

1 **Biotechnological applications of extremophiles, extremozymes and extremolytes**

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1 **Abstract**

2 In the last decade, attention to extreme environments has increased because of interests to isolate
3 previously unknown extremophilic microorganisms in pure culture and to profile their metabolites.
4 Microorganisms that live in extreme environments produce extremozymes and extremolytes that
5 have the potential to be valuable resources for the development of a bio-based economy through
6 their application to white, red and grey biotechnologies. Here, we provide an overview of
7 extremophile ecology, and we review the most recent applications of microbial extremophiles and
8 the extremozymes and extremolytes they produce to biotechnology.

9

10 **Key words:** extremophilic/extremotolerant prokaryotes, extremozymes, extremolytes,
11 biotechnology, bioeconomy.

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1 **Introduction**

2 Biocatalysts are whole microbial cells or enzymes that can be used in biochemical reactions of
3 modern biotechnology. Some of these reactions have optimized or even replaced existing processes
4 (Wohlgemuth, 2010; Resch et al., 2011). Interest in biocatalysts has recently increased with the
5 growth and development of biotechnology as a strategy towards attaining a bio-based economy.
6 White (or industrial) biotechnology aims to resolve environmental and economic concerns
7 associated with increasing energy and fuel demands and subsequent prices of petroleum-based
8 products. It uses biocatalysts to convert renewable resources, such as wastes and byproducts, into
9 fine chemicals, biopolymers, biomaterials and biofuels. Grey (or environmental) biotechnology
10 applies biocatalysts to bioremediate contaminated sites, while red (or medical/pharmaceutical)
11 biotechnology exploits microorganisms to produce pharmaceuticals. To date, the majority of
12 enzymes on the market are of bacterial or fungal origin, while few are derived from archaea, most
13 of which have been produced by mesophilic microorganisms who are often inhibited under the
14 extreme conditions of many industrial processes. Thus, the search for new sources of isolation,
15 experimental procedures and analytical methods is recently growing to identify robust biocatalysts.
16 Specifically, extremophiles are receiving increasing attention, several have been obtained in pure
17 culture, their genomes analyzed and their enzymes characterized by either academic or industrial
18 laboratories (Cárdenas et al., 2010; López-López et al 2014; Yildiz et al., 2015).

19 Extremophilic microorganisms thrive in the harsh environments where other organisms cannot even
20 survive. Extremophiles are taxonomically widely distributed and are a functionally diverse group
21 (Cowan et al., 2015) that includes thermophiles, psychrophiles, acidophiles, alkalophiles,
22 halophiles, barophiles/piezophiles, metalophiles and radiophiles. Extremophiles have the potential
23 to produce biomolecules of high relevance for white, grey and red biotechnological sectors. These
24 microorganisms produce extremophilic enzymes (extremozymes) and protective organic
25 biomolecules (extremolytes) that convey characteristics for survival in extreme environmental
26 conditions. Here, we present an overview of the potential applications of these microorganisms and
27 their products in biotechnology. We briefly describe the ecology of extremophilic prokaryotes and
28 review the most recent reports on the application of extremozymes and extremolytes derived from
29 extremophilic and extremotolerant microorganisms in various biotechnological processes.

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31 **Ecology and classification of extremophiles**

32 Extremophiles are organisms that have adapted to thrive in ecological niches that are uninhabitable
33 to others, for example, deep-sea hydrothermal vents, hot springs, sulfataric fields, soda lakes, inland
34 saline systems, solar salterns, hot and cold deserts, environments highly contaminated with nuclear

