microbial biotechnology

Microbial Biotechnology (2011) 4(4), 449–460 doi:10.1111/j.1751-7915.2011.00258.x

Minireview

Biotechnological uses of enzymes from psychrophiles

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Summary

The bulk of the Earth's biosphere is cold (e.g. 90% of the ocean's waters are ≤ 5°C), sustaining a broad diversity of microbial life. The permanently cold environments vary from the deep ocean to alpine reaches and to polar regions. Commensurate with the extent and diversity of the ecosystems that harbour psychrophilic life, the functional capacity of the microorganisms that inhabit the cold biosphere are equally diverse. As a result, indigenous psychrophilic microorganisms provide an enormous natural resource of enzymes that function effectively in the cold, and these cold-adapted enzymes have been targeted for their biotechnological potential. In this review we describe the main properties of enzymes from psychrophiles and describe some of their known biotechnological applications and ways to potentially improve their value for biotechnology. The review also covers the use of metagenomics for enzyme screening, the development of psychrophilic gene expression systems and the use of enzymes for cleaning.

Characteristics of enzymes from psychrophiles

The flexible structures of enzymes from psychrophiles (cold-adapted enzymes) compensates for the low kinetic energy present in cold environments. Because of their inherent flexible structure, cold-adapted enzymes show a reduction in activation enthalpy ($\Delta H^{\#}$) and a more negative activation entropy ($\Delta S^{\#}$) compared with mesophilic and thermophilic homologues (Siddiqui and Cavicchioli, 2006). As a consequence, when temperature is decreased the reaction rate of enzymes from psychrophiles tends to decrease more slowly compared with equivalent enzymes from thermophiles. This balance of thermodynamic activation parameters is translated into relatively high catalytic activity (k_{cat}) at low temperatures and a concomitant low structural stability compared with enzymes from mesophiles or thermophiles. The gain in enzymatic activity would be enormous if the reduction in $\Delta H^{\#}$ was not accompanied by a concomitant decrease in $\Delta S^{\#}$. For example, a decrease in $\Delta H^{\#}$ of 20 kJ mol^{-1} would result in ~ 50 000-fold increase in k_{cat} at 15°C at constant $\Delta S^{\#}$. However, in practice such a vast increase in activity is not observed as a result of enthalpy-entropy compensation (Lonhienne *et al*., 2000; Siddiqui and Cavicchioli, 2006). This is reflected in the activity-stability-flexibility characteristics of many thermally adapted enzymes (Table 1).

The compositional and structural features that confer high flexibility to thermolabile cold-adapted enzymes are generally opposite to that of more rigid and stable mesophilic and thermophilic homologues (Siddiqui and Cavicchioli, 2006; Feller, 2008). For example, psychrophilic enzymes tend to possess various combinations of the following features: decreased core hydrophobicity, increased surface hydrophobicity, lower arginine/lysine ratio, weaker inter-domain and inter-subunit interactions, more and longer loops, decreased secondary structure content, more glycine residues, less proline residues in loops, more proline residues in α-helices, less and weaker metal-binding sites, a reduced number of disulfide bridges, fewer electrostatic interactions (H-bonds, salt-bridges, cation–pi interactions, aromatic–aromatic interactions), reduced oligomerization and an increase in conformational entropy of the unfolded state. Genomic comparisons of psychrophiles vs. thermophiles have also revealed that distinct biases in amino acid composition is a trademark of thermal adaptation (Saunders *et al*., 2003; Siddiqui and Cavicchioli, 2006).

In certain enzymes such as a zinc metalloprotease from an Arctic sea ice bacterium, the whole structure of the

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Table 1. Activity-stability-flexibility relationships in a select range of thermally adapted enzymes.

Enzyme (source)	k_{cat} (min^{-1})	T_{opt} (°C)	T_m (°C)	$\Delta H^{\#}$ ($kJ\ mol^{-1}$)	$T\Delta S^{\#}$ ($J\ mol^{-1}\ K^{-1}$)	${}^{a}K_{sv}$ (M^{-1})	Reference
α-amylase (10°C)							D'Amico *et al.* (2003)
Psychrophile (*P. haloplanktis*)	17 640	28	44	35	–23	9	
Mesophile (Pig)	5 820	53	52	47	–12	6.8	
Thermophile (*B. amyloliquefaciens*)	840	84	86	70	7.5	3.2	
Family 8 glucanases (10°C)							Collins *et al.* (2003)
Psychrophile (*P. haloplanktis*)	30 930	35	53	21	–33	5.9	
Mesophile (*Streptomyces* sp)	3 570	62	64	58	–2	nd	
Thermophile (*Clostridium thermocellum*)	222	80	81	62	–4	5	
Aminopeptidase (10°C)							Huston *et al.* (2008)
Psychrophile (*Colwellia psychrerythraea*)	950	39	47	66	3	Higher	
Mesophile (human leukotriene A_4 hydrolase)	114	49	58	73	5	lower	

a. K_{SV}, Stern-Volmer Constant, measure of the permeability of a small quencher molecule (acrylamide) to the interior of a protein. Flexibility is directly proportional to the permeability.
nd, not detectable.

