Chapter 13
Antarctic Yeasts as a Source of Enzymes for Biotechnological Applications

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13.1 Introduction

Psychrophilic and psychrotrophic microorganisms present an enzymatic metabolism of outstanding characteristics arising from the extraordinary environmental challenges with which they must deal. This fact makes cold-adapted microorganisms a relevant target for scientific research and its possible use in several industrial processes (Cavicchioli et al. 2011). These enzymes present several advantages for use in industrial applications. Among these advantages is their thermolability, which makes them useful when a selective thermal inactivation step is necessary. In addition, as they are synthesized at low temperatures, there is a potential reduction in energy costs during their technological production and, additionally, their use in processes occurring at low temperatures greatly reduces the chances of contamination with mesophilic microorganisms (Javed and Qazi 2016).

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In the past 30 years, the interest in cold-adapted microorganisms, the main source for cold-active enzymes, has grown considerably. Nevertheless, most of the works report the prospecting and characteristics of enzymes obtained from prokaryotic microorganisms (De Maayer et al. 2014; Santiago et al. 2016), while enzymes from psychrophilic or psychrotolerant eukaryotes are reported less (Duarte et al. 2018). Even though yeasts are able to grow using several carbon sources and some groups are adapted to live in cold and extreme environments, the information on cold-active enzymes produced by yeasts is quite scarce. (Buzzini et al. 2012). As was suggested by Margesin et al. (2003), fungi, and yeasts among them, are better adapted than bacteria for living in cold environments.

13.2 Structural Features and Action Mechanism of Cold-Adapted Extremozymes

When mesophilic enzymes are exposed to low temperatures, the kinetic energy available for reaction is too low; it is insufficient to reach the activation energy for catalysis, and hence enzymatic activity is scarce. In addition, at low temperatures, proteins tend to denature because of the decrease in water molecules' availability, as these become more ordered and less associated with proteins (Karan et al. 2013).

Cold-active enzymes present a number of specific structural adaptations that result in a more flexible structure than their mesophilic and thermophilic counterparts. These adaptations allow higher catalytic activities at low temperatures and reduce their thermostability (Siddiqui and Cavicchioli 2006).

Based on previous reviews of cold-active enzymes, Sarmiento et al. (2015) listed the following features considered as important adaptations for maintaining high flexibility and high activity at low temperatures: decreased core hydrophobicity, increased surface hydrophobicity, changes in amino acid compositions (i.e., lower arginine/lysine ratio, more glycine residues for better conformational mobility, fewer proline residues in loops but more in α-helices, more non-polar residues on the protein surface), weaker protein interactions (such as interdomain and inter-subunit interactions, less disulphide bridges, fewer hydrogen bonds and other electrostatic interactions), decreased secondary structures and oligomerization (but an increase in the number and size of loops), and, finally, increased conformational entropy of the unfolded protein. Feller (2018) reported that one interesting consequence of these special characteristics is that the reaction rates of psychrophilic enzymes tend to decrease slowly when compared to similar enzymes from mesophilic or thermophilic microorganisms in response to a temperature decrease.

13.3 Biotechnological Potential of Cold-Active Enzymes

High activity at low temperatures and thermolability are valuable characteristics for different biotechnological applications in a wide variety of industries such as food, beverage, and household (Sarmiento et al. 2015). For this reason, psychrophilic enzymes are replacing mesophilic enzymes in several industrial processes (Fig. 13.1).

The food industry is one of the main potential users of cold-active enzymes, with special interest in dairy products, juice, meat, and baking industries. Intolerance to lactose, a disaccharide sugar naturally present in milk, represents a common health concern worldwide. Cold-active β-galactosidases are useful to reduce the lactose amount at low temperatures in the milk-processing industry, allowing intolerant people to consume milk and milk derivatives (Erich et al. 2015).

Throughout the world, pectinases are used in the fruit juice industry during the juice extraction process to reduce viscosity and refine the final product. Cold pectinases could be also used in wineries, where most of the fermentations are performed