1 waste or heavy metals as well as lithic or rock environments. Psychrophiles are extremophiles that
2 are adapted to extreme cold, and halophiles describe those that thrive in the presence of high salt
3 concentrations; each type of microorganism uses different survival strategies to be successful in
4 their environment (Oren 2013; De Maayer et al., 2014). Psychrophilic prokaryotes are widespread
5 among bacteria and archaea and can be found within the genera *Aletromonas*, *Halobacterium*,
6 *Shewanella*, *Psychrobacter*, *Pseudoalteromonas*, *Arthrobacter*, *Colwellia*, *Gelidibacter*,
7 *Marinobacter*, *Psychroflexus*, *Pseudomonas*, *Methanlobus* and *Methanococcoides* (De Maayer et
8 al., 2014). In addition to adaptations for acidic environments, acidophiles are also typically adapted
9 to environments with high temperatures, high salinity or heavy metal concentrations because these
10 conditions often co-occur, for example, in areas of acid drainage (Cárdenas et al., 2010; Navarro et
11 al., 2013; Dopson and Holmes, 2014). Meanwhile, alkalophiles thrive in alkaline environments such
12 as gypsum-based soils or soda lakes and are often halophiles. They encompass bacteria from
13 different genera including among others *Bacillus*, *Halomonas* and *Pseudomonas* (Sarethy et al.,
14 2011) as well as archaea belonging to the genera *Halalkalicoccus*, *Halobiforma*, *Halorubrum*,
15 *Natrialba*, *Natronococcus* and *Natronorubrum* (Bowers and Wiegel, 2011). Deep-sea and deep
16 subsurface environments host piezophiles (barophiles), a group of extremophiles that produce
17 compatible solutes and polyunsaturated fatty acids and form multimeric and antioxidant proteins
18 that enable them to survive under extremely high hydrostatic pressures (Kawamoto et al., 2011;
19 Zhang et al., 2015). Most piezophiles are psychrophilic gram-negative bacterial species that belong
20 to the genera *Shewanella*, *Psychromonas*, *Photobacterium*, *Colwellia*, *Thiopfundum* and
21 *Moritella*, but some are archaea derived and can be found among the genera *Thermococcus*,
22 *Sulfolobus* and *Pyrococcus* (Zhang et al., 2015). Adaptation to high concentrations of heavy metals
23 (otherwise essential as trace elements) allow metalophiles to thrive in metal-polluted sites (Johnson,
24 2014; Orell et al., 2013). Metalophiles are also acidophiles and include both bacteria from the
25 genera *Acidithiobacillus*, *Leptospirillum*, *Alicyclobacillus*, *Acidiphilium*, *Acidimicrobium*,
26 *Ferrimicrobium* and *Sulfobacillus* and archaea from the genera *Ferroplasma*, *Acidiplasma*,
27 *Sulfolobus*, *Metallosphaera* and *Acidianus* (Johnson, 2014; Dopson and Holmes, 2014). In
28 environments of high oxidative stress and radiation (UV, gamma and X-rays), radiophiles thrive
29 because of their ability to repairing extensive DNA damage. Radiophiles are found among various
30 microbial groups and species including bacteria from the genera *Deinococcus*, *Bacillus*,
31 *Rubrobacter* and *Kineococcus*; the family *Geodermatophilaceae* and cyanobacteria including the
32 genera *Nostoc* and *Chroococcidiopsis* (Brim et al., 2003; Gtari et al., 2012; Bagwell et al., 2008;
33 Gabani and Singh 2013).

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1 **Potential applications of extremophilic/extremotolerant biocatalysts**

2 Owing to their unique enzymatic features and physiological properties, the potential
3 biotechnological applications of whole-cell extremophilic biocatalysts range from the
4 bioremediation of toxic pollutants from water and/or sediments to the production of biomolecules
5 for medical and industrial purposes. Because of their adaptation to high concentrations of heavy
6 metals, metalophiles/acidophiles are currently being used for bioremediation and biomining
7 (Navarro et al., 2013; Johnson 2014; Orell et al., 2013), while radiophiles are suited for application
8 in the management of nuclear-waste-polluted environments (Brim et al., 2003; Appukuttan et al.,
9 2006). Applications can also be envisaged in agriculture where desert bacterial extremophiles that
10 are able to cope with low water activity conditions can be used to improve the management of water
11 by plants under drought stress (Marasco et al., 2012; Rolli et al., 2014).

12 In addition to entire microbial cells, extremozymes are enzymes that have developed molecular
13 mechanisms (Hough and Danson 1999) of adaptation to extreme physico-chemical conditions that
14 have relevant applications as biocatalysts in industrial biotransformation processes. Enzymes
15 produced by psychrophiles have been shown to display high catalytic efficiency in the detergent and
16 food industries and for the production of fine chemicals (Cavicchioli et al., 2011). Karan et al.
17 (2013) reported on the purification and characterization of β -galactosidase from the cold-adapted
18 haloarchaeon *Halorubrum lacusprofundi*. This enzyme was overexpressed in the model
19 haloarchaeon, *Halobacterium* sp. NRC-1, and was shown to be active in high salinity environments
20 (with maximal activity in either 4-M NaCl or KCl) across a wide temperature range (-5 to 60°C).
21 Its functionality is conserved in the presence of 10-20% (v/v) organic solvents, including methanol,
22 ethanol, n-butanol and isoamyl alcohol, suggesting its suitability for the synthesis of
23 oligosaccharides under low water activity and cold temperatures.