enzyme appears to be uniformly flexible (global flexibility) as a result of an overall decrease in H-bonding (Xie *et al.*, 2009). However, in other enzymes flexibility has been shown to be localized in the structures surrounding or comprising the active site. Cold-adapted enzymes with local flexibility include a carbonic anhydrase (Chiuri *et al.*, 2009) and an α-amylase (D'Amico *et al.*, 2003). The multi-domain α-amylase from *Pseudoalteromonas haloplanktis* loses activity at a temperature that is lower than the temperature of unfolding of its overall structure (D'Amico *et al.*, 2003). Moreover, its active site appears to unfold at a urea concentration that is lower than what is required to unfold other structural elements (Siddiqui *et al.*, 2005). In a cold-adapted citrate synthase, local areas of flexibility were identified in other regions of the enzyme structure, but not at the active site (Bjelic *et al.*, 2008). These findings illustrate the specific ways in which flexibility can manifest in cold-adapted enzymes.

Overview of the use of cold-adapted enzymes for biotechnological application

The biotechnological value of cold-adapted enzymes stems from their high k_{cat} at low to moderate temperatures, their high thermolability at elevated temperatures and their ability to function in organic solvents (Gerday *et al.*, 2000; Cavicchioli *et al.*, 2002; Cavicchioli and Siddiqui, 2006; Siddiqui and Cavicchioli, 2006; Marx *et al.*, 2007; Margesin and Feller, 2010). Cold-adapted enzymes can provide economic benefit by being more productive than mesophilic or thermophilic homologues at low temperature, thereby providing energy savings to the processes that the enzymes are used in (Table 2). As a result, cold-adapted enzymes have found application in industries as diverse as household detergents, molecular biology and baking.

The use of cold-adapted enzymes can minimize undesirable chemical reactions that can occur at higher temperatures, the enzymes can be rapidly inactivated by heating, and they can be used to transform substrates that require enzyme reactions to be performed at low temperature because substrates are heat-sensitive (Jeon *et al.*, 2009a). These properties are of particular relevance to the food and feed industry where it is important to avoid spoilage, and change in nutritional value and flavour of the original heat-sensitive substrates and products (Russell, 1998; Gerday *et al.*, 2000; Cavicchioli *et al.*, 2002; Tutino *et al.*, 2009).

In addition to the food industry, cold-adapted enzymes are useful for the molecular biosciences because of the need to use enzymes in sequential reactions, and the need to inactivate each enzyme after it has performed its function. Heat-labile enzymes enable heat inactivation to be performed at temperatures that do not cause double-stranded DNA to melt, and the use of heat-labile enzymes obviates the need to use chemical extraction processes.

The most valuable psychrophilic DNA modifying enzyme is alkaline phosphatase. It is used for dephosphorylating DNA vectors before cloning to prevent self-ligation (re-circularization), and for the removal of phosphates at the 5' termini of DNA strands before end-labelling by T4 polynucleotide kinase. However, persistence of alkaline phosphatase activity can interfere with subsequent steps. Commercially available alkaline phosphates, such as calf intestinal alkaline phosphatase and *E. coli* alkaline phosphatase, are resistant to heat inactivation, and therefore require inorganic extraction methods. Alkaline phosphatase from Arctic shrimp can be irreversibly inactivated at 65°C. However, alkaline phosphatases from Antarctic bacteria are superior because they can be completely inactivated at lower temperatures (50–55°C) after a shorter period of heating (Kobori *et al.*, 1984; Rina *et al.*, 2000).

Recently, a novel cold-adapted cellulase complex from an earthworm living in a cold environment was discovered that contained both endo-β-1,4-D-glucanase and β-1,4-

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Table 2. Selected potential biotechnological applications of cold-adapted enzymes.

