA was applied for the synthesis of petroleum, food, and cosmetic products (butyl and oleic esters) in a nonpolar solvent. The successful synthesis of butyl acetate, butyl butyrate, and oleic acid butyl ester was confirmed by gas chromatography analysis.

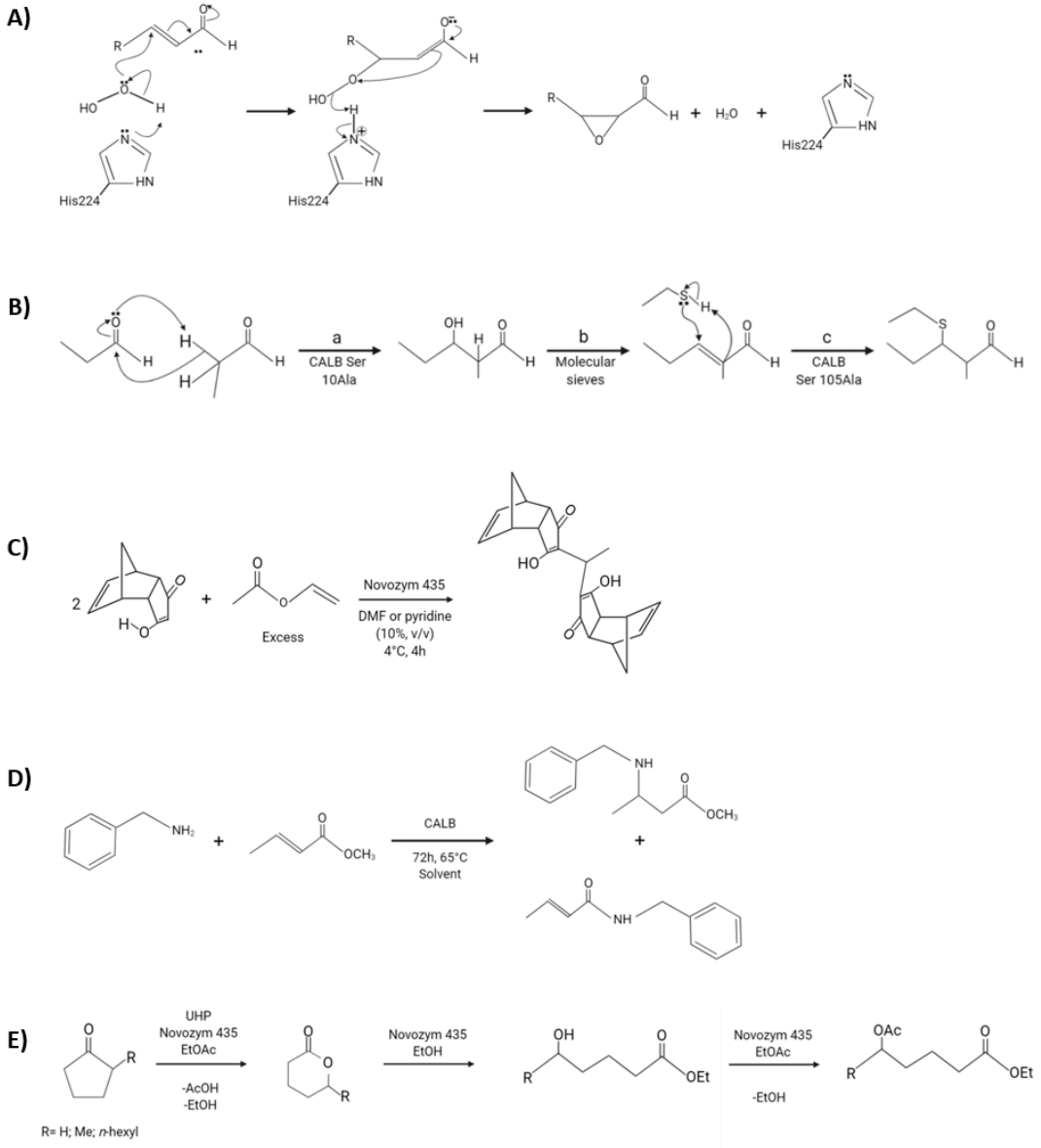

Figure 1. Reactions catalyzed by *Candida antarctica* lipase B. (**A**) Epoxidation of α,β-unsaturated aldehydes with hydrogen peroxide, (**B**) aldol condensation (a and b) and Michael addition (c), (**C**) formation of bis-product, (**D**) formation of Michael addition and aminolysis product, and (**E**) Baeyer–Villiger oxidation reaction.

