Chapter 17
Miscellaneous Cold-Active Yeast Enzymes of Industrial Importance

Aneta Białkowska and Marianna Turkiewicz

Abstract Cold-adapted organisms, thriving permanently at near-zero temperatures, synthesize cold-active enzymes to sustain their cell cycle. These enzymes are already used in many biotechnological applications requiring high activity at mild temperatures or fast heat-inactivation rate. In this chapter, we describe the main properties of enzymes from cold-adapted yeasts and describe some of their potential biotechnological applications. The useful applications of these enzymes are widespread to a large number of industries like textile industry, food and dairy industry, brewing and wine industry, laundry, etc. Cold-active hydrolytic enzymes like lipases (reviewed in Chap.16), proteases, cellulases, and amylases can be used as an active agent in detergents applied for cold washing. Other potential applications of psychrophilic enzymes, apart from these, are in processes such as the hydrolysis of lactose in milk using β-galactosidases, extraction and clearing fruit juices using pectinases, meat tenderization or taste improvement of refrigerated meat using proteases, betterment of bakery products using glycosidases (e.g., amylases, xylanases).

Keywords Psychrophilic enzymes · Cold-active yeast enzymes · Cold-adapted yeasts · Biotechnological potential

Contents

A. Białkowska · M. Turkiewicz (✉)
Institute of Technical Biochemistry, Faculty of Biotechnology and Food Sciences, Lodz University of Technology, Stefanowskiego Street, 4/10, 90-924 Lodz, Poland
e-mail: marianna.turkiewicz@p.lodz.pl

P. Buzzini and R. Margesin (eds.), *Cold-Adapted Yeasts*,
DOI: 10.1007/978-3-642-39681-6_17,

17.1 Introduction

The unique properties of the enzymatic apparatus of psychrophilic and psychrotrophic microorganisms, resulting from the special environmental challenges they face, make them an interesting subject of scientific research and lead to their increasingly widespread use in industry (Cavicchioli et al. 2011). The advantages of cold-active enzymes are unquestionable. Being thermolabile, they can be selectively inactivated. Thanks to low-temperature processing, they allow for the reduction of the energy costs of technological processes and prevent infection with mesophilic bacteria thus ensuring a better quality of the final product (Margesin and Schinner 1994).

Interest in psychrophilic microorganisms, which are a source of unique cold-active biocatalysts, has grown considerably over the past twenty years. However, it should be noted that the majority of works on the structure and properties of these proteins, the general mechanism of low-temperature catalysis, and its biological potential, concern enzymes derived from prokaryotic organisms, mostly psychrophilic and psychrotrophic bacteria (Feller and Gerday 1997; Russel 2000; Zecchinon et al. 2001; D'Amico et al. 2002; Feller and Gerday 2003; Feller 2003; Hoyoux et al. 2004; Georlette et al. 2004; Siddiqui and Cavicchioli 2006; Struvay and Feller 2012), while there are few papers dealing with the enzymatic properties of psychrophilic eukaryotes (Shivaji and Prasad 2009; Cavicchioli et al. 2011; Buzzini et al. 2012). Undoubtedly, the least information is available on cold-adapted yeasts, even though they are a versatile group of eukaryotic microorganisms characterized by varied feeding preferences and a surprising ability to survive in extreme environments with diverse physical and geochemical parameters (Buzzini et al. 2012). It has even been suggested that in psychrophilic habitats yeasts are better adapted to low temperatures than bacteria (Margesin et al. 2003). To date, research into these yeasts has mainly focused on their growth conditions, interactions with the abiotic environment, and taxonomical classification. Over the last two decades, there has been increasing interest in psychrophilic yeasts, as it is believed that due to their exceptional ability to grow and maintain metabolism at low temperatures, they produce unique biocatalysts that may be valuable for industrial applications. Examples include lipases A and B synthesized by *Candida antarctica* (now *Pseudozyma antarctica*), which are used in many biotransformations in the food, pharmaceutical, and cosmetic industries (Shivaji and Prasad 2009).

17.2 Biotechnological Potential of Cold-Active Yeast Enzymes

The potential commercial users of cold-active enzymes are primarily the food and household chemical industries. Some of these biocatalysts may also prove a valuable tool for low-temperature biotransformations and in molecular biology (Cavicchioli et al. 2011). Cold-active enzymes may also find applications in the chemical industry for manufacture of extremely volatile organic compounds that can only be modified at low temperatures. Low temperature may facilitate product separation and reduce the process costs (Brenchley 1996).

Among the psychrophilic yeast enzymes explored to date, most attention has been given to hydrolases. However, these enzymes (perhaps except for lipases, discussed at length in Chap. 16) have been studied to varying extent: some authors report characteristics of purified homogeneous protein preparations, while others determine crude enzymatic preparations. Some other works are limited to enzymatic activity screening without in-depth analysis (Brizzio et al. 2007; Khan Pathan et al. 2010; Carrasco et al. 2012; Loperena et al. 2012; Singh and Singh 2012).

17.2.1 Amylases, Xylanases, Chitinases, and Lysozymes

Cold-adapted amylases have emerged as some of the leading biocatalysts with proven potential to be used in a wide array of industrial applications (Kuddus et al. 2011). Most of the cold-adapted amylases studied to date have been derived from prokaryotic organisms. There is only one report concerning these enzymes isolated from psychrophilic yeasts (De Mot and Verachtert 1987), that is, two amylases produced by *C. antarctica* (now *P. antarctica*) CBS 6678, which were purified to homogeneity using various chromatographic techniques and analyzed kinetically. Both of these enzymes, that is, α-amylase and glucoamylase, are monomeric glycoproteins hydrolyzing soluble starches at optimum temperatures of 62 and 57 °C, respectively. The optimum temperature of glucoamylase is in the range of mesophilic yeast glucoamylases (50–60 °C), while the optimum temperature of the other amylolytic enzyme is much higher than that of most of its yeast homologs (below 50 °C). Amylolytic preparations from *C. antarctica* exhibit high specificity for high-molecular-weight substrates, including raw starches. α-Amylase is also active toward cyclodextrins, hydrolyzing α-1,4-glycosidic bonds, while glucoamylase catalyzes the cleavage of α-1,6-glycosidic bonds in dextran, pullulan, and glycogen. Furthermore, De Mot and Verachtert (1987) emphasize the similarity of the amylolytic enzymes from *C. antarctica* CBS 6678 to their mold homologs in terms of glycosylation levels, substrate preferences, and sensitivity to inhibitors, and discuss the potential use of the strain producing these enzymes as a source of genetic material that could be cloned into mesophilic yeasts of the genus *Saccharomyces* to improve the fermentative properties of these microorganisms.

According to Kuddus et al. (2011), cold-active α-amylases have a wide range of potential applications. In the food industry, they may be used for the production of maltotetraose syrups, which are sweeteners and substitutes for sucrose that do not affect the initial taste and aroma of the product. These syrups are characterized by a high ability to bind water, thanks to which they ensure the proper level of humidity and texture in foodstuffs. Cold-active α-amylases may also be applied in the production of maltose and high-molecular-weight branched dextrins, which are used as fillers in the manufacture of bulk foods. In the cotton industry, they may be used for desizing raw fabric and processing denim textiles and in the detergent industry for cold washing. Other potential applications of cold-active α-amylases include the fruit industry (improved juice clarity), the baking industry (delayed bread staling), the paper industry (lower starch viscosity for appropriate coating of paper), the pharmaceutical industry (in dietary supplements to aid digestive processes), and in the treatment of food-processing wastewater (removal of starch waste). The application of psychrophilic enzymes in the above processes brings substantial benefits in terms of reduced energy consumption (and, by the same token, reduced costs) and environmental protection due to the fact that enzymes are biodegradable.