Applications	Enzymes	Reference
Food and Feed industry:		
Animal feed for the improvement of digestibility and assimilation	Lipase, protease, phytase, glucanases, xylanase	Collins *et al.* (2005); Hatti-Kaul *et al.* (2005); Huang *et al.* (2009); Tutino *et al.* (2009); Ueda *et al.* (2010)
And removal of hemicellulosic material from feed	Protease	Wang *et al.*, 2010a
Meat tenderizing	Chitinase	Dahiya *et al.* (2006)
Single-cell protein from shellfish waste	α-amylase, glucoamylase	Gerday *et al.* (2000)
Starch hydrolysis	Pectinase, xylanase	Nakagawa *et al.* (2004); Collins *et al.* (2005)
Clarification of fruit, vegetable juices and wine	Pectate lyase, pectinase	Truong *et al.* (2001)
Cheese ripening	α-amylase, xylanase	Gerday *et al.* (2000); Collins *et al.* (2005);
Dough fermentation, bakery products	β-galactosidase	Białkowska *et al.* (2009)
Removal of lactose from milk, conversion of lactose in whey into glucose and galactose in dairy industry	Laccase	Kunamneni *et al.* (2008)
Wine and beverage stabilization	Feruloyl esterase	Aurilia *et al.* (2008)
Production of vanillin as a food precursor		
Detergents and cleaning industry:		
Additive to detergents for washing at room temperature	Lipase, protease	Tutino *et al.* (2009); Wang *et al.* (2010a)
Fine chemical synthesis by reverse hydrolysis in organic solvents:		
Flavour modification, optically active esters	Lipase, esterase	Joseph *et al.* (2008)
Asymmetric chemical synthesis	Dehydrogenase	Cavicchioli *et al.* (2002)
Peptides, oligosaccharides	Protease, feruloyl esterase	Aurilia *et al.* (2008); Wang *et al.* (2010a)
Epoxides	Epoxide hydrolase	Kang *et al.* (2008)
Organic compounds	Peroxidases	Ferreira-Leitao *et al.* (2003)
Environmental Biotechnology:		
Bioremediation, degradation and removal of xenobiotics and toxic compounds	Lipase, protease, hydrocarbon degrading enzyme, xylanase, peroxidase	Joseph *et al.* (2008); Wang *et al.* (2010a); Margesin *et al.* (2003); Collins *et al.* (2005) Ferreira-Leitao *et al.*, 2003
Tanning and hide industry	Collagenase (deseasin)	Zhao *et al.* (2008)
Biobleaching in paper and pulp industry	Xylanase	Collins *et al.* (2005)
Biofuels and energy production:		
Biodiesel production by *trans*-esterification of oils and alcohols	Lipase	Tutino *et al.* (2009)
Conversion of chitin to ethanol	Chitinase and yeast	Dahiya *et al.* (2006)
Conversion of cellulose to ethanol	Cellulase-β-glucosidase complex	Ueda *et al.* (2010)
Bioethanol production from dairy waste	β-galactosidase	Hildebrandt *et al.* (2009)
Pharmaceutical, medical and domestic industry:		
Hydrolysis of chitin to chitosan, chitooligosaccharides, glucosamine	Chitinase	Dahiya *et al.* (2006)
Anti-fungal drug and additive for anti-fungal creams and lotions	Chitinase	Dahiya *et al.* (2006)
Mosquito control at larval stage	Endo-chitinase and lipase	Dahiya *et al.* (2006)
Synthesis of citronellol laurate	Lipase	Joseph *et al.* (2008)
Cosmetics	Lipase, laccase	Joseph *et al.* (2008); Kunamneni *et al.* (2008)
Anti-bacterial agent	Lysozyme	Sotelo-Mundo *et al.* (2007)
Anti-microbial, antioxidant, photoprotectant (ferulic acid)	Feruloyl esterase	Aurilia *et al.* (2008)
Antibiotic degradation	β-lactamase	Michaux *et al.* (2008)
Chiral resolution of drugs to increase potency and spectrum	Esterase	Jeon *et al.* (2009a,b)
Chiral resolution and synthesis of chemicals (such as dyes)	Peroxidase	Ferreira-Leitao *et al.* (2003)
Manufacture of anti-cancer drugs	Laccase	Kunamneni *et al.* (2008)
Preparation of precursors of antibiotics	Imidase (cyclic imide hydrolase)	Huang and Yang (2003)
Textile industry		
Stone washing	Cellulase	Ueda *et al.* (2010)
Desizing denim jeans	α-amylase	Gerday *et al.* (2000)
Retting of flax, jute ramie, hemp etc	Xylanase	Collins *et al.* (2005)

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glucosidase activities that could convert cellulose directly into glucose (Ueda *et al.*, 2010). The conversion of cellulose to ethanol is typically performed at relatively high temperatures (50–60°C), which can increase energy consumption and production costs. The use of the cold-adapted cellulase complex with yeast was able to produce ethanol directly from cellulosic material at low temperature (Ueda *et al.*, 2010). This may be an important step towards the efficient production of biofuels from cellulosic waste at low temperatures.

