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) compensate for the low kinetic energy present in cold environments. Because of their inherent flexible structure, cold-adapted enzymes show a reduction in activation enthalpy (ΔH°) and a more negative activation entropy (ΔS°) 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 (kcat) 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 ΔH° was not accompanied by a concomitant decrease in ΔS°. For example, a decrease in ΔH° of 20 kJ mol⁻¹ would result in ~ 50 000-fold increase in kcat at 15°C at constant ΔS°. 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 thermostable 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–π 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...
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., 2002) and an α-amyase (D’Amico et al., 2003). The multi-domain α-amyase 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 deposphorylating 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 phosphatases, 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-
| Applications                                                                 | Enzymes                                  | Reference                                      |
|-----------------------------------------------------------------------------|------------------------------------------|------------------------------------------------|
| **Food and Feed industry:**                                               | Lipase, protease, phytase,              | Collins et al. (2005); Hatti-Kaul et al. (2005); Huang et al. (2009); Tutino et al. (2009); Ueda et al. (2010) |
| Animal feed for the improvement of digestibility and assimilation          | glucanases, xylanase                    | Wang et al. (2010a)                            |
| And removal of hemicellulosic material from feed                           | Protease                                |                                              |
| Meat tenderizing                                                            | Chitinase                               | Dahiya et al. (2006)                          |
| Single-cell protein from shellfish waste                                   | α-amylace, 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-amylace, xylanase                     | Gerday et al. (2000); Collins et al. (2005);  |
| Removal of lactose from milk, conversion of lactose in whey into           | β-galactosidase                         | Biakowska et al. (2009)                       |
| Wines and beverage stabilization                                            | Laccase                                 | Kunamneni et al. (2008)                       |
| Production of vanillin as a food precursor                                  | Feruloyl esterase                       | Aurilia et al. (2008)                         |
| **Detergents and cleaning industry:**                                      | Lipase, protease                        | Tutino et al. (2009); Wang et al. (2010a)     |
| Additive to detergents for washing at room temperature                     |                                           |                                               |
| **Fine chemical synthesis by reverse hydrolysis in organic solvents:**     | Lipase, esterase                        | Joseph et al. (2008)                          |
| Flavour modification, optically active esters                              | Dehydrogenase                           | Cavicchioli et al. (2002)                     |
| Asymmetric chemical synthesis                                              | Protease, feruloyl esterase             | Aurilia et al. (2008); Wang et al. (2010a)    |
| Peptides, oligosaccharides                                                 | Epoxide hydrolase                       | Kang et al. (2008)                            |
| Organic compounds                                                          | Peroxidases                             | Ferreira-Leitao et al. (2003)                 |
| **Environmental Biotechnology:**                                            | Lipase, protease, hydrocarbon degrading | Joseph et al. (2008); Wang et al. (2010a); Margesin et al. (2003); Collins et al. (2005) |
| Bioremediation, degradation and removal of xenobiotics and toxic compounds | enzyme, xylanase, peroxidase           | 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:**                                        | Lipase                                  | Tutino et al. (2009)                          |
| Biodiesel production by transesterification of oils and alcohols           | Chitinase                               | Dahiya et al. (2006)                          |
| Conversion of chitin to ethanol                                            | Cellulase-β-glucosidase complex         | Ueda et al. (2010)                            |
| Conversion of cellulose to ethanol                                         | β-galactosidase                         | Hildebrandt et al. (2009)                     |
| Bioethanol production from dairy waste                                     |                                           |                                               |
| **Pharmaceutical, medical and domestic industry:**                         | Chitinase                               | Dahiya et al. (2006)                          |
| Hydrolysis of chitin to chitosan, chito-oligosaccharides, glucosamine      | Endo-chitinase and lipase              | Dahiya et al. (2006)                          |
| Anti-fungal drug and additive for anti-fungal creams and lotions           | Lipase                                  | Joseph et al. (2008)                          |
| Conversion of chitin to ethanol                                            | Lysozyme                                | Sotelo-Mundo et al. (2007)                    |
| Conversion of cellulose to ethanol                                         | Feruloyl esterase                       | Aurilia et al. (2008)                         |
| Bioethanol production from dairy waste                                     | β-lactamase                             | Michaux et al. (2008)                         |
| **Mosquito control at larval stage**                                       | Esterase                                | Jeon et al. (2008a,b)                         |
| **Synthesis of citronellol laurate**                                       | Peroxidase                              | Ferreira-Leitao et al. (2003)                 |
| **Cosmetics**                                                              | Laccase                                 | Kunamneni et al. (2008)                       |
| **Anti-bacterial agent**                                                   | Imidase (cyclic imide hydrolase)        | Huang and Yang (2003)                         |
| **Anti-microbial, antioxidant, photoprotectant (ferulic acid)**            |                                          |                                               |
| **Antibiotic degradation**                                                 |                                          |                                               |
| **Chiral resolution of drugs to increase potency and spectrum**            |                                          |                                               |
| **Chiral resolution and synthesis of chemicals (such as dyes)**            |                                          |                                               |
| **Manufacture of anti-cancer drugs**                                       |                                          |                                               |
| **Preparation of precursors of antibiotics**                               |                                          |                                               |
| **Textile industry**                                                       |                                          |                                               |
| Stone washing                                                              | Cellulase                               | Ueda et al. (2010)                            |
| Desizing denim jeans                                                       | α-amylace                               | Gerday et al. (2000)                          |
| Retting of flax, jute, ramie, hemp etc                                     | Xylanase                                | Collins et al. (2005)                         |
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 cellulotic material at low temperature (Ueda et al., 2010). This may be an important step towards the efficient production of biofuels from cellulotic 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 stereospecificity 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 (t1/2) and activity (kcat) of a cold-adapted Lipase B from Candida antarctica were significantly improved; a mutant displayed an increase in t1/2 from 8 to 211 min and kcat 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 (Topt: ΔT15–20°C) and t1/2 (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 kcat, Km, t1/2, Topt and melting temperature (Tm) 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 been 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 alkan 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 amyllose, soluble starch, glycogen and maltose, was isolated by screening libraries constructed from Himalayan soil (Sharma et al., 2010). Soil (from Antarc-tica) 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 celliotriose 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).
Table 3. Metagenome derived-cold adapted enzymes.

| Enzyme          | Environment                                      | Host/Vector                  | Positive 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 chemicals, 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, 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% activity at 20) | 8        | Protein extraction, MALDI-TOF analysis, °C, pH, substrate specificity                  | Coulou et al. (2010)                         |
| Esterase        | Deep sea sediment (Papua New Guinea)             | 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 chemicals, 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       | Protein purification, temperature, pH, substrate specificity, 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 Desulfoaltera 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 (Mitewa 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 (Mitewa 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 (Poole 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 (Zahlle 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 CO₂ 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 (Poole and van der Graaf, 2005), and cleaning of equipment in brewing (Zahlle 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
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 (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.

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