Many companies are interested in financing research on α-amylases despite the high costs and risk that it entails. Cooperation with commercial partners has led to identification of some new amylases from Antarctic microorganisms, which may have potential applications in industry. For instance, *Bacillus licheniformis* amylase was found to be highly active in the temperature range of 10–60 °C in a study by Borchert et al. (2004) sponsored by the company Novozymes. This discovery is the subject of US Patent no. 6673589, although most patents in this field concern processes rather than the substances isolated. Given the above, it should be mentioned that the results of research on amylases have not been commercialized to date.

Other identified cold-active eukaryotic glycosidases are xylanases from the yeasts *Cryptococcus adeliensis* (formerly *Cryptococcus adeliae*) and *Cryptococcus albidus*, which may be applied in low-temperature recycling of farm, industrial, and sewage waste (Shivaji and Prasad 2009). Furthermore, these enzymes are particularly useful in the hydrolysis of xylan, which is the main component of hemicelluloses in plant cell walls. The products of enzymatic degradation of this polysaccharide are used in biotechnological processes for, e.g., manufacture of fuels and organic solvents (Amoresano et al. 2000). The low-temperature activity of xylanases is also taken advantage of in the baking industry. The first tests on cold-adapted bacterial xylanases showed that their high activity at low temperatures and mechanism of action improve the quality of bread by increasing its volume. Some commercial preparations of psychrophilic bacterial xylanases are already available from the company Puratos (Grand-Bigard, Belgium) (Feller 2012).

Gomes et al. (2000) optimized culture conditions for *Cryptococcus adeliensis* with a view to efficient production of xylanase, reaching an activity of 400 nkat ml^{-1} during 168 h of culture at 4 °C. Among 13 tested sources of carbon, xylanolytic activity was induced to the highest degree by xylan, while the most

preferred source of nitrogen was yeast extract. In the presence of other sources of carbon in the medium, the level of enzymatic activity was of constitutive nature. An unpurified preparation of xylanase from *C. adeliensis* revealed optimum activity at a pH of 5.0–5.5 and was stable in the pH range of 4–9 (21 h at 4 °C). Despite the fact that this protein exhibited optimum activity at a relatively high temperature (45–50 °C), it was very thermolabile with a half life of 78 min at 35 °C, while temperatures in the range of 40–50 °C led to a loss of 71–95 % of its initial activity within 5 min. The authors suggested that the low thermal stability of *C. adeliensis* xylanase may result from the increased structural flexibility of the molecule. Similar conclusions were advanced by Petrescu et al. (2000), who studied psychrophilic xylanase from the strain *C. adeliensis* ATCC 201412. The mature glycosylated enzyme consists of 338 amino acid residues and is 84 % identical to the amino acid sequence of its mesophilic homolog from *C. albidus*. Similar to the cold-adapted xylanase described above, the enzyme produced by the strain ATCC 201412 is thermolabile with a half life of 60 min at 30 °C, while the activity of its mesophilic homolog is not affected at this temperature. The kinetic adaptation of psychrophilic xylanase has also been confirmed in a study using differential scanning calorimetry (DSC), which revealed a difference between the melting points of these proteins (48 °C and 62 °C for psychro- and mesophilic xylanase, respectively). *C. adeliensis* and *C. albidus* xylanases differed in the degree of their activity in hydrolysis of xylan conducted at 5 °C ($k_{cat} \sim 14.8$ and 4.9 s^{-1} for psychro- and mesophilic xylanase, respectively) as well as in the activation energy of the catalyzed reaction ($E_a = 47.7$ and 52.2 kJ mol^{-1} for C. *adeliensis* and *C. albidus* xylanase, respectively). The suggestion that the considerable thermolability of the cold-adapted xylanase results from increased molecule flexibility was partially confirmed by the analysis of a three-dimensional model of this enzyme. The authors observed some subtle differences between the structures of the psychro- and mesophilic proteins, such as a less tightly packed hydrophobic core, a lack of one salt bridge, and destabilization of α-helix macrodipoles in the psychrophilic enzyme. The spatial structure of the xylanase derived from the Antarctic yeast *Cryptococcus albidosimilis* (*C. albidus* TAE85) was resolved by a more accurate method, using mass spectometry (Amoresano et al. 2000). This enzyme was shown to be a protein composed of 338 amino acids, *N*- or *O*-glycosylated with GalGalNAc only at residue Asn254 in the polypeptide chain. It should be noted that this was the first report concerning the structural characteristics and glycosylation mechanism in a eukaryotic psychrophilic protein.

Furthermore, assay of the enzymatic activity of *C. adeliensis* grown in submerged culture revealed that this yeast produces endoglucanases, β-arabinofuranosidases, β-xylosidases, and β-glucosidases, which are less active than xylanases, as well as β-mannanases (Gomes et al. 2000). The authors suggest that *C. adeliensis*, which produces a wide range of hydrolytic enzymes useful for degradation of plant poly- and oligosaccharides, may be applied as a probiotic ingredient or therapeutic agent in feed.

To produce cold-active enzymes on a larger scale, researchers aim at expression of their genes in mesophilic microorganisms, whose culture is technologically

simpler. An example here is the gene of a psychrophilic chitinase from the strain *Glaciozyma antarctica* PI12 with an optimum temperature of 15 °C, which was transferred to a mesophilic host, *Komagataella pastoris* (formerly *Pichia pastoris*). The latter gives a high yield of this enzyme in the presence of 1 % methanol at 25 °C (Ramli et al. 2011). This recombinant yeast enzyme was purified to homogeneity using immobilized metal affinity chromatography and characterized with a view to industrial applications. Based on bioinformatic analysis, it was classified as a class II chitinase, glycosyl hydrolase family 18. The regions forming the active site of *G. antarctica* PI12 chitinase also turned out to be highly conservative revealing strong identity to the catalytic domains of 5 eukaryotic homologous enzymes. The main advantages of this psychrophilic enzyme are its high activity at low temperatures (T_{opt} 15 °C; at 5 °C the enzyme exhibits over 20 % of its maximum activity) and ability to efficiently hydrolyze chitin at acidic pH (pH_{opt} 4.0). Just as the majority of typical cold-adapted enzymes, *G. antarctica* PI12 chitinase is characterized by low thermal stability (following 30 min incubation at 25 °C it lost 30 % of its initial activity). In turn, its activity rose by up to 20 % in the presence of 1 mM K^+, Mn^{2+}, and Co^{2+} ions. Examination of the substrate preferences of this enzyme revealed that its activity was highest in the hydrolysis of colloidal chitin, as compared to swollen chitin, carboxymethyl chitosan, and glycol chitosan. This was confirmed by kinetic experiments which showed that *G. antarctica* PI12 chitinase has higher V_{max} and k_{cat} values in low-temperature degradation of colloidal chitin than for other substrates. The authors proposed that due to its biochemical and kinetic properties, the enzyme should be an attractive tool for the production of chitooligosaccharides and in other biotechnological applications, such as biocontrol of microbial spoilage of refrigerated foods and mycoparasitic activity against phytopathogenic fungi in cold environments (Ramli et al. 2011, 2012).

Another interesting example is a lysozyme from the cold-adapted yeast *Debaryomyces hansenii* (muramidase, EC 3.2.1.17) (Wang et al. 2012). It cleaves the *β*-(1,4)-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan, the major bacterial cell wall polymer, and acts as a nonspecific innate immunity molecule against the invasion of bacterial pathogens.