24 The industrial potential for halophilic enzymes resides in their ability to be active and stable under
25 low water activity, and in many cases, also in the presence of organic solvents (Raddadi et al., 2013;
26 Datta et al., 2010). Examples of these extremozymes include polysaccharide-hydrolyzing enzymes
27 of high relevance for the hydrolysis of cellulose, xylan and starch (Raddadi et al., 2013; Bhalla et
28 al., 2013; Du et al., 2013; Elleuche et al., 2014). For example, the extremotolerant cellulases
29 produced by *Paenibacillus tarimensis* L88, an isolate obtained from the Sahara Desert in southern
30 Tunisia, have been shown to have high functionality across a broad pH range (3.0 to 10.5), at high
31 temperatures (80°C) and high salt concentrations (up to 5-M NaCl) (Raddadi et al., 2013).
32 Carboxymethyl cellulase activity has been detected in the presence of 40% (v/v) 1-butyl-3-
33 methylimidazolium chloride or 20% (w/v) 1-ethyl-3-methylimidazolium acetate ionic liquids and
34 was maintained after exposure to organic solvents, detergents, heavy metals and even under high

1 alkalinity. *Paenibacillus tarimensis* is an optimal candidate for the production of cellulases with
2 promising applications in detergent, textile and pulp and paper industries; it also has potential for
3 simultaneous ionic liquid treatment and saccharification of lignocellulose in biorefinery processes
4 (Raddadi et al., 2013). Some halophilic enzymes are lipolytic, such as lipases and esterases, such
5 that they have the ability to hydrolyze long-chain acylglycerols ($\geq C_{10}$) and short-chain fatty esters
6 ($\leq C_{10}$), respectively. These enzymes have a wide range of applications including the production of
7 polyunsaturated fatty acids in the food industry or of biodiesel (Litchfield, 2011; Schreck and
8 Grunden, 2014). For example, lipase from the halophilic bacterium *Idiomarina* sp. was shown to be
9 highly active under a variety of harsh conditions including in the presence of organic solvents and
10 high salt concentrations. Its application for biodiesel production from jatropha oil in free or
11 immobilized forms resulted in 80 and 91% yields, respectively (Li et al., 2014).

12 Bacterial alkaliphiles are mainly exploited for the production of enzymes that are widely applied in
13 the detergent and laundry industries (Sarethy et al., 2011). Although the biotechnological potential
14 of piezophiles is still poorly explored (Abe and Horikoshi, 2001; Mota et al., 2013; Lamosa et al.,
15 2013), they may be valuable to the food industry in processes that require high pressures (Zhang et
16 al., 2015). Moreover, piezophilic bacteria could be a source of essential fatty acids like for example
17 omega-3-polyunsaturated fatty acids since these compounds are produced by the bacteria to
18 stabilize the cell membrane under high pressure (Zhang et al., 2015).

19 Enzymes produced by radiotolerant microorganisms have been shown to be resistant to other
20 stresses. For example, Shao et al. (2013) characterized lipases from the radiation-tolerant bacterium
21 *Deinococcus radiodurans* expressed in *E. coli*. Purified enzymes showed preference for short-chain
22 esters, three of which were thermostable and retained their activities in the presence of surfactants
23 and organic solvents.

24 Thermozyms are extremozymes produced by thermophilic and hyperthermophilic microorganisms.
25 These enzymes are also often able to tolerate proteolysis and harsh conditions like the presence of
26 denaturing agents and organic solvents as well as high salinity. Benefits of using thermozyms
27 include reduced risk of contamination, lower viscosity and higher solubility of substrates. Toplak et
28 al. (2013) identified a gene coding for a subtilase termed proteolysin in the Gram-positive,
29 anaerobic, thermophilic bacterium *Coprothermobacter proteolyticus*. By functionally expressing the
30 gene into *E. coli*, the enzyme could be purified and identified as highly thermostable in the presence
31 of organic solvents and detergents with a high level of activity across a wide pH range at high
32 temperatures (up to 80°C), making it a suitable candidate for application to thermophilic organic
33 solid waste degradation (Toplak et al., 2013). This subtilase is a member of the serine protease
34 family produced by *Bacillus* strains including the largest group of commercial proteolytic enzymes

1 extensively used in food, textile, detergent, pharmaceutical and leather industries. In addition, a
2 thermostable nucleoside phosphorylase has been characterized from hyperthermophilic aerobic
3 crenarchaeon *Aeropyrum pernix* K1 and has been used for the synthesis of nucleoside analogues
4 used in antiviral therapies as an alternative to chemical synthesis (Zhu et al., 2013). Other
5 thermozyms also include proteases like thermolysin used in the synthesis of dipeptides, pretaq
6 protease used to cleanup DNA prior to PCR amplification and starch-processing and DNA-
7 processing enzymes (Bruins et al., 2001; Jayakumar et al., 2012).