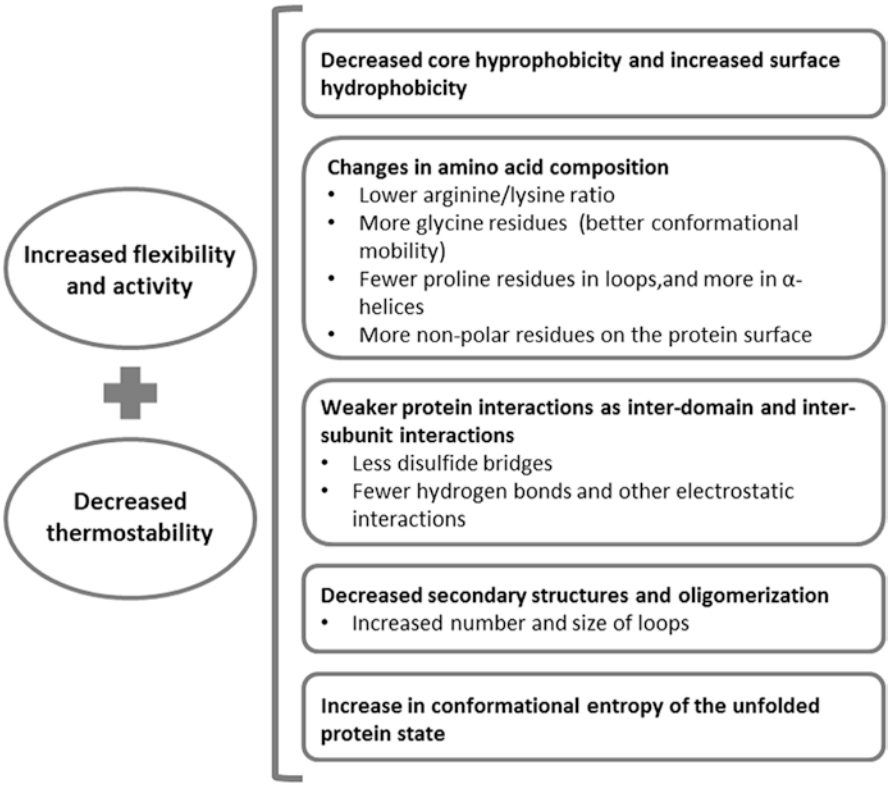

Fig. 13.1 Some relevant adaptations for keeping high flexibility and high activity at low temperatures in cold-active enzymes. (Adapted from Sarmiento et al. 2015)

at temperatures ranging from 15 to 20 °C to avoid evaporation of volatile molecules. Following this thinking, Merin et al. (2011) have proposed the use of psychrotolerant pectinase-producing yeast as adjuvant for wine fermentation. In the meat-processing industry, cold-active proteases are used during the tenderization process, which increases final product quality. Some enzymes, including proteases, amylases, and xylanases, are also helpful in baking processes by reducing the dough fermentation time along with retention of aromas and moisture levels (Kuddus 2018).

In the detergent industry, several enzymes are used for both household and industrial laundry and dishwashing processes. Lipases catalyze the hydrolysis of fats (lipids) and remove fatty stains (butter, oil, and sauces) from fabrics. The trend toward cold-washing to reduce energy consumption (and also to protect fabrics and extend their life) is one of the main reasons why search for the discovery and development of novel cold-adapted lipases from psychrophilic microorganisms has increased rapidly in the past years (Joseph et al. 2008). One example is Lipoclean®, which is a cold-adapted lipase produced by the world's leading producer of enzymes, Novozyme. This product targets stains from triglycerides at low temperatures (above 20 °C) and is very stable in multienzymatic solutions, being, therefore, suitable and effective as a cleaning mixture.

Proteases and celullases are also examples of enzymes used in detergent industries and their cold-active versions are as important as lipases in this industry. Proteases catalyze the hydrolysis of peptide bonds that link amino acids together, digesting proteins into smaller fragments. In the detergent industry, they help in breaking down protein stains, such as blood, egg, grass, cocoa, and human sweat (Joshi and Satyanarayana 2013). Commercial examples of these enzymes are Polarzyme® and (Novozyme), a serine protease for hand washing laundry able to maintain its high activity in a broad range of temperatures ranging from 5 to 60 °C. Purafect® Prime and Properase® are two cold-adapted proteases for laundry detergents with optimal activity for soil stains removal at temperatures ranging from 20 to 40 °C. On the other hand, Excellase® is a product for dishwashing at low or moderate temperatures. All these three enzymes are produced by Genencor.

Some of these biocatalysts may also provide a valuable tool for low-temperature biotransformations and bioremediation. Based on the high catalytic efficiency of cold-active enzymes and their unique specificity at low and moderate temperatures, psychrophilic and psychrotolerant microorganisms are powerful tools for bioremediation purposes. In most of these cases, purification of these enzymes for bioremediation is not necessary, and most of the reported protocols have been carried out using mixed cultures to provide a wide variety of enzymes able to degrade as many pollutants as possible (Welander 2005; Filler et al. 2008). Wastewater treatment and bioremediation of contaminated soils in cold environments also involve the potential application of cold-adapted microorganisms for reducing the amounts of toxic compounds, for example, nitrates, hydrocarbons, aromatic compounds, heavy metals, and biopolymers such as cellulase, chitin, lignin, proteins, and triacylglycerols (Gerday et al. 2000).

13.4 Antarctica as a Source of Yeast with Cold-Active Enzyme Production

Antarctica is one of the harshest environments on Earth, showing several extreme climatic factors including low temperature, low humidity, and high solar irradiation. Additionally, soil and water in Antarctic environment are exposed to successive freezing and thawing cycles, demanding specific adaptation responses to biological systems inhabiting them (Yergeau and Kowalchuk 2008). Considering both, continental and maritime regions, less than 2% of the Antarctic continental surface corresponds to ice-free land areas. Antarctic soils are mostly oligotrophic, except for areas where some macrorganisms, such as penguins or elephant seals settled down and breed, enriching soil with their excretions. Precipitations are scarce, and winds are both strong and variable. All these factors determine a wide range of extreme soil microhabitats that differ in temperature, moisture, organic carbon contents, and levels of nitrogen, phosphorous, and other macronutrients (Martorell et al. 2017). Figure 13.2 shows the scheme of bioprospection pathway for Antarctic yeasts with cold-active enzyme activities.