Culture conditions for this enzyme were optimized using statistical design methods, the Plackett–Burman design (PBD) and the development and validation of a quadratic model. As a result, the maximum lysozyme yield was about 3.5-fold higher than the baseline value that greatly enhances the application potential of this enzyme. In general, cold-adapted lysozymes are successfully used as food preservatives for fish and meat products as well as fruits and vegetables (Wang et al. 2012).

17.2.2 Pectinolytic Enzymes

Psychrophilic yeasts are a good source of pectinolytic enzymes. Many yeast species produce them, usually several types of pectinases at the same time, often

also in the form of isoenzymes. These proteins may be applied in the fruit-processing industry, which would be especially interested in cold-active pectinolytic enzymes for reducing juice viscosity at ambient temperature and for improving the clarity of the final product (Alimardani-Theuil et al. 2011).

All known psychrophilic yeasts with pectinolytic activity (also called PPYs, pectinolytic–psychrophilic yeasts) belong to the genera *Cystofilobasidium*, *Cryptococcus*, and *Mrakia*. In contrast to psychrophilic bacteria, which predominantly synthesize alkaline pectinases, yeasts mostly produce enzymes with optimum activity at acidic pH.

The first yeast strain described in the literature in terms of pectinolytic enzyme production was *Cystofilobasidium capitatum* PPY-1 (Nakagawa et al. 2002). When grown on a pectin or polygalacturonate substrate as a source of carbon at 5 °C and below, this yeast synthesized a wide range of pectinolytic enzymes: pectin methylesterases (PME), polygalacturonases (PG), and pectin lyases (PNL), but it did not produce pectate lyases (PAL). The activity of these enzymes in culture supernatants, determined for hydrolysis of pectins and polygalacturonate at 20 °C, amounted to 85, 205, and 1.7 U mg^{-1} protein, respectively. The authors also tested the ability of *C. capitatum* PPY-1 to degrade pectin substances at 5 °C and obtained activity levels of 267, 101, and 0.01 U mg^{-1} protein for PME, PG, and PNL, respectively. One of these enzymes, that is, PPY-1 PG, was purified to homogeneity using various chromatographic methods and exhibited an activity of 458 U mg^{-1} protein under optimum reaction conditions (T_{opt} 45 °C, pH_{opt} 4.4). At 0 °C, its activity was reduced to about 20 % of its maximum activity, which confirms its adaptation to cold (Nakagawa et al. 2005a). The enzyme was found to be stable in the pH range of 2.5–7.0 and up to about 50 °C. It was shown that the enzyme most effectively hydrolyzes polygalacturonate (degree of esterification (DE) 0 %) and pectins moderately esterified with methanol (DE 60 %). Interestingly, based on identification of polygalacturonate hydrolysis products at the initial stages of enzymatic reaction, the authors suggest that PPY-1 PG exhibits mechanisms of action typical of both endo- and exopolygalacturonases. Nakagawa et al. (2005a) emphasize that the special properties of *C. capitatum* PPY-1 PG make this protein useful for industrial applications and especially for low-temperature pectin degradation in the food industry. PPY-1 PG is quite unique as its N-terminal sequence of 20 amino acids does not show high homology with any sequences of other PG deposited in databases. The highest degree of homology was found for *Saccharomyces cerevisiae* PG (52 %).

Another *C. capitatum* PPY-1 enzyme that has been purified and characterized in terms of cold adaptation was pectin lyase (PNL) (Nakagawa et al. 2005b). An efficient purification procedure was developed for this enzyme using traditional chromatographic techniques. As a result, a homogeneous protein was obtained with a high specific activity (1,230 U mg^{-1} protein). Based on SDS-PAGE, its molecular mass was estimated at 42 kDa and a 25-amino acid N-terminal sequence was determined. PPY-1 PNL exhibits maximum activity in pectin hydrolysis at 40 °C, while in the range of 0–20 °C, it retains about 5–50 % of its maximum activity, similar to its homolog from *Penicillium paxilli*. Moreover, in pectin

hydrolysis, the purified enzyme is characterized by high optimum pH (8.0). Thus, it may be considered a cold-adapted alkaline enzyme. Pectin lyase shows a preference for hydrolysis of highly methoxylated pectins and reduces the viscosity of pectin solutions to that of water after 1.5 h reaction at 5 °C.

Nakagawa et al. (2005b) suggest that both enzymes, that is, *C. capitatum* PPY-1 PG and PNL, which depolymerize pectin substances (but differ in substrate specificity and optimum pH) may be used in the form of a mixture in the fruit and vegetable industry to reduce juice and fruit/vegetable pulp viscosity, enhance clarification, and aid depectinization.

Other psychrophilic yeast strains with pectinolytic activity were isolated by Birgisson et al. (2003) from frozen soil, plant leaves, and branches in southwestern Iceland. Based on rRNA sequencing, they classified the yeasts as *C. capitatum*, *Cryptococcus macerans* (anamorph of *Cystofilobasidium macerans*), *Cystofilobasidium lari-marini* (now *C. capitatum*) and *Cryptococcus aquaticus* (now *Mrakiella aquatica*). They showed that the activity of pectinolytic enzymes largely depends on culture conditions, that is, source of carbon, pH, and temperature. Biosynthesis was the most efficient in the presence of pectin as a source of carbon but was repressed on pure glucose. The optimum temperature for the production of polygalacturonases by *Cystofilobasidium* was 14 °C, while in the case of *Cryptococcus,* it was by 5 °C lower. The optimum pH of culture medium also varied among the strains: it was 3.2 for *C. lari-marini* (about 60 U ml^{-1}), 2.6 for *C. macerans* (about 50 U ml^{-1}), and 3.9 for *C. capitatum* (about 37 U ml^{-1}). The authors conducted preliminary analysis of the pectinolytic enzymes contained in the culture supernatant and found that polygalacturonases were produced most efficiently by *Cystofilobasidium* strains, reaching maximum hydrolytic activity at 40 °C and pH 5.0. The same type of enzymes isolated from *Cryptococcus* was characterized by $T_{opt} = 50$ °C and $pH_{opt} = 4.0$. They appear to be cold adapted, as they exhibit relatively high activity in a low-temperature range (0–20 °C): *Cystofilobasidium* PG retains about 20–40 % of its maximum activity, while its *Cryptococcus* homologs retain only 10–30 %. Just as most other psychrophilic enzymes, they are characterized by low thermal stability and undergo partial inactivation at temperatures above 30 °C. Birgisson et al. (2003) emphasize marked kinetic adaptations of yeast polygalacturonases to a cold environment and indicate their usefulness for low-temperature processes as well as ease of selective inactivation after completing the process. Another advantage attributed to the yeast strains is their ability to biosynthesize polygalacturonase isoenzymes, which makes it possible to select proteins with optimum biochemical properties for industrial applications.

Evaluation of the enzymatic activity of psychrophilic yeasts in submerged cultures revealed that they can synthesize other pectinolytic enzymes, albeit not as active as polygalacturonases: *Cystofilobasidium* strains produce pectin lyase, *C. aquaticus* pectin esterase, and *C. macerans* strains produce pectin lyase, pectate lyase, and pectin esterase (Birgisson et al. 2003).

Pectinolytic activity was also observed in yeast strains isolated from forest soil in Abashiri (Hokkaido, Japan) by Nakagawa et al. (2004). Of particular,

importance was discovery of a new species of psychrophilic yeast with pectinolytic activity, that is, *Mrakia frigida*. On a substrate with 1 % w/v pectin as a source of carbon, at 5 °C, this strain synthesizes pectin methylesterase and polygalacturonase with a yield of 16.0 and 7.6 mU mg^{-1} protein, respectively.

