Chapter 4
Thermophiles as a Promising Source of Exopolysaccharides with Interesting Properties

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1 Introduction

Thermophiles are a type of extremophilic microorganisms able to grow at comparatively high temperatures, between 45 and 122 °C (Takai et al. 2008). Extremophiles not only endure, but are functionally active in some of the harshest conditions of life found on Earth. "Extreme" is a relative term, which means the conditions too harsh for the existence of man (Satyanarayana et al. 2005). The conception for upper temperature for life has changed several times at the end of the last century as thermophilic representatives of the domains *Bacteria* and *Archaea* were isolated from geothermal and hydrothermal habitats at higher and higher temperature. Suggested groups of thermophiles are: facultative thermophiles growing up to 50 °C, obligate thermophiles able to grow between 50 and 70 °C and optimally at 55–65 °C, extreme thermophiles growing in the range 65–80 °C, and hyperthermophiles—optimum is higher than 80 °C (Wiegel and Canganella 2001).

Thermophilic niches include volcanic and geothermal areas (terrestrial, subterranean and marine hot springs), solfataric areas, sun heated refuse, oil reservoirs, and manmade habitats (Fig. 4.1). As high temperature in them limits growth of the representatives of the domain *Eukarya*, the representatives of domains *Bacteria*, *Archaea* and their viruses predominate in hot niches (López-López et al. 2013). Various arguments have been cited in support of the idea that the ancestors of the *Archaea* and *Bacteria* domains seem to be (hyper)thermophiles (Di Giulio 2003). Extensive global research efforts on the diversity and biotechnological potential in these extreme environments revealed unexpected number of taxa and presence of number of novel species. Extremophiles possess novel metabolic properties making

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Fig. 4.1 Geysera, Sapareva Banja, Bulgaria is characterized by water temperature of 103 °C in the hole drilling

them capable of physiologic activity in unfavorable conditions and production of unique metabolites (Rozanov et al. 2014). Such compounds significantly contribute to the development of biotechnology in the recent years.

Thermophilic microorganisms and their thermostable enzymes already have imperative biotechnological recognition because of their unique ability to function at high temperature and stability to a variety of harsh industrial conditions. Among compounds involved in thermophilic adaptation, exocellular polysaccharides are of a special interest due to their structural and functional diversity and their diverse physiological roles in cell. Exopolysaccharides are high-molecular-weight microbial polymers secreted into the surrounding environment. They are composed of identical or differing sugar residues arranged as repeated units within the polymer. Homopolysaccharides are composed of identical residues; different sugar residues form the molecule of heteropolysaccharides.

2 Physiological Role of EPSs in Cell

Accumulation of EPS on cell surface is a common adaptation strategy of extremophiles including participation in cell protection by stabilizing membrane structure. Polysaccharide layer over the cell surface is often several times ticker than the cell dimension. However, the physiological role of EPSs in bacteria is probably more diverse and complex than currently known. Some of these biopolymers perform the same function, whereas others fulfill distinct functions in dependence of taxonomic