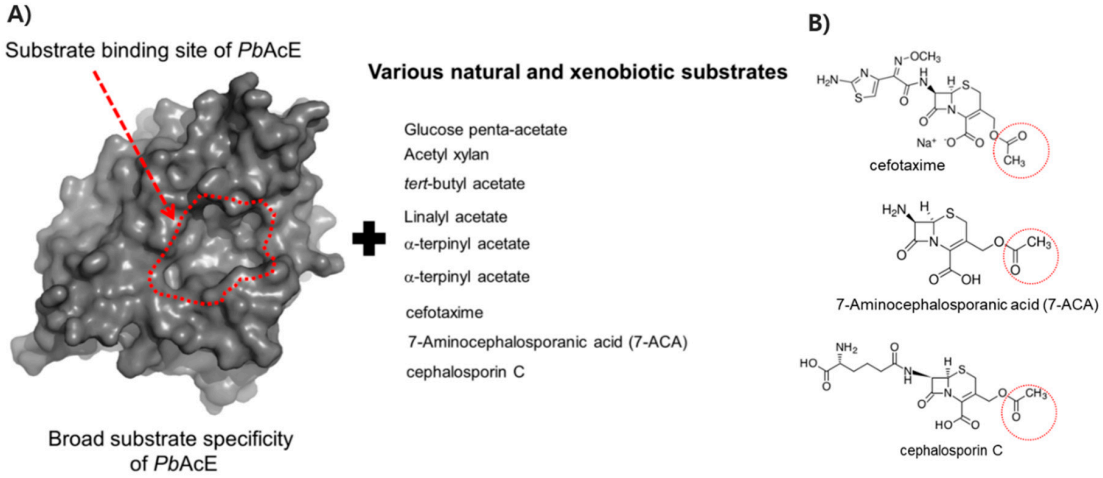

Figure 2. Broad substrate specificity of cold-active acetyl xylan esterase (*Pb*AcE) from *Paenibacillus* sp. (**A**) Relatively larger substrate binding site of *Pb*AcE. *Pb*AcE is displayed space-filled and its substrate-binding site is dotted in red. The substrates modified by *Pb*AcE are listed on the right. (**B**)Deacetylation activity against cephalosporin antibiotics. Acetyl group is circled in red.

Another example is Cytochrome P450 monooxygenases (CYPs). CYPs are heme-containing enzymes that catalyze the hydroxylation of a wide variety of substrate molecules [99]. Bacterial steroid hydroxylase CYPs are particularly useful for effectively introducing hydroxyl groups to a wide range of steroids in the pharmaceutical industry. Thus, these CYPs have received increasing attention in recent years for industrial applications. Kim et al. (2017) reported a newly identified CYP steroid hydroxylase (*Ba*CYP106A2) from the bacterium *Bacillus* sp. PAMC 23377 isolated from Kara Sea of the Arctic Ocean, and its crystal structure was determined at a resolution of 2.7 Å. Furthermore, structural comparisons with other CYPs showed that the α8–α9 loop region of *Ba*CYP106A2 was intrinsically mobile and might be important for broad substrate specificity. The hydroxylase activity of *Ba*CYP106A2 was examined with two different kinds of steroid substrates, 4-androstenedione and nandrolone, and the reaction was analyzed with HPLC and LC-MS [99]. CYPs oxidized 4-androstenedione and nandrolone to 15β-hydroxyandrostenedione and 15β-hydroxynandrolone, respectively. Unlike the mesophilic CYP orthologs from *Bacillus meagterium* [100], *Ba*CYP106A2 seemed to mainly monohydroxylate steroid substrates with high conversion yields (Figure 3). The specific modification of pharmaceutically important compounds by the cold-active CYP sheds light on drug development and production.

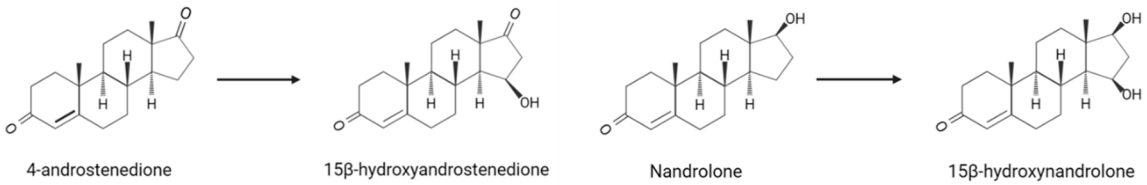

Figure 3. Hydroxylation of 4-androstenedione (**left**) and nandrolone (**right**) by Cytochrome P450 monooxygenases from *Bacillus* sp. PAMC 23377.

In conclusion, structural flexibility of cold-active enzymes could enhance or give rise to enzyme promiscuity. Consequently, cold-active enzymes have merits for the enzymatic modification and semi-synthesis of fine chemicals and medicines by virtue of their broad substrate specificity on top of their existing merits as industrial catalysts. To take advantage of the under-utilized facet of cold-active enzymes, structure-based protein engineering and directed evolution should be employed in the near future to develop enzymes with improved substrate promiscuity for various chemical reactions.

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