Margesin et al. (2005) studied two strains of *M. frigida*, one of which was isolated from Alpine glacier cryoconite (A15), while the other originated from a sediment sample containing mud, spring water, and moss from the Gyda peninsula in northern Siberia (AG25). They analyzed the biochemical properties of pectate lyases (PL) and found that their production in both isolates depended on medium composition, pH, and amount of oxygen dissolved in the medium. PL synthesis by both strains was the most efficient in a medium-containing yeast extract at neutral pH, while optimum temperatures were 5 and 1 °C for A15 and AG25, respectively. Both enzymes exhibited highest activity at 30 °C and were highly active in the temperature range of 0–20 °C (about 20–70 % of the maximum activity). Above the optimum temperature, the activity of the lyases was significantly repressed (after 15 min of incubation at 30 °C they lost about 40 % of their maximum activity, while at 50 °C, they underwent complete denaturation). The cold adaptation of these enzymes is also shown by lower values of E_a and other thermodynamic constants (ΔH*, ΔG*, ΔS*) as compared to their mesophilic homologs. Another interesting biochemical property of *M. frigida* PL is their strong alkaline preference (pH 8.5 and 9.0 for A15 and AG25, respectively) and stability (1 h activity in a pH range of 7–10). The enzymes are completely denatured in the presence of EDTA (10 mmol l^{-1}), SDS (1 %), $KMnO_4$ (10 mmol l^{-1}), and L-cysteine (10 mmol l^{-1}); only Ca^{2+} ions enhance the rate of the reaction they catalyze. According to the authors, the functional advantages of pectate lyases from psychrophilic yeasts include their much greater relative activity at low temperatures as compared to the widely used mesophilic enzymes (by a factor of 4–7) and their alkalinity (Margesin et al. 2005).

17.2.3 Other Glycosidases

Considerably less research has been devoted to cold-active yeast enzymes hydrolyzing di- and oligosaccharides than to enzymes degrading polysaccharides, even though such glycosidases would also prove valuable for industrial applications, especially for food processing and synthesis of probiotic oligosaccharides (Sheik Asraf and Gunasekaran 2010).

From the technological standpoint, of greatest interest are cold-active β-galactosidases, which might be applied in low-temperature hydrolysis of lactose, which is one of the main components of milk and whey (Loveland et al. 1994; Sheridan and Brenchley 2000). Manufacture of lactose-free milk and milk products is of particular importance for consumers with intolerance of this disaccharide, who often suffer from serious gastrointestinal problems. Cold-active β-galactosidases have also been shown to possess transglycosylation activity

whereby lactose hydrolysis takes place with simultaneous transfer of monosaccharides to higher oligosaccharides to form tri- and tetrasaccharides (Karasova-Lipovova et al. 2003; Benesova et al. 2005). Such galacto-oligosaccharides (either produced directly in milk or from whey as an additive in dairy products) can be used as ingredients in probiotic foodstuffs to enhance the growth of bifidobacteria in the large intestine, or as low-calorie sweeteners due to their resistance to metabolism in the small intestine (Huston 2008).

The majority of commercial β-galactosidase preparations contain mesophilic enzymes obtained from yeasts and filamentous fungi. The process of removing lactose from milk or sweet whey using mesophilic β-galactosidases from *Kluyveromyces lactis* or *Kluyveromyces fragilis* (now *Kluyveromyces marxianus*) (Ladero et al. 2000) is conducted for a short time (several hours) at pH 6.0–7.0 and 30–40 °C to prevent infection. Such conditions result in a relatively low degree of lactose degradation. Thus, psychrophilic β-galactosidases would be a perfect alternative to these enzymes, because they exhibit sufficiently high activity at below 20 °C and are not inactivated by sodium or calcium ions or by galactose. The application of such β-galactosidases would reduce the risk of infection with microflora and considerably decrease the energy consumption of the process (Białkowska et al. 2009). Only a few cold-active β-galactosidases have been described to date. They are mostly derived from bacteria of the genera *Arthrobacter*, *Flavobacterium*, *Pseudoalteromonas*, *Rahnella*, *Planococcus*, and *Carnobacterium* (Hoyoux et al. 2001; Turkiewicz et al. 2003a; Wierzbicka-Woś et al. 2011), while two are from yeasts of the genus *Guehomyces*. The first yeast β-galactosidase was isolated from the psychrophilic strain *Guehomyces pullulans* R1 by Nakagawa et al. (2006) in selective tests conducted on agar plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). It was found to be active both in culture supernatants and in biomass. In lactose hydrolysis, the extracellular fraction of the enzyme was characterized by a relatively high optimum temperature for a cold-active enzyme (50 °C), but at 0 °C, it retained as much as 20 % of its maximum activity. Crude enzymatic preparations revealed the highest activity at pH 4.0, with stability in the pH range of 3–7. It is emphasized that the acidic nature of *G. pullulans* R1 β-galactosidase is its unique feature as most of its known psychrophilic homologs hydrolyze lactose most efficiently under near-alkaline pH conditions. Thus, it is suggested that this yeast enzyme may be used for industrial processes conducted in acidic environments. An example of such a process is manufacture of galacto-oligosaccharide syrups from sour whey, where β-galactosidases from filamentous fungi (active in the pH range of 2.5–4.5) are currently applied. β-Galactosidases operating at acidic pH can also reduce the pollution impact and increase the technical usefulness of whey (a product of the cheese industry) by producing glucose- and galactose-rich syrups that can be used as sweeteners in a variety of foodstuffs and substrates that are easily fermented by alcohol-producing microorganisms (Gerday et al. 2005). *G. pullulans* β-galactosidase was found useful for low-temperature hydrolysis of lactose in milk as 10 U ml^{-1} of this enzyme hydrolyzed about 80 % of the disaccharide over 96 h at 10 °C (Nakagawa et al. 2006).

The other yeast β-galactosidase is derived from the psychrotolerant strain *G. pullulans* 17-1, isolated from sea sediment in Antarctica (Song et al. 2010). The biosynthesis of this enzyme, induced by the presence of lactose in the growth medium, is most efficient at pH 4.5. The optimum temperature and pH of the crude protein preparation for hydrolysis of o-nitrophenyl-β-D-galactopyranoside (ONPG) are 50 °C and 4.0, respectively. According to the authors, the cold adaptation of *G. pullulans* 17-1 β-galactosidase is not particularly pronounced if one compares its optimum temperature with those of mesophilic β-galactosidases from the yeasts *K. lactis* ATCC 8583 and *K. fragilis* (T_{opt} = 37 and 30 °C, respectively). However, a major advantage of this enzyme is its extracellular secretion and high hydrolytic activity (25.3 U ml^{-1}) in culture supernatants on a medium scale (2 L bioreactor). Song et al. (2010) reported that this represents the highest activity (in hydrolysis of a synthetic substrate) among all β-galactosidases produced by wild yeast strains obtained to date, even as compared to the activity of β-galactosidases from mesophilic yeasts: under optimum conditions, *K. lactis* synthesizes an enzyme characterized by an activity of 0.9 U ml^{-1}, while the enzyme produced by *K. fragilis* 34440 has an activity of 9.2 U ml^{-1}.