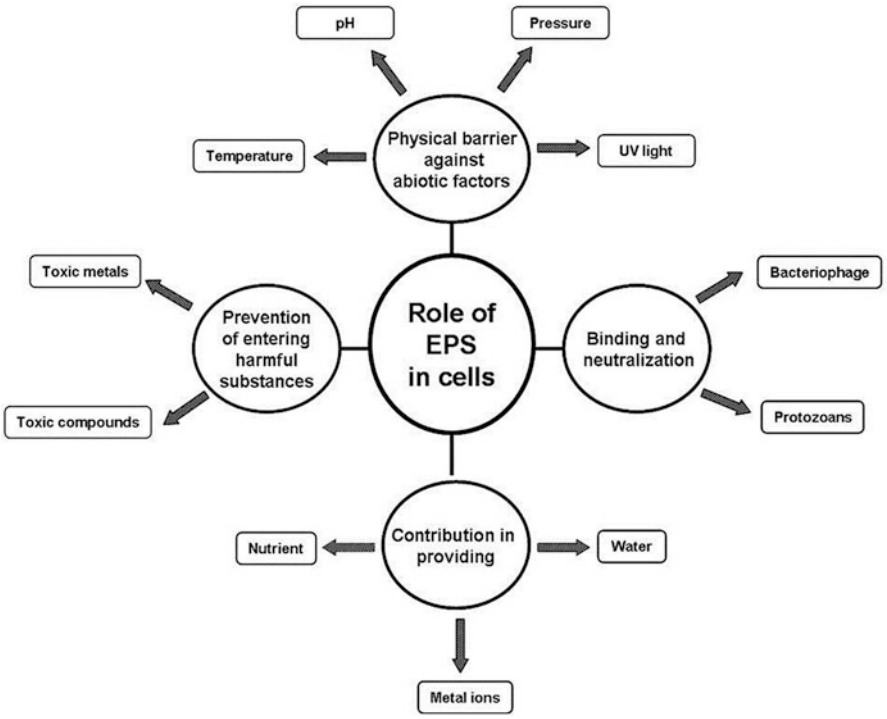

Fig. 4.2 Possible physiological roles of EPS in microbial cells that could contribute surviving in extreme conditions

affiliation and ecological niches (Poli et al. 2011; Nwodo et al. 2012). Despite the fact that the EPS production requires energy representing up to 70 % of total energy reserve, its impact in microbial growth is significantly higher and depends on the environment. Some of the functions are shown below (Fig. 4.2):

- EPSs protect microorganisms as a general physical barrier. Their production reflects selective environmental pressures including osmotic stress, temperature, pH, atmospheric pressure and light intensity and aid in adapting to extreme conditions (Otero and Vincenzini 2003).
- Surrounding of cell by EPS allows retention of water in water-deficient environments maintaining a hydrated microenvironment.
- At oligotrophic conditions like many of the extreme environments are EPSs can sequester nutrient materials from the surrounding environment EPS matrix binds and accumulates biodegradable compounds and cations from the bulk water phase. The anionic nature of the exterior polysaccharides due to the presence of sulphates and uronic acids residues can help interaction with cations such as metals and capture essential minerals and nutrients.
- EPSs may aid the organisms to adhere to surfaces and can serve as flocculants or emulsifiers. Many microorganisms use the synthesis of exopolysaccharides as a

strategy for growing and adhering to solid surfaces by covering the hydrophobic sites on the cell envelope.

- The presence of EPS layer around the cell may affect the diffusion of harmful substances, like antibiotics or toxic compounds (e.g. toxic metal ions, sulphur dioxide, and ethanol) into and out of the cell. Due to their anionic character, the polysaccharides can enhance the immobilization of ions such as Pb^{2+} and Cu^{2+} with a significant ecological impact. EPSs participate in the removal of heavy metals from the environment by flocculation and binding metal ions from solutions (Nicolaus et al. 2010). The polymer from a thermophilic bacterium *Geobacillus tepidamans* was proved to have an anti-cytotoxic activity against avarol (Kambourova et al. 2009).
- Participation of EPSs in cell protection is referred to binding and neutralizing bacteriophage (Vu et al. 2009).
- Although it is commonly accepted that EPSs are not used as energy reserves, and microorganisms are unable to degrade their own EPSs (Donot et al. 2012), EPSs produced by some hyper thermophilic species *(Sulfolobus*, *Thermococcus*, and *Thermotoga)* can act indirectly as extracellular storage polymers in extreme environments poor of other organic source (Nicolaus et al. 1993; Rinker and Kelly 2000).

2.1 Biofilms

In natural environment, matrix of extracellular polymeric substances synthesized by most bacteria comprises surface-associated communities and its integrity is essential for their survival (Moons et al. 2009). Biofilm is involved in the protective role to environmental stress, in adherence of biofilms to surfaces, in cell-cell interactions. Induction of biofilm formation by elevated pH, decreased and increased growth temperature, high salt, and exposure to UV light, oxygen, or antibiotic in *Archaeoglobus fulgidus* and ammonium chloride in *Thermococcus litoralis* demonstrated their protective role (Pysz et al. 2004). Such a way the biofilms create specific microenvironments, and could expand the limits of microbial growth creating more favorable conditions for life (Lowell et al. 2008). Multilayered biofilm structures consist of EPSs, extracellular DNAs (Flemming et al. 2007). Exopolysaccharides are the basis for biofilms, which have been observed in pure cultures or co-cultures. Although many thermophiles have been isolated from hot springs, still the reports of their biofilm EPSs formation are scared. The mechanism of biofilm formation and its importance for microbial survival in natural habitats has attracted increasing interest in recent years. Quorum sensing participation in interspecies interaction was demonstrated by participation of several GGDEF domain proteins in conjunction with a quorum sensing peptide TM0504 in co-cultivation of hyperthermophilic bacterium *Thermotoga maritima* with a methanogenic archaea *Methanococcus jannaschii* (Muralidharan et al. 1997).