8 In addition to the above-mentioned extremozymes, other enzymes are also suitable for use in further
9 industrial processes. For example, alcohol dehydrogenases can be used to synthesize building
10 blocks for the chemical industry, such as optically active alcohols, or for to synthesize cofactors
11 such as NAD and NADP. Meanwhile, nitrile-degrading enzymes are of interest for the
12 transformation of nitriles and carbon-carbon bond forming enzymes like aldolases, transketolases
13 and hydroxynitrile lyases are useful in organic synthesis (Resch et al., 2011; Chen et al., 2009;
14 Egorova and Antranikian, 2005; Demirjian et al., 2001).

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16 **Extremolytes and their biotechnological applications**

17 Extremolytes are organic compounds that can constitute up to 25% of dry cell weight accumulated
18 in microorganisms exposed to stressful environmental conditions. For example, several compounds
19 of polyol derivatives (ectoine, hydroxyectoine and betaine), carbohydrates such as trehalose and the
20 mannose derivatives (mannosylglycerate [firoin] and mannosylglyceramide [firoin-A]),
21 glucosylglucosylglycerate, glucosylglycerate (GG) and various amino acids (Borges et al., 2002;
22 Lentzen and Schwarz, 2006; Singh and Gabani, 2011; Empadinhas et al., 2011; Esteves et al., 2014,
23 Alarico et al., 2013; Lamosa et al., 2013; Bougouffa et al., 2014). Several archaea accumulate
24 negatively charged derivatives of inositol and glycerol such as phosphodiester di-myoinositol-1,1'-
25 phosphate and α -diglycerol phosphate or cyclic 2,3-diphosphoglycerate and trianionic
26 pyrophosphate (Lentzen and Schwarz, 2006; Esteves et al., 2014). Several UV-radiation-protective
27 compounds have been isolated from UV-resistant extremophilic bacteria; for example, scytonemin,
28 mycosporin-like amino-acids (MAAs), ectoines, bacterioruberin and melanin (Singh et al., 2010;
29 Gabani and Singh 2013; Rastogi and Incharoensakdi, 2014).

30 Extremolytes have primarily been used in cosmetics and have the potential for application to the
31 pharmaceutical sector. The behavior of MAAs in the presence of UV radiation make them useful in
32 UV-protective sunscreens in the cosmetics industry, and their potential application as preventative
33 agents of UV-radiation-induced cancers such as melanoma has also been suggested (de la Coba et
34 al., 2009). In the future, MAA compounds may directly be implicated as therapeutic candidates.

1 Scytonemin, a component in sunscreens (Soule et al., 2009), has also been suggested as a potential
2 candidate for the development of a novel pharmacophore to produce protein kinase inhibitors such
3 as antiproliferative and anti-inflammatory drugs (Singh and Gabani, 2011). The bacterioruberin
4 produced by radioresistant microbes (*Halobacterium* and *Rubrobacterium*) has been suggested to
5 have application in preventing human skin cancer because it participates in repairing damaged DNA
6 strands caused by ionizing UV radiation (Singh and Gabani, 2011). Choi et al. (2014) reported that
7 the deinoxanthin isolated from the radioresistant bacterium *Deinococcus radiodurans* induced
8 apoptosis of cancer cells, suggesting that this carotenoid could potentially be useful as a
9 chemopreventive agent.