Turkiewicz et al. (2005) showed *Leucosporidium antarcticum* (now *Glaciozyma antarctica*; Turchetti et al. 2011) to produce cold-active α-glucosidase and β-fructofuranosidase. The former was found to be active in both culture supernatants and biomass, while the latter was not secreted and was strictly limited to the cell. Unfortunately, following release from the cell, invertase underwent prompt and irreversible inactivation. Although the rapidly progressing inactivation was inhibited in the presence of EDTA (2 mM) and Mn^{2+} (1 mM), the protective action of these compounds declined with time of incubation, due to which it was impossible to obtain even partially purified enzymatic preparations. The high *in vitro* sensitivity of *G. antarctica* to inactivation also led to limited pH stability of the enzyme, which retained maximum activity during 30-min incubation at 4 °C within a very narrow acidic pH range (4.3–4.8). For comparison, α-glucosidase from the same strain, secreted to the growth medium, was stable over the same time within a 3-fold wider neutral pH range (6.0–7.5). These enzymes preferred very different pH of the reaction environment and exhibited different optimum temperatures. Both their low thermal stability and high catalytic activity at 0 °C (over 20 % of the maximum activity for invertase) show that *G. antarctica* glycosidases are indeed cold-active.

17.2.4 Proteases

Other cold-adapted yeast hydrolases with a range of applications as wide as that of glycosidases are two exoproteases: an aspartyl protease isolated from the Antarctic strain *Cryptococcus humicola* (formerly *Candida humicola*) and a serine protease from the endemic yeast *G. antarctica*. There are relatively few data on extracellular yeast proteases, even in respect of mesophilic yeasts, as it is thought that

these organisms are not able to synthesize significant amounts of these proteins. It seems that this view largely results from the fact that the predominant yeast species both in research and industrial applications is *S. cerevisiae*. Only a few strains of this yeast exhibit extracellular proteolytic activity and that to a small degree (Sturley and Young 1988).

Psychrophilic proteases have a wide range of industrial and technological applications, e.g., they are used as additives in detergents and foodstuffs, as bioremediators, in biotransformations, and in molecular biology. These enzymes are also used as an alternative to rennet to reduce production costs by accelerating the maturation of slow-ripening cheeses in a low-temperature and low-moisture environment. In seafood processing, cold-active proteases aid in removing scales and skin from fish as well as in extracting carotenoproteins from shellfish. They are also used to impart tenderness to refrigerated meat products and to improve their flavor. Furthermore, proteases active at room temperature may be used as an ingredient in medicinal gels for wound and scar healing and topical treatment of skin infections (Huston 2008).

The discovery of an extracellular protease produced by *C. humicola* by Ray et al. (1992) confirms the fact that aspartyl (rarely cysteine) enzymes predominate among yeasts inhabiting even very distant biotopes. They are often called acidic proteases as they reveal maximum activity in environments with relatively low-pH levels (3.0–4.5). The Antarctic exoprotease was characterized by an even lower optimum pH in hydrolysis of hemoglobin (pH 1–1.2) and additionally exhibited kinetic adaptations to low temperature: it was active in the temperature range of 0–45 °C with a distinct optimum at 37 °C and retained 12–15 % of its maximum activity at 0 °C. It should be stressed that so far this has been the only aspartyl protease isolated from Antarctic yeasts. The majority of aspartyl protease are derived from mesophilic microorganisms, such as *Rhodotorula glutinis* (Kamada et al. 1972), *Cryptococcus albidus* var. *aerius* (Federici 1982), *Candida albicans* (Rüchel 1981), *Candida parapsilosis* (Rüchel et al. 1986), *Candida tropicalis* (Togni et al. 1991), *Candida olea* (Nelson and Young 1987), *Saccharomycopsis lipolytica* (Yamada and Ogrydziak 1983), *Aureobasidium pullulans* (Chi et al. 2007), *Sporidiobolus ruineniae* (Kim 2009), *Pichia farinosa* CO-2 (Kim 2010), *Metschnikowia pulcherrima* IWBT Y1123, and *Candida apicola* IWBT Y1384 (Reid et al. 2012).

Another interesting proteolytic enzyme isolated from psychrophilic yeasts is *G. antarctica* extracellular serine protease (Turkiewicz et al. 2003b). This class of enzymes, degrading protein and synthetic substrates in neutral or alkaline environments, includes only a few extracellular yeast proteases, mostly produced by mesophilic *Yarrowia lipolytica* (Tobe et al. 1976) and *Aureobasidium pullulans* (Ogrydziak 1993). Interestingly, *G. antarctica* is the first psychrophilic subtilase belonging to the proteinase K subfamily (subtilisin family) to be described. An efficient purification procedure was developed for this enzyme using traditional chromatographic methods. The resulting homogeneous protein had a very high specific activity (85 U mg^{-1} protein). It was shown that the enzyme is stable under conditions of storage and purification without protectants and is not activated by

bivalent metal ions. It should be stressed that *G. antarctica* subtilase exhibits a very high specific activity in hydrolysis of protein substrate (hemoglobin denatured with urea), which is much higher than that of many previously isolated microbial or animal psychrophilic proteases. Similar to most other microbial serine proteases, *G. antarctica* 171 subtilase has wide substrate specificity, with the greatest affinity to substrates characteristic of chymotrypsin and chymotrypsin-like enzymes. In this respect, it differs significantly from *Y. lipolytica* AEP, which is of typical trypsin-like nature (Ogrydziak 1993). At 30 °C, the enzyme exhibits an approximately 140-fold higher specificity constant (k_{cat}/K_m) for the hydrolysis of BzTyrOEt (*N*-Benzoyl-L-tyrosine ethyl ester) than for the hydrolysis of BzArgOEt (*N*-Benzoyl-L-arginine ethyl ester) (37.4 and 0.27 s^{-1} mM^{-1}, respectively). Furthermore, *G. antarctica* 171 subtilase displays a significant shift in optimum temperatures toward the low range (25 °C), which is by more than 35 °C lower than the optimum temperature of the best characterized mesophilic subtilisin Carlsberg (T_{opt} = 65 °C; Kulakova et al. 1999). Another unique property of *G. antarctica* 171 subtilase is its high activity at 0 °C (over 20 % of its maximum activity). Interestingly, it remains active at temperatures as low as −10 °C. The enzyme displays high amidase activity (pH_{opt} in the range of 8.0–8.5) against *N*-SucAAPF*p*NA (*N*-Succinyl-AAPF-*p*-nitroanilide) and *N*-SucAAPL*p*NA (*N*-Succinyl-AAPL-*p*-nitroanilide). Similar to most other naturally occurring psychrophilic enzymes, this protease is characterized by high sensitivity to the denaturing action of temperature (half life at 30 °C is 2.5 h). Adaptation to low temperatures is attributed by the authors to the presence of additional loops in the molecule, e.g., a 5-amino acid loop near the N-terminus. Although the gene encoding this 35.5-kDa protein was not isolated, the location of a total of 108 amino acid residues was identified and the N-terminal sequence was determined (58 amino acids; the sequence of 38 was determined by the Edman method, and another 23 by proteomic analysis). Based on the sequences of homologous proteases, the sequences of four peptides defined in proteomic analysis of trypsin-digested *G. antarctica* subtilase were aligned (Turkiewicz, unpublished results), among which are sequences of the catalytic triad of the enzyme Ser224…His69…Asp45 (the two first residues were labeled according to residue numbering in proteinase K), which are highly conservative.

Recently, Zaliha et al. (2012) patented a bifunctional enzyme derived from the strain *L. antarcticum* (now *G. antarctica*) PI12, which exhibited both lipolytic and proteolytic activity (see Chap.16).