Cold-adapted enzymes have potential application in mixed aqueous-organic or non-aqueous solvents for the purpose of organic synthesis. Their utility derives from their inherent flexibility, which counteracts the stabilizing effects of low water activity in organic solvents (Owusu Apenten, 1999; Sellek and Chaudhuri, 1999; Gerday *et al.*, 2000). Cold-adapted esterases and lipases have been found to exhibit a high level of stereospecificity during fine chemical synthesis (Aurilia *et al.*, 2008; Joseph *et al.*, 2008; Tutino *et al.*, 2009). Furthermore, as chiral drugs are twice as potent as a racemic mixture the sterospecificity of cold-adapted enzymes may be useful for synthesizing chiral drugs (Jeon *et al.*, 2009a).

Manipulation of cold-adapted enzymes to generate improvements for industrial applications

Microorganisms are adapted to a range of abiotic conditions. This natural evolution can be exploited for identifying cold-adapted enzymes with other optimal properties, such as activity/stability at specific ranges of pH, salinity and hydrostatic pressure. A cold-adapted subtilase (Yan *et al.*, 2009) and α-amylase (Srimathi *et al.*, 2007) from a *Pseudoalteromonas* sp. displays halophilic characteristics, with high activity and stability in 2–4.5 M NaCl/KCl. Cold-adapted enzymes from organisms living in deep-sea environments have been found to exhibit both high activity and high stability (Saito and Nakayama, 2004; Kato *et al.*, 2008); an unusual property that goes against the trend of trade-off between activity and stability that has been observed for many enzymes (Siddiqui and Cavicchioli, 2006).

Genetic or chemical modification offers useful avenues for modifying the properties of enzymes to enhance their performance or augment their properties. Using directed evolution, both the half-life of enzyme inactivation ($t_{1/2}$) and activity (k_{cat}) of a cold-adapted Lipase B from *Candida antarctica* were significantly improved; a mutant displayed an increase in $t_{1/2}$ from 8 to 211 min and k_{cat} from 84 min^{-1} to 1900 min^{-1} (Zhang *et al.*, 2003). This is a further example of how an activity/stability trade-off can be overcome. Chemical modification of the same enzyme produced similar improvements (Siddiqui and Cavicchioli, 2005).

Starting with a thermophilic subtilase from *Bacillus sp.*, directed evolution combined with site-directed mutagenesis was used to generate a mutant with a sixfold increase in caseinolytic activity, and a lowering of the optimal temperature of activity (T_{opt}: Δ15–20°C) and $t_{1/2}$ (from 60 to 4 min) (Zhong *et al.*, 2009). Chemical modification using Ficol or dextran has also proven useful for generating a fivefold improvement in enzyme productivity at low temperature (5 or 15°C) using a mesophilic protease present in a commercial formulation (Siddiqui *et al.*, 2009).

The latter study demonstrated that improved productivity at low temperature could be achieved by reducing uncompetitive substrate inhibition (Siddiqui *et al.*, 2009). This modified property is particularly valuable for industrial processes that operate with high substrate concentrations as the modified enzyme is not subject to substrate inhibition. For biotechnology purposes the formation of product or disappearance of substrate over an extended period of time (productivity) is a better indicator of enzyme performance than initial rate measurements. This is because productivity reflects the ability of the enzyme to perform under conditions more relevant to an industrial process where the enzyme is continually affected by substrate/product inhibition (Siddiqui *et al.*, 2009) and enzyme unfolding (Siddiqui *et al.*, 2010). As a result of the biotechnological relevance of productivity parameters, it would be valuable if studies were to report data of this type, in addition to k_{cat}, Km, $t_{1/2}$, T_{opt} and melting temperature (T_m) values that are typically reported.

Discovery through enzyme screening

Enzyme screening has led to the commercialization of a number of cold-adapted enzymes, notably an alkaline phosphatase from New England Biolabs and lipase 435 from Novozymes. Patents have also be filed for cold-adapted enzymes that include a β-galactosidase that efficiently hydrolyses lactose in milk at low temperature (Hoyoux *et al.*, 2001) and a xylanase for use in the baking industry (Collins *et al.*, 2006). Many other potentially valuable proteases (Wang *et al.*, 2010a), polysaccharide degrading enzymes (Ma *et al.*, 2007; Zhang and Zeng, 2007; Stefanidi and Vorgias, 2008), lipases (Zhang and Zeng, 2008) and β-galactosidases (Białkowska *et al.*, 2009) have been discovered by screening psychrophilic microorganisms directly on diagnostic media or by PCR amplifying and cloning genes expressed heterologously in *E. coli*. The availability of complete genome sequences for a limited number of cultured psychrophiles (Lauro *et al.*, 2010) also provides a rational means of *in silico* bioprospecting.