3 Thermophilic EPS-Producing Prokaryotes

Synthesis of EPSs is in response to adaptation to an extreme environment, biotic stress (e.g., inter and intra species competition for substrate, water or growth factors), abiotic stress factors (e.g., temperature, light intensity, pH, salinity) (Donot et al. 2012). Thermophilic microorganisms could be found in almost every phylum of *Archaea* and *Bacteria*.

3.1 Bacterial Producers

Thermophilic bacteria belonging to obligate thermophilic genera *Bacillus, Geobacillus, Brevibacillus,* and *Aeribacillus,* extremely thermophilic genus *Thermus* and hyperthermophilic genus *Thermotoga* were reported as good thermophilic producers of EPSs. They were isolated from continental hot springs or shallow marine vents (Table 4.1). Hyperthermophilic bacterial species *Thermotoga maritima* is isolated originally from geothermally heated sea floor (Huber et al. 1986). This microorganism is strictly anaerobic heterotroph with optimal growth temperature of 80 °C. As microorganisms seldom behave in an isolated manner, mutually beneficial relationship probably expands the scope of natural hydrothermal environments (Kolter and Losick 1998). Accumulation of H_2 by methanogens is a preposition for often observed co-cultures of heterotrophs and methanogens in natural environments (Muralidharan et al. 1997). The co-cultivation of methanogenic archaea *Methanococcus jannaschii* and extremely thermophilic fermentative anaerobic bacteria *Thermotoga maritima* results in increasing the cell density. A novel extracellular polysaccharide from the biofilm of *Thermus aquaticus* YT-1 was isolated (Lin et al. 2011).

Despite of the enormous exploration of thermophilic bacilli as sources for thermostable enzymes, the knowledge on their ability to produce EPSs is still in its childhood. *Bacillus licheniformis* B3-15 and T14 (Maugeri et al. 2002; Spanò et al. 2013) and strains belonging to the genus *Geobacillus* isolated from shallow hydrothermal vents and terrestrial geothermal springs (Manca et al. 1996; Nicolaus et al. 2000, 2002, 2003, 2004; Kambourova et al. 2009) were described as producers of EPSs. Two different EPSs were produced by *Aeribacillus pallidus* 418 (Fig. 4.3) (Radchenkova et al. 2013).

3.2 Archaea

Discovery of extremophilic archaea has had a great significance to biocatalysis, as their enzymes allow improvements in multiple sectors of industry (Węgrzyn and Żukrowski 2014). Another type of biopolymer from *Archaea* with a potential impact for biotechnological industry is EPS.

Table 4.1 Exopolysaccharide production by thermophilic prokaryotes

Domain	Microorganism	Isolation source	Growth temperature (°C)	Carbon source	Maximum yield (mg/L)	Functional properties and potential application	Reference
Bacteria	*Thermus aquaticus* YT-1	Hot spring, Yellowstone, US	60	n.d.	n.d.	Immunomodulatory effects	Lin et al. (2011)
	Thermotoga maritima	Geothermal heated marine sediment at Vulcano, Italy	88	Maltose	120	n.d.	Rinker and Kelly (2000)
	Geobacillus thermoantarcticus	Crater of Mount Melbourne, Antarctica	65	Mannose	400	n.d.	Manca et al. (1996); Nicolaus et al. (2004)
	Bacillus licheniformis B3-15	Shallow marine hot spring, Vulcano, Italy	45	Glucose	165	Antiviral and immunoregulatory effects	Maugeri et al. (2002); Arena et al. (2006)
	Bacillus licheniformis T14	Shallow hydrothermal vent, Panarea Island, Italy	50	Sucrose	366	Antiviral and immunomodulatory effects	Spanò et al. (2013); Gugliandolo et al. (2014)
	Geobacillus sp. 4001	Shallow marine hydrothermal vents of Flegrean Ares, Italy	65	Galactose, sucrose	55	n.d.	Nicolaus et al. (2002); Nicolaus et al. (2004)
	Geobacillus sp. 4004	Shallow marine hydrothermal vents of Flegrean Ares, Italy	65	Trehalose, sucrose	65	n.d.	Nicolaus et al. (2002); Nicolaus et al. (2004)
	Geobacillus thermodenitrificans B3-72	Shallow hydrothermal vent, Vulcano island, Italy	65	Glucose, sucrose	70	EPS2: antiviral and immunomodulatory effects	Nicolaus et al. (2000); Arena et al. (2009)
	Geobacillus tepidamans V264	Hot spring, Velingrad	60	Maltose	111.4	High thermostability; anti-cytotoxic activity	Kambourova et al. (2009)
	Aeribacillus pallidus 418	Hot spring, Rupi basin, Bulgaria	55	Maltose	170	High thermostability; emulsifying properties	Radchenkova et al. (2013); Radchenkova et al. (2014)
	Brevibacillus thermoruber 423	Hot spring, Blagoevgrad region, Bulgaria	55	Maltose	897	Biomedical applications	Yasar Yildiz et al. (2014)