Such a profusion of different environmental conditions constitutes a huge challenge for the survival of microorganisms, which need to possess several physiological and metabolic adaptations to cope with these extreme conditions (Ruisi et al.

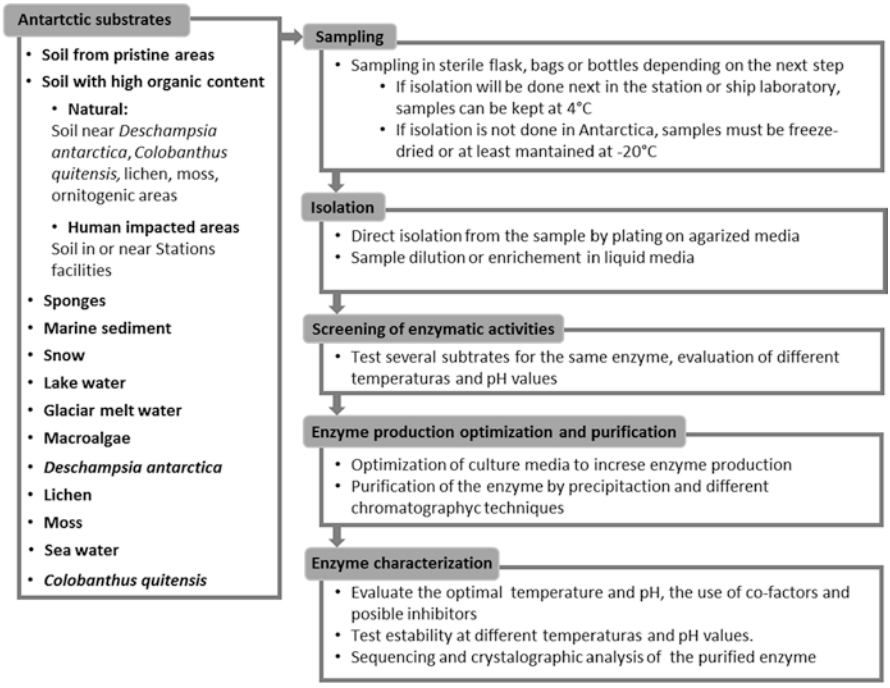

Fig. 13.2 Bioprospection pathway for Antarctic yeasts with cold-active enzyme activities

2007). Among these adaptations, the expression of a variety of cold-adapted enzymes able to work in a wide range of low temperatures is the main reason why in the recent decades Antarctic psychrophilic microorganisms (bacteria, archaea, algae, and, less frequently, fungi) and their enzyme production have been the focus of numerous investigations (Yárzabal 2016; Duarte et al. 2018). This chapter focuses on yeasts isolated from Antarctica and the cold-active enzymes produced by them. Table 13.1 lists, for the enzymes discussed in this chapter, the most promising yeasts isolated from Antarctica during the last 20 years and the main characteristics of the cold-active enzymes produced by them.

13.5 Lignocellulolytic Enzymes

Lignocellulose is the most abundant biomass on earth. Agricultural, forest, and agro-industrial activities generate tons of lignocellulosic waste annually, which present readily procurable, economically affordable, and renewable feedstock for various lignocellulose-based applications. Cellulose, hemicellulose, pectin (carbohydrate), and lignin (non-carbohydrate) polymers are the main substrates of lignocellulose-degrading enzymes (cellulases, hemicelullases, xylanases, pectinases, and laccases, among others) (Saini et al. 2015). The search of a replacement for fossil fuels reinforced the interest on processes that allow the use of renewable energy sources and there is another niche for cold-active lignocellulolytic enzymes (Budsberg et al. 2016).

13.5.1 Cellulases

Several substrates such as sponges, ornithogenic soils, soil near decaying wood and other organic materials have been reported as the source of fungi producing cold-active cellulases (Krishnan et al. 2011; Carrasco et al. 2012; Duncan et al. 2008; Vaca et al. 2013; Martorell et al. 2017, 2019). As a consequence of human activities, wood and other organic materials were introduced into Antarctica. Allochthonous fungi with the ability to degrade those organic compounds were introduced as well. These exogenous fungi faced the challenge of adapting to low temperature or dying.