10 Extremolytes can also be used to stabilize macromolecules such as proteins and nucleic acids.
11 Protein instability is a central challenge for administering therapeutic protein-based medicines,
12 particularly in aqueous formulations. Owing to their ability to stabilize proteins *in vivo* and *in vitro*,
13 extremolytes offer an attractive solution for the stabilization and storage of sensitive proteins in the
14 absence of other protein stabilizers (Avanti et al., 2014). Moreover, extremolytes can inhibit protein
15 misfolding and/or aggregation and hence, are interesting candidates for the development of drugs
16 for several diseases (Ryu et al., 2008; Faria et al., 2013; Kanapathipillai et al., 2005). For example,
17 ectoine is currently used in skin care products (Pastor et al., 2010) and firoin and ectoine have
18 recently been shown to reduce signal-dependent events resulting from exposure to carbon
19 nanoparticles *in vitro* and *in vivo*, widening the fields of application for these compatible solutes.
20 Such events include indeed the activation of mitogen-activated protein kinases or the upregulation
21 of pro-inflammatory cytokines, apoptosis and proliferation in lung epithelial cells, which could lead
22 to lung cancer, chronic obstructive pulmonary disease and fibrosis (Autengruber et al 2014).
23 Furthermore, extremolytes have the potential for application in the food industry for the production
24 of functional foods, food products that have an added positive health benefit by enhancing short-
25 term well being/performance ability or by the long-term mitigation of certain diseases (Cencic and
26 Chingwaru, 2010). For example, in some cheeses that have been treated with *Brevibacterium linens*
27 for surface ripening of the product, ectoine has been reported to accumulate (up to 89 mg/100 g of
28 product) (Rattray and Fox 1999; Klein et al, 2007). Investigating whether ectoine accumulates in
29 other fermented food products would be worthwhile towards evidencing extremolytes as functional
30 food ingredients.

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32 **Concluding remarks and perspectives**

33 Extremophilic/extremotolerant microbes have the potential to make a great impact on
34 biotechnology via the compounds they produce (i.e., extremozymes and extremolytes) that enable

1 them to thrive in harsh environments. The economic potential of extremozymes is considerable for
2 their application to agriculture, food and beverages and feed, pharmaceutical, detergent, textile,
3 leather, pulp and paper and biomimic industries. Although only a few extremozymes are currently
4 being produced and used at the industrial level, the development of new industrial processes based
5 on these enzymes is motivated by important results obtained in the field of extremophile research,
6 the increasing demand of biotech industries for novel biocatalysts and the rapid progress of new
7 omics techniques such as metagenomics, proteomics, metabolomic gene-directed evolution and
8 gene/genome shuffling (Egorova and Antranikian, 2005; Ferrer et al., 2007). For example,
9 extremozymes have been identified in metagenomes as overcoming bottlenecks related to the
10 uncultivability of extremophiles in some cases (Ferrer et al., 2007, López-López et al 2014). To
11 date, extremolytes have primarily been used in pharmaceutical and cosmetic sectors. At the
12 industrial level, ectoine and its derivatives are produced using the ‘bacterial milking’ process
13 (Pastor et al., 2010), and research initiatives directed at developing additional strategies to improve
14 the productivity of other compatible solutes are underway. For example, genetic engineering and
15 encapsulation of glucosylglycerate (GG)-producing cyanobacteria in gels that aim to concentrate
16 and secrete extremolytes into the extracellular environment have been performed (Tan et al., 2015).
17 The authors report successful growth of *Synechocystis* and improved production and secretion of
18 GG in the encapsulating gels after salt stress.

19 In addition to extremozymes and extremolytes, other metabolites, including exopolysaccharides
20 (Raveendran et al., 2015), biosurfactants, biopolymers and peptides, from
21 extremophilic/extremotolerant microorganisms have great economic-industrial potential. For
22 example, in agriculture, biosurfactants could substitute chemical surfactants as adjuvants in
23 herbicide and pesticide formulations, enhance bioremediation of soils or be applied to the biocontrol
24 of phytopathogens owing to their antimicrobial activity and stimulation of plant defense (Sachdev
25 and Cameotra, 2013). Moreover, biosurfactants could improve arid-zone soil structure and quality
26 due to hydrophilization of soils, which improves wettability and finally to reduce water infiltration.
27 Subsequently, sustainable agriculture could be expanded in arid conditions. Radiophiles produce
28 compounds with the potential for use as radioprotective drugs; however, because only a few studies
29 of these microbes have been performed, their exploitation remains limited. Moreover, challenges
30 associated with the specific nutritional needs and growing conditions of extremophiles has made
31 their isolation and maintenance difficult; isolation of purified extremolytes is among the limiting
32 factors in developing these compounds for therapeutic purposes.

1 In conclusion, extremophilic/extremotolerant microorganisms are sustainable resources that could
2 be better exploited in several biotechnological sectors towards the development of a biobased
3 economy.

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1 **Ethical statement**

2 The authors declare that they have no conflict of interest. This review article has been prepared
3 following principles of ethical and professional conduct.

4

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