17.2.5 Phytases and Other Esterases

Of industrial importance is the cold-active phytase isolated from the Antarctic yeast *Cryptococcus laurentii* AL_{27} (*myo*-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) (Pavlova et al. 2008). It is a phosphatase that catalyzes phytate hydrolysis to phosphorylated *myo*-inositol derivatives and inorganic phosphate and

the first known yeast-derived Antarctic phytase. This enzyme is used as a dietary ingredient that plays a significant role in feeding monogastric animals. Its main task is to reduce inorganic phosphorus in animal fodder to prevent excessive concentrations of this element in animal waste. Furthermore, this phosphatase increases assimilation of some dietary elements (Ca, Cu, Zn, Mn, and Fe), proteins, and amino acids. Over the past several years, special attention has been given to the products of phytase activity, that is, *myo*-inositol products with varying degrees of phosphorylation, due to the potential application in the pharmaceutical industry (Pavlova et al. 2008).

The strain *C. laurentii* AL_{27} was chosen as a result of a 2-step selection procedure as the best phytase producer among 124 strains of Antarctic yeasts. The first step involved strain cultivation on calcium- and phytate-containing solid medium, and the second step consisted of culturing the isolates selected in the first step in a liquid medium containing glucose as a source of carbon (20 g l^{-1}). *C. laurentii* AL_{27}, grown at 24 °C, was characterized by the highest intracellular phytase activity (23.8 U g^{-1} biomass) on a medium containing sucrose (40 g l^{-1}) and KH_2PO_4 (at a concentration supplying phosphorus at 5 mg l^{-1}). Pavlova et al. (2008) indicate that Antarctic phytase plays a useful role by efficiently degrading phytates directly in the stomachs of monogastric animals (37–40 °C, pH 2–3). The major advantages of the *C. laurentii* enzyme include its relatively low optimum temperature (40 °C as compared to 45–60 °C for most other phytases), an optimum pH of 4.8, and stability over a wide pH range (2.0–5.0; at pH 2.0 the protein preserves about 50 % of its maximum activity).

An interesting cold-active yeast enzyme was described by Zimmer et al. (2006), who isolated an esterase from *Rhodotorula mucilaginosa*. The yeast was grown on *(2R,3R)*-(−)-di-*O*-benzoyl-tartrate, which it stoichiometrically hydrolyzed to monoesters. The monoesters were subsequently broken into benzoates and tartrates and metabolized. The enzyme was obtained from the culture supernatant, purified, and determined to be a monomeric glycosylated 86 kDa benzoyl esterase. Its optimum pH was 7.5, and the optimum temperature was 45 °C. The psychrophilic properties of this esterase were proven by its high activity at 0 °C, which amounted to 20 % of its activity at 30 °C. This shows that the enzyme may be used in low-temperature settings, e.g., in manufacturing volatile aromas and fragrances. *R. mucilaginosa* esterase hydrolyzed short-chain *p*-nitrophenyl-alkyl esters as well as some benzoyl esters, including benzoyl-methyl ester, phenyl-benzoyl ester, ethylene–glycol-dibenzoyl ester, 1,5-anhydro-D-fructose-tribenzoyl ester, and cocaine. The enzyme is potentially useful for hydrolysis of 1,5-anhydro-D-fructose-tribenzoyl ester, which could be used as a reagent for specific insertion or release of protecting groups in sugars (benzoylated carbohydrates are precursors in the synthesis of, e.g., beta-L-nucleoside analogs with antiviral activity). Thus, *R. mucilaginosa* esterase seems to be a useful low-temperature reagent in the production of benzoylated compounds for the pharmaceutical, cosmetic, and fine chemical industries (Zimmer et al. 2006).

Another cold-active esterase is produced by *Y. lipolytica* CL180 (rYli180). It preferentially hydrolyzes the *S*-enantiomer of racemic ofloxacin ester (Kim et al.

2007). The gene of this protein was cloned into *Escherichia coli*, and the product of its expression, with a mass of 53 kDa, was purified to homogeneity by means of immobilized metal affinity chromatography. Based on the determination of its biochemical properties, it was shown that this esterase preferentially hydrolyzes *p*-nitrophenyl esters of fatty acids with short-chain lengths ($\leq$10 carbon atoms) and is a cold-active protein (T_{opt} = 35 °C; 40–60 % of maximum activity in the range of 0–20 °C). According to the authors, these features make it a very attractive enzyme for potential application as a biocatalyst, especially in the manufacture of thermolabile chemicals.

Sabri et al. (2001) described an acyl-CoA thioesterase produced by the psychrophilic Antarctic yeast *Rhodotorula aurantiaca*. Its application potential is not as yet known, albeit it plays an important role in the metabolism of myristoyl-CoA, and, indirectly, also in the regulation of yeast growth at different temperatures.

17.3 Conclusions and Future Prospects

Literature data show cold-active enzymes to have several advantages over their mesophilic and thermophilic homologs. Despite some difficulties linked to their relatively high cost, low activity and stability under typical process conditions, and the low degree of biodiversity of the psychrophilic microbes known to date, cold-active enzymes may successfully replace traditional ones in a range of industrial applications. Some relatively new recombinant DNA technologies, including site-directed mutagenesis and metatranscriptomics, enable the production of an increasing number of recombinant enzymes. Thanks to this, it is possible to obtain cheaper cold-tolerant enzymes with novel or modified activity. It seems advisable to pursue commercially viable production of such enzymes in heterologous hosts and their modification by chemical or protein engineering methods with a view to obtaining higher activity levels and greater robustness. Some strains could be genetically improved to make them more suitable for the expression of cold-adapted enzymes, which would have significant positive implications for their use in various fields of biotechnology and industry.

While research into useful cold-active yeast enzymes is largely limited to hydrolases, it seems that other classes of enzymes could be equally relevant for industrial applications. For instance, oxidoreductases are playing an increasingly important role in biotransformations conducted in organic solvents (microaqueous systems) (Straathof et al. 2002; Burton 2003; Solano et al. 2012). Enzymes that could be particularly suited to such applications include those produced by yeasts from cold biotopes with low water activity, such as, e.g., yeasts inhabiting some Antarctic environments. The only cold-active oxidoreductase from psychrophilic yeasts that has been discovered to date is D-galacturonate reductase from *Cryptococcus diffluens* (Hamada et al. 2011).

References

Alimardani-Theuil P, Gainvors-Claise A, Duchiron F (2011) Yeasts: An attractive source of pectinases—from gene expression to potential applications: a review. Proc Biochem 46:1525–1537

Amoresano A, Andolfo A, Corsaro MM, Zocchi I, Petrescu I, Gerday C, Marino G (2000) Structural characterization of a xylanase from psychrophilic yeast by mass spectrometry. Glycobiology 10:451–458

Benesova E, Markova M, Kralova B (2005) α-glucosidase and β-glucosidase from psychrotrophic strain *Arthrobacter* sp C2–2. Czech J Food Sci 23:116–120

Białkowska AM, Cieśliński H, Nowakowska KM, Kur J, Turkiewicz M (2009) A new β-galactosidase with a low temperature optimum isolated from the Antarctic *Arthrobacter* sp. 20B: gene cloning, purification and characterization. Arch Microbiol 191:825–835

Birgisson H, Delgado O, García Arroyo L, Hatti-Kaul R, Mattiasson B (2003) Cold-adapted yeasts as producers of cold-active polygalacturonases. Extremophiles 7:185–193

Borchert TV, Svendsen A, Andersen C, Nielsen B, Lauesgaard Nissen T, Kjerulff Sorren (2004) Amylase mutants. US Patent 6,673,589

Brenchley JE (1996) Psychrophilic microorganisms and their cold-active enzymes. J Industr Microbiol 17:432–437

Brizzio S, Turchetti B, de Garcia V, Libkind D, Buzzini P, van Broock M (2007) Extracellular enzymatic activities of basidiomycetous yeasts isolated from glacial and subglacial waters of northwest Patagonia (Argentina). Can J Microbiol 53:519–525