While screening enzymes from axenic cultures is unquestionably valuable, this approach is limited as a result of the small fraction (typically ≤ 1%) of culturable

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microorganisms (Amann *et al.*, 1995). Psychrophiles have the added disadvantage of requiring specialized temperature controlled equipment (and associated energy costs for operation) to enable growth (Hoag, 2009). As a result, the use of recombinant DNA methods to characterize enzymes from microorganisms offers potential benefits. Environmental samples can be used for DNA extraction and construction of clone libraries for direct enzyme screening or random shot-gun sequencing (metagenomics). Table 3 describes cold-adapted enzymes identified from the screening of metagenome libraries and/or metagenome data representing a range of cold environments. DNA sequence data representing a broad range of microbial (cultured and uncultured) sources are publically available (e.g. in GenBank), offering good opportunities for bioinformatic-based discovery.

Using hybridization probes or degenerate primer sets designed to target consensus regions of specific genes (Uchiyama and Miyazaki, 2009), a chitinase gene was isolated from lake sediment (Xiao *et al.*, 2005) and an alkane monooxygenase gene from the sediment of a bay (Kuhn *et al.*, 2009) in Antarctica. Both genes sequences showed sequence identity (< 75%) with known genes sequences.

An advantage of analysing DNA sequence data acquired from shot-gun sequencing of environmental samples is the capacity to rapidly search a potentially large number of gene candidates. Screening can be performed by searching for primary sequence identity and motifs, and by evaluating predicted protein structures and putative catalytic sites that match to known enzymes. The analysis of Arctic permafrost metagenome data identified trehalase, chitinase, β-glucosidase and β-galactosidase genes (Yergeau *et al.*, 2010). The main limitations of this approach are the capacity to only identify targets matching known genes, and the high level of coverage required to identify targets, which are likely to represent only a small proportion of the genes within the dataset (Vieites *et al.*, 2009). An advantage of function-based enzyme screening is the potential to identify candidates that have novel properties without prior knowledge of the gene sequence, and a number with biotechnological potential have been identified using agar- and microtitre plate-based assays (Streit and Schmitz, 2004; Li *et al.*, 2009; Steele *et al.*, 2009; Ferrer *et al.*, 2009a,b).

By being able to select, rather than screen for activity, the use of host strains that require heterologous complementation for viability has been found to be an effective means for isolating genes with DNA polymerase activity (Simon *et al.*, 2009). Nine different genes were isolated from metagenomic libraries constructed from glacial ice, and have potential for use as molecular biology enzymes (Simon *et al.*, 2009).

There is a high demand for lipases for use in biofuel production (Tuffin *et al.*, 2009) and the potential application of a cold-adapted lipase for performing a transesterification reaction in the production of biodiesel at low temperature has been described (Luo *et al.*, 2006). Novel cold-adapted lipases and esterases from diverse environments have been reported, including deep sea sediment (Hardeman and Sjoling, 2007; Park *et al.*, 2007; Jeon *et al.*, 2009b), soil (Elend *et al.*, 2007; Wei *et al.*, 2009), tidal flat sediment (Kim *et al.*, 2009), mangrove sediment (Couto *et al.*, 2010), Arctic sediment (Jeon *et al.*, 2009a) and Antarctic soil (Heath *et al.*, 2009). Screening for lipases and esterases has been successfully performed by manual and high-throughput screening using trybutyrin, *p*-nitrophenyl esters or tricaprylin. Typically, *E. coli* clones were grown at room temperature or 37°C before being incubated at 4°C for phenotypic screening, resulting in lipases and esterases identified with temperature optima ranging from 20–55°C (Table 3).

Highlighting the value of functional screening, a new family of bacterial lipolytic enzymes (Lee *et al.*, 2006a), and a cold-adapted, alkaline lipase that had essentially no amino acid similarity to known lipolytic enzymes (Kim *et al.*, 2009), were both identified from samples of tidal sediments. A cold-adapted lipase isolated by screening libraries generated from oil contaminated soil exhibited a high preference for esters of primary alcohols and a high selectivity for (R) enantiomers of pharmaceutically important substrates (Elend *et al.*, 2007), and an esterase with enantioselective resolution of racemic ofloxacin esters was isolated from Arctic sediment (Jeon *et al.*, 2009a).