A specific screening for cellulase-producing fungi can be done by adding CMC (carboxy methyl cellulose) (Bradner et al. 1999) to enrichment cultures. Another approach is to test already isolated fungi for cellulase activities in specific solid media supplemented with CMC and then measure the haloes of CMC consumption as a qualitative and preliminary estimation of the activity. Yeasts isolated from Antarctica with cellulase activity belong to several genera such as *Cryptococcus*, *Guehomyces*, *Leucosporidium*, *Mrakia*, *Leuconeurospora*, *Exophiala*, *Dioszegia*, *Leucosporidiella*, *Rhodotorula*, *Nadsonia*, *Filobasidium*, *Holtermanniella*, *Microglossum*, *Hiphozyma*, *Cystobasiduium*, *Fellozyma*, *Wickerhamomyces*,

Table 13.1 Some cold-active enzymes from yeasts isolated in Antarctica

Enzyme	Yeast	Substrate	Temperature of growth (°C)	Qualitative screening	Quantitative analysis	Comments	Reference
Xylanase	*Candida davisiana*	ND	15	ND	0.75 U mL^{-1} at 120 h	A	Duarte et al. (2013)
	Cryptococcus adeliensis	ND	15	ND	0.43 U mL^{-1} at 120 h	A	
	Guehomyces pullulans	ND	15	ND	0.43 U mL^{-1} at 120 h	A	
	Cryptococcus laurentii	ND	15	ND	1.09 U mL^{-1} at 120 h	B	
	Guehomyces pullulans	soil	15	6	ND	C	Martorell et al. (2017)
	Cryptococcus adeliae	ND	10	ND	385 nkat mL^{-1} at 111 h	D	Gomes et al. (2000)
Protease	*Rhodotorula mucilaginosa*	ND	25	ND	11.12 U mL^{-1} at 120 h	–	Duarte et al. (2013)
	Wickerhamomyces anomalus	Melt water	30	5	ND	C	Carrasco et al. (2012)
	Mrakia frigida	Human impacted lake	4	1.8	ND	E	Krishnan et al. (2011)
	Sporobolomyces roseus	Soil	22	8	ND	F	Troncoso et al. (2017)
	Leucosporidium creatinivorum		15	5	ND	C	Martorell et al. (2017)
	Mrakia frigida		15	5	ND	C	
	Rhodotorula muscorum		15	7	ND	C	
	Rhodotorula sp.		15	6	ND	C	

(continued)

Table 13.1 (continued)

Enzyme	Yeast	Substrate	Temperature of growth (°C)	Qualitative screening	Quantitative analysis	Comments	Reference
Lipase	*Cryptococcus laurentii*	ND	20	ND	0.143 U L^{-1} at 120 h	–	Duarte et al. (2013)
	Cryptococcus adeliensis	ND	20	ND	0.113 U mL^{-1} at 144 h	–	
	Leucosporidium scottii	ND	20	ND	0.230 U mL^{-1} at 120 h	–	
	Cystobasidium pallidum	Soil	30	8	ND	F	Troncoso et al. (2017)
Amylase	*Dioszegia fristingensis*	Soil	22	7	ND	C	Carrasco et al. (2012)
	Dioszegia sp.		15	7	ND	C	
	Cryptococcus victoriae		22	5	ND	F	Troncoso et al. (2017)
	Cryptococcus sp.		22	6	ND	F	
	Hyphozyma sp.		15	6	ND	F	
Cellulase	*Mrakia bollopsis*	Soil	15	8	ND	C	Carrasco et al. (2012)
	Mrakia psychrophila		10	10	ND	C	
	Mrakia frigida	Human impacted lake	4	1,7	ND	E	Krishnan et al. (2011)
	Cryptococcus victoriae	Soil	10	3	ND	F	Troncoso et al. (2017)
	Hyphozyma sp.		15	5	ND	F	
	Cryptococcus victoriae		15	6	ND	C	Martorell et al. (2017)
	Guehomyces pullulans		15	5	ND	C	

Enzyme	Yeast	Substrate	Temperature of growth (°C)	Qualitative screening	Quantitative analysis	Comments	Reference
	Mrakia frigida		15	5	ND	C	
Esterase	*Cryptococcus victoriae*	Soil	15	5	ND	C	Carrasco et al. (2012)
	Dioszegia fristingensis		22	6	ND	C	
	Dioszegia sp.		15	6	ND	C	
	Leuconeurospora sp.		15	6	ND	C	
	Mrakia psychrophila		10	7	ND	C	
	Cystobasidium pallidum		30	3	ND	F	Troncoso et al. (2017)
	Cryptococcus victoriae		22	10	ND	F	
	Cystobasidium laryngis		15	7	ND	F	
	Cryptococcus victoriae		15	7	ND	C	Martorell et al. (2017)
	Cystobasidium laryngis		15	5	ND	C	
	Guehomyces pullulans		15	6	ND	C	
	Holtermanniella sp.		15	6	ND	C	
	Mrakia frigida		15	7	ND	C	
	Pichia caribbica		15	6	ND	C	
Pectinase	*Dioszegia fristingensis*	Soil	22	7	ND	C	Carrasco et al. (2012)
	Dioszegia sp.		15	6	ND	C	
	Leuconeurospora sp.		15	6	ND	C	
	Sporobolomyces salmonicolor		22	6	ND	C	

(continued)

Table 13.1 (continued)

Enzyme	Yeast	Substrate	Temperature of growth (°C)	Qualitative screening	Quantitative analysis	Comments	Reference
	Cryptococcus sp.		10	20	ND	F	Troncoso et al. (2017)
	Cryptococcus adeliensis		15	6	ND	C	Martorell et al. (2017)

A = endoxylanase activity was measured, B = xylosidase was measured, C = corresponds to the halo of activity measured from the edge of the colony to the edge of the halo in mm, D = the enzyme was partially characterized, E = the numbers represent a relative activity (diam. of halo-diam. of colony/diam. of halo), F = corresponds to the halo (measured in mm) formed by the culture supernatant deposited into wells made in agarized media
ND not determined

Candida, and *Pichia* (Krishnan et al. 2011; Vaz et al. 2011; Carrasco et al. 2012; Vaca et al. 2013; Troncoso et al. 2017; Martorell et al. 2017).