Burton SG (2003) Oxidizing enzymes as biocatalyst. Trends Biotechnol 21:543–549

Buzzini P, Branda E, Goretti M, Turchetti B (2012) Psychrophilic yeasts from worldwide glacial habitats: diversity, adaptation strategies and biotechnological potential. FEMS Microbiol Ecol 82:217–241

Carrasco M, Rozas JM, Barahona S, Alcaíno J, Cifuentes V, Baeza M (2012) Diversity and extracellular enzymatic activities of yeasts isolated from King George Island, the sub-antarctic region. BMC Microbiol 12:251–260

Cavicchioli R, Charlton T, Ertan H, Mohd Omar S, Siddiqui KS (2011) Biotechnological uses of enzymes from psychrophiles. Microb Biotechnol 4:449–460

Chi Z, Ma C, Wang P, Li HF (2007) Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. Biores Technol 98:534–538

D'Amico S, Claviere P, Collins T (2002) Molecular basis of cold adaptation. Phil Trans R Soc Lond B 357:917–925

De Mot R, Verachtert H (1987) Purification and characterization of extracellular α-amylase and glucoamylase from the yeast *Candida antarctica* CBS 6678. Eur J Biochem 164:643–654

Federici F (1982) A note on milk clotting ability in the yeast genera *Cryptococcus* and *Rhodotorula*. J Appl Bacteriol 52:293–296

Feller G (2003) Molecular adaptations to cold in psychrophilic enzymes. Cell Mol Life Sci 60:648–662

Feller G (2012) Psychrophilic enzymes: from folding to function and biotechnology. Scientifica 2013, ID 512840

Feller G, Gerday C (1997) Psychrophilic enzymes: molecular basis of cold-adaptation. Cell Mol Life Sci 53:830–841

Feller G, Gerday C (2003) Psychrophilic enzymes: hot topics in cold adaptation. Nature Rev Microbiol 1:200–208

Georlette D, Blaise V, Collins T, D'Amico S, Gratia E, Hoyoux A, Marx JC, Sonan G, Feller G, Gerday C (2004) Some like it cold: biocatalysis at low temperatures. FEMS Microbiol Rev 28:25–42

Gerday C, Hoyoux A, Francois J M, Dubois P, Baise E, Jennes I, Genicot S (2005) Cold-active β-galactosidase, the process for its preparation and the use thereof. US Patent 2,005,196,835

Gomes J, Gomes I, Steiner W (2000) Thermolabile xylanase of the Antarctic yeast *Cryptococcus adeliae*: production and properties. Extremophiles 4:227–235

Hamada S, Seike Y, Tanimori S, Sakamoto T, Kishida M (2011) Characterization of *D*-galacturonate reductase purified from the psychrophilic yeast species *Cryptococcus diffluens*. J Biosci Bioeng 111:518–521

Hoyoux A, Jennes I, Dubois P, Genicot S, Dubail F, François JM, Baise E, Feller G, Gerday C (2001) Cold adapted *β*-galactosidase from the Antarctic psychrophile *Pseudoalteromonas haloplanktis*. Appl Environ Microbiol 67:1529–1535

Hoyoux A, Blaise V, Collins T, D'Amico S, Gratia E, Huston AL, Marx J, Sonan G, Zeng Y, Feller G, Gerday Ch (2004) Extreme catalysts from low-temperature environments. J Biosci Bioeng 98:317–330

Huston AL (2008) Biotechnological aspects of cold-adapted enzymes. In: Margesin R, Schinner F, Marx JC, Gerday C (eds) Psychrophiles: from biodiversity to biotechnology. Springer, Berlin, pp 347–363

Kamada M, Oda K, Murao S (1972) The purification of the extracellular acid protease of *Rhodotorula glutinis* K-24. Agric Biol Chem 36:1103–1108

Karasová-Lipovová P, Strnad H, Spiwok V, Spiwok V, Malá S, Králová B, Russell NJ (2003) The cloning, purification and characterisation of a cold-active *β*-galactosidase from the psychrotolerant Antarctic bacterium *Arthrobacter* sp. C2–2. Enzyme Microb Technol 33:836–844

Khan Pathan AA, Bhadra B, Begum Z, Shivaji S (2010) Diversity of yeasts from puddles in the vicinity of midre lovenbreen glacier, arctic and bioprospecting for enzymes and fatty acids. Curr Microbiol 60:307–314

Kim JY (2009) Isolation of *Sporidiobolus ruineniae* CO-3 and characterization of its extracellular protease. J Korean Soc Appl Biol Chem 52:1–10

Kim JY (2010) Isolation of protease-producing yeast, *Pichia farinosa* CO-2 and characterization of its extracellular enzyme. J Korean Soc Appl Biol Chem 53:133–141

Kim JT, Kang SG, Woo JH, Lee JH, Jeong BC, Kim SJ (2007) Screening and its potential application of lipolytic activity from a marine environment: characterization of a novel esterase from *Yarrowia lipolytica* CL180. Appl Microbiol Biotechnol 74:820–828

Kuddus M, Roohi Arif JM, Ramteke PW (2011) An overview of cold-active microbial α-amylase: adaptation strategies and biotechnological potentials. Biotechnology 10:246–258

Kulakova L, Galkin A, Kurihara T, Tohru Y, Nobuyoshi E (1999) Cold-active serine protease from psychrophilic *Shewanella* strain Ac10: gene cloning and enzyme purification and characterization. Appl Environ Microbiol 65:611–617

Ladero M, Santos A, Garcia-Ochoa F (2000) Kinetic modeling of lactose hydrolysis with an immobilized *β*-galactosidase from *Kluyveromyces fragilis*. Enzyme Microb Technol 27:583–592

Loperena L, Soria V, Varela H, Lupo S, Bergalli A, Guigou M, Pellegrino A, Bernardo A, Calvino A, Rivas F, Batista S (2012) Extracellular enzymes produced by microorganisms isolated from maritime Antarctica. World J Microbiol Biotechnol 28:2249–2256

Loveland J, Gutshall K, Kasmir J, Prema P, Brenchley JE (1994) Characterization of psychrotrophic microorganisms producing *β*-galactosidase activities. Appl Environ Microbiol 60:12–18

Margesin R, Schinner F (1994) Properties of cold-adapted microorganisms and their potential role in biotechnology. J Biotechnol 33:1–14

Margesin R, Gander S, Zacke G (2003) Hydrocarbon degradation and enzyme activities of cold-adapted bacteria and yeasts. Extremophiles 7:451–458

Margesin R, Fauster V, Fonteyne PA (2005) Characterization of cold-active pectate lyases from psychrophilic *Mrakia frigida*. Lett Appl Microbiol 40:453–459

Nakagawa T, Yamada K, Miyaji T, Tomizuka N (2002) Cold-active pectinolytic activity of psychrophilic basidiomycetous yeast *Cystofilobasidium capitatum* strain PPY-1. J Biosci Bioeng 94:175–177

Nakagawa T, Nagaoka T, Taniguchi S, Miyaji T, Tomizuka N (2004) Isolation and characterization of psychrophilic yeasts producing cold-adapted pectinolytic enzymes. Lett Appl Microbiol 38:383–387

Nakagawa T, Nagaoka T, Miyaji T, Tomizuka N (2005a) Cold-active polygalacturonase from psychrophilic basidiomycetous yeast *Cystofilobasidium capitatum* strain PPY-1. Biosci Biotechnol Biochem 69:419–421

Nakagawa T, Nagaoka T, Miyaji T, Tomizuka N (2005b) A cold-active pectin lyase from the psychrophilic and basidiomycetous yeast *Cystofilobasidium capitatum* strain PPY-1. Biotechnol Appl Biochem 42:193–196