Recombinant screening of environmental DNA from low temperature (14°C) wastewater from a dairy farm (Lee *et al.*, 2006b) and a goat rumen (Wanga *et al.*, 2011) identified cold-adapted xylanases with properties distinguishing them from other cold-adapted xylanases isolated from Antarctic krill (Turkiewicz *et al.*, 2000) and a range of bacteria (Petrescu *et al.*, 2000; Collins *et al.*, 2002; Lee *et al.*, 2006c). An α-amylase with activity at 10°C to 30°C against amylose, soluble starch, glycogen and maltose, was isolated by screening libraries constructed from Himalayan soil (Sharma *et al.*, 2010). Soil (from Antarctica) was again the source for a novel cellulase, which lacked a cellulose-binding domain and appeared to only be active with carboxymethyl cellulose as substrate, producing cellobiose and cellotriose as products (Berlemont *et al.*, 2009). Soil (from an oil field) was also the source for three clones (out of ~12 000 screened) with β-galactosidase activity against o-nitrophenyl-β-D-galactopyranoside, with one of the enzymes being cold-adapted and the gene exhibiting high cellular levels when expressed in the yeast *Pichia pastoris* (Wang *et al.*, 2010b).

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Table 3. Metagenome derived-cold adapted enzymes.

Enzyme	Environment	Host/ Vector	Postive clones/ Number of screened clones	Screening technique	T_{opt} (°C)	pH_{opt}	Level of characterization	Reference
Lipase	Baltic sea sediment	*E. coli*/ fosmid	70/ > 7000	Agar-based assay	35	na	Protein purification, temperature, substrate specificity, kinetic analysis	Hardeman and Sjoling (2007)
Lipase	Oil contaminated soil (Northern Germany)	*E. coli*/ cosmid	na	Agar-based assay	30	7	Protein purification, temperature, pH, effects of metals ions, solvent and various chemical, substrate specificity	Elend *et al.* (2007)
Lipase	Deep sea sediment of Edison Seamount (Papua New Guinea)	*E. coli*/ fosmid	1/8823	Agar-based assay	25	8	Protein purification, temperature, pH, substrate specificity, effects of metal ions and detergent	Jeon *et al.* (2009b)
Lipase	Intertidal flat sediment (Korea)	*E. coli*/ fosmid	1/6000	Agar-based assay	30	8	Protein purification, temperature, pH, effects metals ions and detergents, substrate specificity, conformational stability	Kim *et al.* (2009)
Lipase	Soil from different altitude of Taishan (China)	*E. coli*/ Plasmid	2/na	Agar-based assay	20	7 to 9	Protein purification, °C, pH, substrate specificity, effects of metal ions, kinetic analysis	Wei *et al.* (2009)
Lipase	Mangrove sediment (Brazil)	*E. coli*/ fosmid	1/2400	Agar-based assay	35, (61% acticity at 20)	8	Protein extraction, MALDI-TOF analysis, °C, pH, substrate specificity	Couto *et al.* (2010)
Esterase	Deep sea sediment (Papua New Guiney)	*E. coli*/ fosmid	1/na	Agar-based assay	50–55 (high activation energy at 10–40)	10 to 11	Protein purification, temperature, pH, effects of metal ions and detergent, substrate specificity	Park *et al.* (2007)
Esterase	Antarctic desert soil	*E. coli*/ fosmid	3/100 000	Agar-based assay	40, (active at 7–54)	Alkaline	Protein purification, temperature, pH, substrate specificity	Heath *et al.* (2009)
Esterase	Arctic seashore sediment	*E. coli*/ fosmid	6/60 132	Agar-based assay	30	8	Protein purification, temperature, pH, substrate specificity, enantioselective resolution of racemic ofloxacin esters	Jeon *et al.* (2009a)
Amylase	Soil of Northwestern Himalayas	*E. coli*/ cosmid	1/350 000	Agar-based assay	40	6.5	Protein purification, temperature & pH, effects of metal ions	Sharma *et al.* (2010)
Cellulase	Antarctic soil	*E. coli*/BAC	11/10 000	Agar-based assay	10 to 50	6 to 9	Protein purification, protein purification, temperature, pH, effects of various chemical, substrate specificity, viscometric assay	Berlemont *et al.* (2009)
β-galactosidase	Topsoil of oil field (China)	*E. coli/* plasmid	3/1200	Agar-based assay	38, 54% activity at 20	7	Protein expression in *Pichia pastoris*, protein purification, temperature, pH, effects of metal ions, substrate specificity, kinetics	Wang *et al.* (2010b)
Xylanase	Waste lagoon of dairy farm (California)	*E. coli*/ phagemid	1/5 000 000	Agar-based assay	20	6 to 7	Protein purification, temperature, pH, substrate specificity, kinetic analysis	Lee *et al.* (2006b)
Chitinase	Lake sediment, Ardley Island, Antarctica	*E. coli*/ plasmid	295/na	PCR amplification	na	na	RFLP, gene sequencing	Xiao *et al.* (2005)
Alkane monooxygenase	Sediment from Admiralty Bay, King George Island, Antarctica	*E. coli*/ plasmid	177/na	PCR amplification	na	na	Gene sequencing	Kuhn *et al.* (2009)
DNA polymerase 1	Glacial ice (Germany)	*E. coli*/ plasmid and fosmid	15/23 000 And 1/4 000	Growth assay	na	na	Subcloning into expression vector	Simon *et al.* (2009)