13.5.2 Xylanases

Fungi able to produce xylanases have been isolated from several Antarctic samples, mainly from ornithogenic soils and marine invertebrates (Carrasco et al. 2012; Duarte et al. 2013; Martorell et al. 2017). For the screening of xylanase producers, xylan is added to solid media as a substitute for the original carbon source (glucose in most media). Martorell et al. (2017, 2019) reported the use of Yeast Morphology Media (YM) for the search of several enzyme activities, replacing the carbon source (glucose) for a particular substrate, in this case xylan. This strategy proved to be an efficient and simple way to analyze the presence of several enzymes using just one simple and standardized culture media. The most reported xylanase-producing yeasts genera isolated from Antarctica belong to *Candida*, *Cryptococcus*, *Mrakia*, *Wickerhamomyces*, *Dioszegia*, *Fellomyces*, *Guehomyces*, *Bullera*, *Leucosporidium*, *Phenoliferia*, and *Pichia* (Gomes et al. 2000; Carrasco et al. 2012; Duarte et al. 2013; Martorell et al. 2017).

13.5.3 Pectinases

Pectinases comprise a large group of enzymes that break down pectic polysaccharides of plant tissues into simpler molecules, such as galacturonic acid. Pectinases represent an important fraction of the food-processing market, where they are used for degradation of pectin and are involved in different processing steps such as liquefaction, clarification, and juice extraction. The industry currently uses pectinases from mesophilic or thermophilic microorganisms that are well established, but recently, there has been is a new trend in the food industry to adopt low-temperature processing. This trend is due to the potential economic and environmental advantages that the industry envisages for the near future. To achieve this change, cold-active pectinases and the microorganisms that produce them need to be studied more deeply (Adapa et al. 2014). In this sense, psychrophilic pectinases derived from cold-adapted yeasts are known to function at low to freezing temperatures and may be an alternative to address the problem. The isolation media for these enzymes generally contains a specific substrate for pectinase activity. Most researchers use pectin (Fenice et al. 1997; Vaz et al. 2011; Carrasco et al. 2012; Martorell et al. 2017); nevertheless, other compounds, such as polygalacturonic acid, have been used (Margesin 2000).

In Antarctica, yeasts able to produce these kinds of enzymes were mainly found in the marine environment or in soils surrounding *Deschampsia antarctica*, mosses, or lichens. Most of the isolated pectinase producer yeasts belong to *Cryptococcus*,

Wickerhamomyces, *Rhodotorula*, *Microglossum*, *Leuconeurospora*, *Leucosporidiella*, *Leucosporidium*, *Dioszegia*, and *Mrakia* (Vaz et al. 2011; Carrasco et al. 2012; Duarte et al. 2013; Martorell et al. 2017). All the isolates of *Mrakia frigida* reported by Martorell et al. (2017) presented pectinase activity, in accordance with two strains of *M. frigida* isolated from the European Alps and North Siberia by Margesin et al. (2005). These authors characterized the enzymes and concluded that both strains are capable of maximal enzyme production at temperatures below 5 °C, reflecting their adaptation to permanent cold natural place they inhabit. Maximal activity of the purified enzymes was observed at 30 °C, but the stability of both enzymes sharply decreased in the 10–40 °C range, classifying the enzymes as thermolabile. Similar characteristics are expected from the Antarctic strains of *M. frigida*.

13.5.4 Laccase, Lignin Peroxidase, and Decolorizing Enzymes

Rovati et al. (2013) evaluated the presence of these kinds of lignocellulolytic enzymes in yeasts isolated from King George Island in Antarctica. They used lignin, guaiacol, and a mixture of textile dyes as substrates to evaluate lignin peroxidase, laccase, and decolorizing activities, respectively. Lignin peroxidase activity was detected in yeasts belonging to *Leucosporidiella*, *Rhodotorula*, *Dioszegia*, *Debariomyces*, *Mrakia*, and *Cryptococcus*. Laccase activity was present in yeasts belonging to *Leucosporidiella*, *Rhodotorula*, *Dioszegia*, *Debariomyces*, *Bullera*, *Cylindrobasidium*, and *Cryptococcus*. Finally, yeasts with decolorizing activity belonged to *Leucosporidiella*, *Rhodotorula*, *Debariomyces*, *Mrakia*, *Bullera*, *Exophiala*, and *Cryptococcus*.