Nakagawa T, Ikehata R, Uchino M (2006) Cold-active acid β-galactosidase activity of isolated psychrophilic-basidiomycetous yeast *Guehomyces pullulans*. Microbiol Res 161:75–79

Nelson G, Young TW (1987) Extracellular acid and alkaline proteases from *Candida olea*. J Gen Microbiol 133:1461–1469

Ogrydziak DM (1993) Yeast extracellular proteases. Crit Rev Biotechnol 13:1–55

Pavlova K, Gargova S, Hristozova T, Tankova Z (2008) Phytase from antarctic yeast strain *Cryptococcus laurentii* AL_{27}. Folia Microbiol 53:29–34

Petrescu I, Lamotte-Brasseur J, Chessa JP, Ntarima P, Claeyssens M, Devreese B, Marino G, Gerday Ch (2000) Xylanase from the psychrophilic yeast *Cryptococcus adeliae*. Extremophiles 4:137–144

Ramli AN, Mahadi NM, Rabu A, Murad AM, Bakar FD, Illias RM (2011) Molecular cloning, expression and biochemical characterization of a cold-adapted novel recombinant chitinase from *Glaciozyma antarctica* PI12. Microb Cell Fact 10:94–107

Ramli AN, Mahadi NM, Shamsir MS, Rabu A, Joyce-Tan KH, Murad AM, Illias RM (2012) Structural prediction of a novel chitinase from the psychrophilic *Glaciozyma antarctica* PI12 and an analysis of its structural properties and function. J Comput Aided Mol Des 26:947–961

Ray MK, Uma Devi K, Seshu Kumar G, Shivaji S (1992) Extracellular protease from the antarctic yeast *Candida humicola*. Appl Environ Microbiol 58:1918–1923

Reid VJ, Theron LW, du Toit M, Divol B (2012) Identification and partial characterization of extracellular aspartic protease genes from *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384. Appl Environ Microbiol 78:6838–6849

Rüchel R (1981) Properties of a purified proteinase from the yeast *Candida albicans*. Biochem Biophys Acta 659:99–113

Rüchel R, Boning B, Borg M (1986) Characterization of a secretory proteinase of *Candida parapsilosis* and evidence for the absence of the enzyme during infection *in vitro*. Infect Immun 53:411–419

Russell NJ (2000) Toward a molecular understanding of cold activity of enzymes from psychrophiles. Extremophiles 4:83–90

Sabri A, Baré G, Jacques P, Jabrane A, Ongena M, Van Heugen JC, Devreese B, Thonart P (2001) Influence of moderate temperatures on myristoyl-CoA metabolism and acyl-CoA thioesterase activity in the psychrophilic antarctic yeast *Rhodotorula aurantiaca*. J Biol Chem 276:12691–12696

Sheik Asraf S, Gunasekaran P (2010) Current trends of β-galactosidase research and application. In: Mendez-Vilas A (ed) Current research, technology and education topics in applied microbiology and microbial biotechnology. Formatex Research Center, Badajoz, Spain, pp 880–890

Sheridan PP, Brenchley JE (2000) Characterization of a salt-tolerant family 42 β galactosidase from a psychrophilic antarctic *Planococcus isolate*. Appl Environ Microbiol 66:2438–2444

Shivaji S, Prasad GS (2009) Antarctic yeasts: biodiversity and potential applications. In: Satyanarayana T, Kunze G (eds) Yeast biotechnology: diversity and applications. Springer, Berlin, pp 3–16

Siddiqui KS, Cavicchioli R (2006) Cold-adapted enzymes. Annu Rev Biochem 75:403–433

Singh P, Singh SM (2012) Characterization of yeast and filamentous fungi isolated from cryoconite holes of Svalbard, Arctic. Polar Biol 35:575–583

Solano DM, Hoyos P, Hernáiz MJ, Alcántara AR, Sánchez-Montero JM (2012) Industrial biotransformations in the synthesis of building blocks leading to enantiopure drugs. Biores Technol 115:196–207

Song Ch, Chi Z, Li J, Wang X (2010) β-galactosidase production by the psychrotolerant yeast *Guehomyces pullulans* 17–1 isolated from sea sediment in Antarctica and lactose hydrolysis. Bioprocess Biosyst Eng 33:1025–1031

Straathof AJJ, Panke S, Schmidt A (2002) The production of fine chemicals by biotransformations. Curr Opin Biotechnol 13:548–556

Struvay C, Feller G (2012) Optimization to low temperature activity in psychrophilic enzymes. Int J Mol Sci 13:11643–11665

Sturley SL, Young TW (1988) Extracellular protease activity in a strain of *Saccharomyces cerevisiae*. J Inst Brew London 94:23–27

Tobe S, Takami T, Ikeda S, Mitsugi K (1976) Production and some enzymatic properties of alkaline proteinase *Candida lipolytica*. Argic Biol Chem 40:1087–1092

Togni G, Sanglard D, Falchetto R (1991) Isolation and nucleotide sequence of the extracellular acid protease gene (ACP) from yeast *Candida tropicalis*. FEBS Lett 286:181–185

Turchetti B, Hall SRT, Connell LB, Branda E, Buzzini P, Theelen B, Müller WH, Boekhout T (2011) Psychrophilic yeast from Antarctica and European glaciers: description of *Glaciozyma* gen. nov., *Glaciozyma martinii* sp.nov. and *Glaciozyma watsonii* sp. nov. Extremophiles 15:573–586

Turkiewicz M, Kur J, Białkowska A, Cieśliński H, Kalinowska H, Bielecki S (2003a) Antarctic marine bacterium *Pseudoalteromonas* sp. 22b as a source of cold-adapted β-galactosidase. Biomol Eng 20:317–324

Turkiewicz M, Pazgier M, Kalinowska H, Bielecki S (2003b) A cold-adapted extracellular serine proteinase of the yeast *Leucosporidium antarcticum*. Extremophiles 7:435–442

Turkiewicz M, Pazgier M, Donachie SP, Kalinowska H (2005) Invertase and α-glucosidase production by the endemic antarctic marine yeast *Leucosporidium antarcticum*. Pol Polar Res 26:125–136

Wang Q, Hou Y, Yan P (2012) Optimization of cold-adapted lysozyme production from the psychrophilic yeast *Debaryomyces hansenii* using statistical experimental methods. J Food Sci 77:337–342

Wierzbicka-Woś A, Cieśliński H, Wanarska M, Kozłowska-Tylingo K, Hildebrandt P, Kur J (2011) A novel cold-active β-galactosidase from the *Paracoccus* sp. 32d—gene cloning, purification and characterization. Microb Cell Fact 10:108–120

Yamada T, Ogrydziak DM (1983) Extracellular acid proteases produced by *Saccharomycopsis lipolytica*. J Bacteriol 154:23–31

Zaliha RN, Salleh AB, Basri M, Mohamad Ali MSB (2012) Cold active enzyme and method thereof. US Patent 2012/0058514 A1

Zecchinon L, Claverie P, Collins T, D'Amico S, Delille D, Feller G, Georlette D, Gratia E, Hoyoux A, Meuwis MA, Sonan G, Gerday Ch (2001) Did psychrophilic enzymes really win the challenge? Extremophiles 5:313–321

Zimmer Ch, Platz T, Cadez N, Giffhorn F, Kohring GW (2006) A cold-active (2*R*,3*R*)-(-)-di-*O*-benzoyl-tartrate hydrolyzing esterase from *Rhodotorula mucilaginosa*. Appl Microbiol Biotechnol 73:132–140