na, not applicable or not available.

Expression systems for cold-adapted enzymes

Some types of enzymes pose difficulties for screening (Fernández-Arrojo *et al*., 2010), and the development of low-temperature expression systems provide a number of advantages: (i) an obvious advantage is being able to maintain the stability of heat-labile cold-adapted enzymes thereby enabling effective enzyme purification of enzymes from psychrophiles (Feller *et al*., 1991; 1998; Gerike *et al*., 1997). A good example of a biotechnologically relevant enzyme is alkaline phosphatase (Kobori *et al*., 1984; Rina *et al*., 2000) where the enzymes start to lose activity at 15°C (in the absence of substrate), (ii) low-temperature expression can reduce the formation of inclusion bodies, thereby facilitating the production of soluble proteins (Vasina and Baneyx, 1997), (iii) the construction of a low-temperature expression system will facilitate genetic manipulation studies of the host psychrophile (Tutino *et al*., 2001) and (iv) while not directly relevant to cold-adapted enzymes, by being able to thermally suppress enzyme activity (e.g. of a thermophilic enzyme) a low-temperature expression system would enable the production of enzymes that are otherwise harmful to the cell (e.g. proteases).

Low-temperature expression systems have been developed by utilizing plasmids native to psychrophiles, including the Gram-negative Antarctic bacteria, *Psychrobacter* sp. (Tutino *et al*., 2000), *P. haloplanktis* (Tutino *et al*., 2001) and *Shewanella livingstonensis* (Miyake *et al*., 2007). The origin of replication from the *P. haloplanktis* multicopy plasmid, pMtBL was used to construct an *E. coli* shuttle vector utilizing a commercial pGEM plasmid (Tutino *et al*., 2001). This shuttle vector was able to be stably maintained in five cold-adapted Gram-negative bacteria and was used to express a heat-labile α-amylase in *P. haloplanktis* (Tutino *et al*., 2001). For the *S. livingstonensis* system, low temperature-upregulated promoter regions from *S. livingstonensis* were fused to a β-lactamase reporter gene from *Desulfotalea psychrophila* and cloned into the broad host range plasmid pJRD215 (Miyake *et al*., 2007). Varying levels of expression were obtained for genes encoding a chaperonin GroES, alkyl hydroperoxide reductase and two proteases, relative to T7-controlled expression in pET21 in *E. coli*. A low-temperature *E. coli* expression system has also been developed by utilizing *groEL* from the Antarctic bacterium *Oleispira antarctica* to enable *E. coli* to grow and overexpress effectively at low temperature (Ferrer *et al*., 2003; Margesin and Feller, 2010).

Development of a low-temperature expression system for Gram-positive bacteria has also been initiated utilizing a psychrophilic *Arthrobacter* sp. isolated from a Greenland glacier (Miteva *et al*., 2008). The plasmid p54 from the *Arthrobacter* sp. was used with the commercial *E. coli* plasmid pUC18 to construct a shuttle vector that was able to be transformed (but not necessarily stable) into four other high G + C Gram-positive bacteria (Miteva *et al*., 2008).

Use of cold-adapted enzymes for cleaning

The ability of enzymes to hydrolyse substrates has proven useful for cleaning applications in a wide range of industries, including laundry and dishwasher (Aehle, 2007), food, dairy and brewing (Li and Chen, 2010; Lowry, 2010), medical devices (Rutala and Weber, 2004) and water treatment (Poele and van der Graaf, 2005). The use of enzymes as cleaning agents has been motivated by increased regulatory demands and commercial requirements for improved efficacy and environmental sustainability (Laugesen, 2010; McCoy, 2011). In particular, the implementation of life cycle assessments to evaluate the effects that a product has on the environment over the entire period of its life is directly impacting the development of business cases for product commercialization (Horne *et al*., 2009). Life cycle assessments of cleaning methods have been reported for dairy (Eide *et al*., 2003), water treatment (Tangsubkul *et al*., 2006), detergent (Nielsen, 2005) and brewing industries (Zahller *et al*., 2010).