13.6 Lipolytic Enzymes

Microbial lipases are, besides proteases, the group of microbial enzymes showing the highest biotechnological potential. They catalyze hydrolytic reactions in media with low water activity. They show high regiostereo- and chemoselectivity. Many of them were obtained as crystals and, therefore, their structures have been deeply understood (Jaeger and Eggert 2002). These enzymes are used in pharmaceutical, food, and household chemicals industries and for the treatment of environmental pollution. Lipases production is increasing constantly, and it accounts for more than one-fifth of the global enzyme market nowadays (Szczęsna-Antczak et al. 2014).

It is important to highlight that special attention was paid to lipases and esterases producers, as these are enzymatic activities related to hydrocarbons degradation, a relevant catabolic ability with potential application in bioremediation processes of

cold polluted environments, as well as in biodiesel production. Although microorganisms able to degrade hydrocarbons efficiently have also been isolated from uncontaminated environments, their numbers, including fungi, significantly increase in oil-contaminated sites. In this sense, Aislabie et al. (2001) attributed the significant enhancement in numbers of cultivable yeasts and filamentous fungi in oil-contaminated cold soils to the important role of these microorganisms in the degradation of hydrocarbons or their metabolites (Fernandez et al. 2017). The production of esterases and lipases by Antarctic fungi may be associated with the mechanisms of cold tolerance, since the maintenance of the adequate cell membrane fluidity, essential to cellular survival, may be achieved by increasing the fraction of unsaturated fatty acids (Duarte et al. 2018).

To isolate or select yeasts with lipase activity, solid media is supplemented with olive oil and rhodamine B. After growth, a fluorescent halo under UV light is used to identify the colonies that exhibited lipase activity (Martorell et al. 2017). While this is a simple method used by most researchers, other substrates can be used for this purpose, such as vegetable and fish oils, animal fats, and synthetic triacylglycerides (Duarte et al. 2018).

13.6.1 Lipases

Reported Antarctic yeasts having lipase activity belong to the genera *Candida*, *Cryptococcus*, *Guehomyces*, *Hyphozyma*, *Hamamotoa*, *Kriegeria*, *Cystobasidium*, *Rhodotorula*, *Fellomyces*, *Sporidiobolus*, *Leucosporidium*, *Mrakia*, *Leuconeurospora*, *Wickerhamomyces*, *Exophiala*, *Phenoliferia*, and *Pichia* (Vaz et al. 2011; Carrasco et al. 2012; Duarte et al. 2013; Martorell et al. 2017; Troncoso et al. 2017). The most studied lipases from an Antarctic yeast are lipases A and B from *Pseudozyma* (*Candida*) *antarctica*, which have been thoroughly analyzed at molecular level and subjected to many genetic and chemical modifications, including immobilization (Szczęsna-Antczak et al. 2014). Lipase B is produced by Novozymes (Bagsvaerd, Denmark) and commercialized as Novozyme 435. This enzyme is involved in many organic synthesis applications related to food and feed processing, pharmaceuticals, and cosmetics (Yang et al. 2013; Duarte et al. 2018).

As was pointed out by Szczęsna-Antczak et al. (2014) in their extensive review on Lipases A and B from *Candida antarctica*, even though the number of reports and patents from Lipases A and B are impressive and increasing, the major portion have not been fully translated into industrial implementations, mainly because of the relatively high cost of the enzymes preparations, both in free or immobilized forms. The authors highlighted that the effort on the development of these enzymes must be focused on making them cheap, stable, and reusable.

13.6.2 Esterases

Unlike lipases, which prefer long-chain triglycerides, esterases hydrolyze short acyl-chain soluble esters (less than ten carbons) (Hashim et al. 2018). Esterase activity plays a major role in the degradation of natural materials and industrial pollutants, cereal wastes, plastics, and other toxic chemicals. They are useful for the synthesis of optically pure compounds, perfumes, and antioxidants (Panda and Gowrishankar, 2005).

For esterase activity screening from Antarctic yeasts, Tween 80 is the preferred substrate (Vaz et al. 2011; Carrasco et al. 2012; Martorell et al. 2017). Genera with esterase activity were reported by Vaz et al. (2011), Carrasco et al. (2012), Martorell et al. (2017), and Troncoso et al. (2017). It is a large group of microorganisms, comprising members of *Cryptococcus*, *Guehomyces*, *Leucosporidium*, *Mrakia*, *Leuconeurospora*, *Exophiala*, *Phenoliferia*, *Dioszegia*, *Glaciozyma*, *Leucosporidiella*, *Mrakia*, *Rhodotorula*, *Metchniskowia*, *Nadsonia*, *Cystobasidium*, *Holtermanniella*, *Protomyces*, *Hyphozyma*, *Kriegeria*, *Fellomyces*, and *Pichia.* Martorell et al. (2017) reported that esterase was the enzyme produced by most of the Antarctic isolated yeasts (72%). This result was in accordance with Carrasco et al. (2012), who reported the same situation for lipase and esterase. Interestingly, it was the only enzyme produced by all the *Cystobasidium larynges* isolates in the screening performed by Martorell et al. (2017).