Archaea	*Thermococcus litoralis*	Shallow marine thermal spring, Naples, Italy	88	Maltose	180	Production of a mannan-like compound typically produced by eukaryotes	Rinker and Kelly (1996); Rinker and Kelly (2000)
	Sulfolobus solfataricus MT4	Hot acidic spring, Agnano, Italy	88	Glucose	8.4	n.d.	Nicolaus et al. (1993)
	Sulfolobus solfataricus MT3	Hot acidic spring, Agnano, Italy	75	Glucose	7	n.d.	Nicolaus et al. (1993)
	Sulfolobus tokodaii	Beppu hot springs, Japan	76	n.d.	n.d.	n.d.	Koerdt et al. (2010)
	Sulfolobus acidocaldarius	Hot spring, Yellowstone, US	76	n.d.	n.d.	n.d.	Koerdt et al. (2010)

n.d. no data

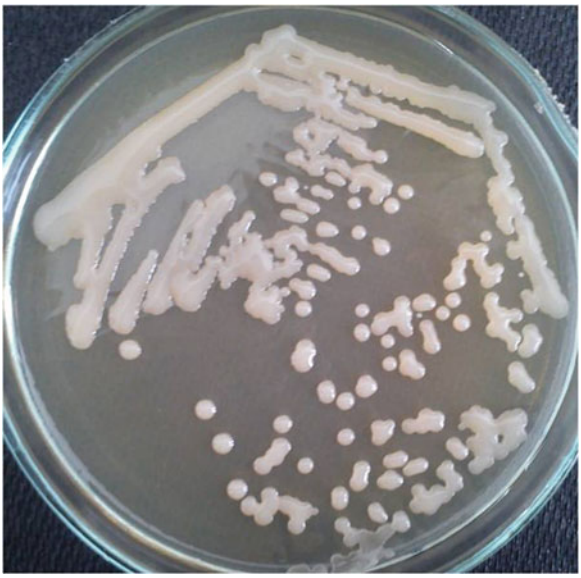

Fig. 4.3 Slime production by an obligate thermophile *Aeribacillus pallidus* 418 on peptone-yeast extract agar medium, 24 h culture

Archaea representatives are often observed component of biofilm communities from many different environments (Krüger et al. 2008; Zhang et al. 2008), but few studies report on biofilm formation by *Archaea*. The ability of *Sulfolobus solfataricus* to produce EPS was first studied more than 20 years ago (Nicolaus et al. 1993) and further *Sulfolobus acidocaldarius* and *Sulfolobus tokodaii* were reported to form biofilm (Koerdt et al. 2010). Since similar biofilms were observed for *Archaeoglobus profundus*, *Archaeoglobus fulgidus* (Lapaglia and Hartzell 1997), *Thermococcus litoralis* (Rinker and Kelly 1996), *Methanococcus jannaschii*, (Lapaglia and Hartzell 1997) and *Methanothermobacter thermoautotrophicus* (Thoma et al. 2008), the biofilm formation might be a common stress response mechanism among the *Archaea* (Hartzell et al. 1999).

Representatives of a genus *Sulfolobus* (phylum *Crenarchaeota*) exist all over the world in acidic, mostly muddy, hot springs in which the sharp variations in temperature, pH and geochemical conditions are often observed suggesting the need in a quick adaptation or survive undisturbed the changing conditions (Koerdt et al. 2010).