The link between a reduced wash temperature and improved energy conservation has been recognized by detergent manufacturers (Proctor and Gamble, 2009; Laugesen, 2010), with a reduction in wash temperature from 40°C to 30°C reported to produce a 30% reduction in electricity used, equating to a reduction of 100 g of CO_2 per wash (Nielsen, 2005). Proteases, amylases, lipases and cellulases, such as Alcalase, Natalase and Lipolase Ultra from Novozymes have been used for low temperature (≥ 20°C) washing (Aehle, 2007). While the effectiveness of cleaning typically increases with the temperature of the cleaning solution (Li and Chen, 2010), the ability of enzymes to clean effectively in detergents at low temperature has seen a reduction in temperature used for washing procedures in a range of industries; examples include automated dishwashers (Aehle, 2007), the cleaning of membranes for water treatment (Poele and van der Graaf, 2005), and cleaning of equipment in brewing (Zahller *et al*., 2010) and dairy (Eide *et al*., 2003). Enzymes from psychrophiles, such as proteases from *Serratia rubidaea* and *Stenotrophomonas maltophilia* (Doddapaneni *et al*., 2007; Kuddus and Ramteke, 2009) and an amylase identified by metagenomic screening of glacial water (Sharma *et al*., 2010), are the types of enzymes that have potential to extend the effectiveness of enzyme-based, low-temperature cleaning formulations.

Surfaces that are at ambient temperatures, such as buildings, carpets and benches, cannot easily be heated

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or immersed in cleaning solutions and tend to be cleaned using sprays or wipes, providing good avenues for the use of cold-adapted enzymes. Illustrating the value of enzymes, a lipase and glucose hydrolase have been used in a cleaning solution in a building conservation project to improve the removal of mould from stone and reduce the damage normally associated with the use of standard cleaning agents (Valentini *et al*., 2010).

The maintenance of food processing plants relies on the frequent cleaning of equipment without the dismantling of the manufacturing plant (referred to as 'Clean-in-Place'). Reducing the need to cycle between cool (operating) and warm/hot (cleaning) temperatures by using cold-adapted enzymes would save both energy costs and down time (Marshall *et al*., 2003; Arizona Department of Health Services, 2011). In addition, the use of enzymes in cleaners in the food industry has been somewhat constrained by concerns over enzyme activity remaining after cleaning that might cause product degradation (Lowry, 2010). The relatively high thermolability of cold-adapted enzymes may therefore be advantageous as their activity could be minimized by rinsing using heated water. An interesting avenue for the application of cold-adapted enzymes in the food processing industry is their potential use as a co-cleaner to complete the cleaning process where crushed ice is forced through pipelines to physically remove materials causing soiling (Quarini *et al*., 2002). This application would extend to cleaning industrial heat exchangers (Shire *et al*., 2009) and water supply systems (Quarini *et al*., 2010).

The solvent tolerance of cold-adapted enzymes may be useful for cleaning purposes. Organic solvents are often used in cleaning formulations, with over one-quarter of cleaners in a database of formulations for dairy, food and industrial cleaners containing alcohols (Flick, 2006). This property may extend to the petroleum industry, where microbial biofilms can cause microbially induced corrosion and fuel contamination in storage tanks (Bento and Gaylarde, 2001), automotive fuels (Rodriguez-Rodriguez *et al*., 2009), aviation fuel (Rauch *et al*., 2006) and pipelines (Rajasekar *et al*., 2007). However, while control measures are being explored for surfactant and biocide emulsions (Muthukumar *et al*., 2007a,b), the use of solvent tolerant enzymes in detergents that can function at the interface of organic/aqueous phases (e.g. lipases) have not been reported. Formulations that can hydrolyse ester-containing components in the extracellular matrix of biofilms (Flemming and Wingender, 2010) may be particularly useful.

Enzymes have already contributed to improved cleaning efficacy and environmental sustainability of cleaning formulations in a wide range of industries. The use of enzymes from psychrophiles in cleaning formulations has gained recognition for some industries (e.g. food) and has potential for a growing number of others (e.g. membrane filtration, petroleum). With advances particularly in metagenomic screening and protein engineering, there are good opportunities for exploiting the properties of new cold-adapted enzymes (high activity at low temperature, heat lability enabling heat inactivation, and solvent tolerance) in cleaning formulations.

Acknowledgements

This research was supported by the Australian Research Council.

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