Hashim et al. (2018) reported a new cold-active esterase-like protein with putative dienelactone hydrolase (GaDlh) activity from the psychrophilic yeast *Glaciozyma antarctica*. These authors characterized the purified recombinant GaDlh, which presented an optimal temperature of 10 °C and an optimum pH of 8.0. The behavior of the enzyme with its specific substrate, dienelactone, remains unknown due to the lack of the substrate commercially. Several cold-adapted features were observed in the predicted protein structure. Nevertheless, isolation, heterologous expression, and characterization of cold-active enzymes from Antarctic yeasts, as is the case of esterase from *G. antarctica*, are always appreciated and have become a key stone in the bioprospection of cold-active enzymes.

13.7 Amylases

Cold-active amylase activity was detected in members of several genera isolated in Antarctica including *Cryptococcus, Guehomyces*, *Leucosporidium*, *Mrakia*, *Leuconeurospora*, *Exophiala*, *Phenoliferia*, *Dioszegia, Glaciozyma, Hyphozyma, Hamamotoa, Kriegeria, Fellozyma, Leucosporidiella, Rhodotorula*, *Holtermanniella*, *Fellomyces*, and *Pichia* (Krishnan et al. 2011; Vaz et al. 2011; Carrasco et al. 2012; Vaca et al. 2013; Troncoso et al. 2017; Martorell et al. 2017). To evidence amylase activity, starch in agarized media is the main substrate used for screening. After incubation, revealing decolorization haloes with Lugol solution helps identify amylase-positive yeast.

De Mot and Verachtert (1987) purified α-amylase and a glucoamylase from *Candida antarctica* CBS. Both enzymes were monomeric glycoproteins with different amino acid composition. Kinetic analyses indicated that both enzymes preferentially hydrolyzed high-molecular-mass substrates, including some raw starches. α-Amylase was active on cyclodextrins, whereas debranching activity was demonstrated for glucoamylase.

Ramli et al. (2013) also purified and sequenced an *α-amylase from Glaciozyma antarctica* PI12. They developed and analyzed a 3D model for the α-amylase AmyPI12 structure and identified several novel characteristics of the newly isolated cold-adapted protein. They suggested that the AmyPI12 model developed for this enzyme could be used for further comparative structural analyses to understand the structure and activity relationships and may contribute to a better understanding of the structures and functions of other cold-adapted proteins in nature.

Cold-active amylases can be used in a wide range of processes, such as desizing operation (removal of starch) in textile industry, cold-washing with low-temperature detergents, direct fermentation of starch to ethanol, manufacture of maltotetraose syrup, manufacture of maltose, manufacture of high-molecular-weight branched dextrins, treatment of starch-containing wastewater, etc. (Kuddus et al. 2011). Also, cold-active but thermolabile amylases are important in the food industry, as their use can prevent substrates' and products' modification after enzymatic treatment (Kuddus et al. 2012).

13.8 Proteases

Antarctic yeasts showing cold-active protease activity belong to the genera *Candida*, *Cryptococcus*, *Exophiala*, *Guehomyces*, *Glaciozyma*, *Leuconeurospora*, *Leucosporidium*, *Mrakia*, *Rhodotorula*, *Leucosporidiella*, *Nadsonia*, *Pichia*, *Sporidiobolus*, and *Wickerhamomyces* (Krishnan et al. 2011; Vaz et al. 2011; Carrasco et al. 2012; Duarte et al. 2013; Vaca et al. 2013; Martorell et al. 2017; Troncoso et al. 2017). Ray et al. (1992) isolated a cold-active acidic protease from *Candida humicola*. These authors reported that the secretion of this protease was dependent on medium composition, being higher when the medium was supplemented with proteins. In addition, enzyme secretion during exponential growth was greater at low temperatures than at higher temperatures. Nevertheless, the extracellular protease isolated from *C. humicola* was active at temperatures ranging from 0 to 45 °C, with an optimum activity at 37 °C. Due to these particular features, this protease provides a powerful tool for processes where similar activity is required under temperature changes.

In 2003, Turkiewicz et al. (2003) reported the purification to homogeneity and characterization of an extracellular serine proteinase, lap2, from Antarctic yeast *Leucosporidium antarcticum* 171. This proteinase showed to be halotolerant, whereas its activity and stability were dependent neither on Ca^{2+} nor on other metal ions. It showed an optimal temperature as low as 25 °C, poor thermal stability, rela-

tively small values of free energy, enthalpy and entropy of activation, and high catalytic efficiency in the range 0–25 °C. The 35 N-terminal amino acid residues of lap2 presented homology with subtilases of the proteinase K subfamily (clan SB, family S8, subfamily C). This proteinase lap2 was the first psychrophilic subtilase in this family.

The production, purification, and characterization of an extracellular protease released by *Rhodotorula mucilaginosa* L7 were reported by Lario et al. (2015). The enzyme production started at the beginning of the exponential growth phase and reached a maximum after 48 h. The purified protease presented optimal catalytic activity at pH 5.0 and 50 °C. In addition, the enzyme was stable in the presence of high concentrations of NaCl, in accordance with the marine environment from where isolation of the yeast was done.

Nowadays, around 60% of the total enzyme market is represented by proteases used in various industries. Proteases are applied in diverse fields such as detergent industry, leather processing, silk degumming, food and dairy, baking, pharmaceutical industries, silver recovery from x-ray films, waste management, and others (Joshi and Satyanarayana 2013).

13.9 Patents

Patenting is one of the ways for developments and discoveries to reach industrial use or market. As was described in this chapter, enzymes from cold-adapted yeast were reported as "powerful" tools for industry. Do they jump the hit from "potential biotechnological tools" to real applied products? The analysis of patents related to cold enzymes could be helpful to answer this question. Duarte et al. (2018) made and exhaustive research on patents (or patent requests) related to enzymes from Antarctic fungi from information deposited in 14 databases from around the world. Despite the high number of keywords used, their survey did not retrieve many relevant results, indicating that this environment has an enormous biotechnological potential, but it has not been reflected in patented products or process yet. They also reported that patents' records or requests are associated mostly with bacteria, such as the lipase from *Bacillus pumilus* from Antarctica (10–1596435-0000), the cold-adapted protease HSPA-2 from a marine bacterium (WO2013177834), and anti-tumor and anti-microbial compounds from Lakes Schirmacher Oasis' bacteria, in East Antarctica (US20110301216).

When it comes to patents of cold-adapted enzymes produced by Antarctic yeasts, they are all related to lipase produced by *Candida antarctica*. Several companies and organizations own patents and commercialize this enzyme as AstraZeneca (UK/Sweden), Du Pont (US), Novo Nordisk (Denmark), DSM NV (The Netherlands), Korea Ocean Research and Development Institute (Korea), Shin Dong Bang Corp. (Korea), and Nagata Sangyo (Japan) (Duarte et al. 2018).

13.10 Antarctic Regulation for Bioprospection

Bioprospecting in Antarctic environments is a relatively new phenomenon that generates both challenges and doubts, especially when the particular characteristics that rules Antarctica, as established by the Antarctic Treaty System (ATS 1959) and its Protocol, are taken into account. The guideline establishes that it is crucial to evaluate both all possible benefits and consequences from bioprospecting, looking for the protection of Antarctic ecosystems. The aim of the ATS is to keep the white continent as a territory used exclusively for peaceful purposes (Article I of the ATS). Commercial activities are not allowed, with the exception of tourism and fishery. Mining is forbidden, as well as the exploitation of other natural recourses. There have been multiple proposals by researchers and their countries, which have been presented in the ATS. However, the challenges are such that there is still a long way to go in the research field as well as in the ATS (Villamizar-Lamus 2015).

The first question to answer is: Does bioprospection endanger Antarctic ecosystems? The second one is: Does bioprospection represent a commercial exploitation of Antarctic life resources? To respond to these questions is not a simple task. Different approaches should be considered. So far, a regulated Antarctic bioprospect guideline has been gradually generated, with a starting point based on the requirement of channeling information about bioprospection activities to the Antarctic community to make proper decisions in the near future (ATCM 28, ATCM 36). As expressed in the documents signed during those meetings, the ATS is convinced of the benefits of scientific research in the field of biological prospecting for the progress of humankind and that scientific observations and results from Antarctica must be exchanged and made available for all countries.

13.11 Conclusions and Future Perspectives

Yeasts isolated from the Antarctic Continent provide a significant source for enzyme production. Enzymes from Antarctic yeast can be potentially applied in a myriad of industrial processes, with several examples of real and effective applications. In this chapter, we mainly reviewed hydrolases and oxidoreductases. Beyond bioprospection, it is important to consider the ecological role of Antarctic yeast and their battery of enzymes; through these enzymes Antarctic yeasts make essential contributions to nutrient cycling and organic matter mineralization in Antarctica, where nutrients are scarce.

Most of the cold-enzyme bioprospection in Antarctic yeasts has been done using cultivable, already isolated yeasts, with the consequent and beneficial creation of several culture collections in most of the countries with a research program focused on microorganisms.

As these studies started about 50 years ago and because it is difficult to carry out sampling in Antarctica, the knowledge about microorganisms and their metabolites

in general, and about yeasts in particular, is quite scarce. However, the use of metagenomics and culture-independent techniques is improving day by day, leading to an improvement in the understanding of the diversity and ecology of the Antarctic microbiota and an increase in the prospection of possible new metabolites, including enzymes.

Besides yeasts isolation and enzyme screening, more research on the purification and characterization of these enzymes needs to be done. Culture collections are rich and their hidden enzymatic potential is supposed to be abundant, but the number of cold enzymes that show effective application is not very large. Finally, as pointed out by the Antarctic Treaty, the white continent must remain a territory that is used exclusively for peace and science. The knowledge and benefits obtained from bioprospecting in Antarctica should be shared and made available for all countries.

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