EPS production is not limited to *Crenarchaeota*; some species belonging to the phylum *Euryarchaeota* have this ability too. The cells of euryarchaeon *Pyrococcus furiosus* (notable for its optimum growth temperature of 100 °C and often used as a model organism for hyperthermophiles) are interconnected by flagella in microcolonies in biofilm-like structures (Näther et al. 2006). This microorganism was able to adhere also to biotic surfaces; it was proved to form biofilm in co-culture with another hyperthermophilic euryarchaeon, *Methanopyrus kandleri* habituating similar niches (temperature close to that of boiling water and anaerobic conditions). *Pyrococcus furiosus* and *Methanopyrus kandleri* cells were able to form archaeal

bi-species biofilm under laboratory conditions in less than 24 h (Schopf et al. 2008). The heterotrophic facultative sulfur-dependent hyperthermophile *Thermococcus litoralis* (*Euryarchaeota*) isolated from shallow marine thermal spring also formed a biofilm on hydrophilic surfaces under a variety of conditions. Baker-Austin et al. (2010) observed two distinct biofilm morphologies in the extremely acidophilic *Euryarchaeote, Ferroplasma acidarmanus*. The ability of an anaerobic marine hyperthermophilic euryarchaeon *Archaeoglobus fulgidus* to colonize widely separated areas was related to biofilm formation as a stress mechanism for surviving variations in conditions like concentrations of nutrients, temperature, and potentially toxic compounds (Lapaglia and Hartzell 1997).

4 Chemical and Structural Composition of Thermophilic Exopolysaccharides

In contrast to plant polysaccharides, accumulated knowledge on the structural properties of bacterial exopolysaccharides and especially EPSs from thermophilic microorganisms is scarce. The EPSs synthesized by microbial cells are characterized by different composition and hence, different chemical and physical properties. Comparison the data for carbohydrate structures of mammals and bacteria accumulated in multiple databases showed diversity in bacteria that is 10 times higher for monosaccharides and 9 times higher for glycosidic bonds compared to those reported from mammals (Herget et al. 2008). Most EPSs are heteropolysaccharides consisting of three or four different monosaccharides forming groups of 10 or less to form the repeating units (Poli et al. 2011). The most commonly monosaccharides are linked in the backbones by strong 1,4-β-, 1,3-β-, or 2,6-β linkages and more flexible 1,2-α- or 1,6-α-linkages. Several linkages can occur at the same time in one polysaccharide. The repeating sugar units are mainly composed of glucose, galactose, mannose, uronic acids, N-acetyl glucosamine, N-acetyl galactosamine and rhamnose, in variable ratios. The composition and structure of the polysaccharides determine their primary conformation. Secondary configuration comprises aggregated helices; acyl substituents influence the transition from random coil to ordered helical aggregates (Sutherland 1994). The rigidity of glycosaminoglycans in EPS provides structural integrity of molecules and cells. Forming of random coils that tend to form helical aggregates by the acetyl groups of N-acetyl galactosamines in EPSs from *Thermus aquaticus* YT-1 was observed by Lin et al. (2011). Its presence contributes to a regular and stable structure. Often heteropolysaccharides contain non-sugar components like acetates, pyruvates, succinates, phosphates, sulphates, methyl esters, proteins, nucleic acids and lipids (Nicolaus et al. 2010) which components may play an important role, for example, the over-sulphation of EPSs can modify their biological activity (Courtois et al. 2014). Some EPSs are neutral macromolecules, polyanionic nature of many EPSs due to uronic acids or ketal-linked pyruvate or inorganic residues in the molecule (Nicolaus et al. 2010).

The polysaccharides from *Thermococcus litoralis* and *Geobacillus thermoantarcticus* are homopolymers composed by mannose (Rinker and Kelly 1996; Manca et al. 1996). Almost pure glucan is EPS synthesized by *Geobacillus tepidamans* (Kambourova et al. 2009).

The main sugar for most of the heteropolysaccharides synthesized by thermophilic microorganisms is glucose or mannose; however also fructose or galactose was reported (Table 4.2). Three *Geobacillus* strains isolated from shallow marine vent is reported to produce EPSs with different composition (Nicolaus et al. 2003). EPSs isolated from two *Geobacillus* sp. strains contained as main sugars glucose, galactose and mannose in different proportions and the third strain from the same genus contained glucosamine and arabinose together with galactose and mannose. EPS from the thermophilic bacterium *Brevibacillus thermoruber* 423 is composed by five different sugars (with glucose as a major monomer unit) (Yasar Yildiz et al. 2014). *Aeribacillus pallidus* 418 produced two high molecular weight EPSs consisting of high variety of sugars (six for EPS 1 and seven for EPS 2) with mannose as a major component (Radchenkova et al. 2013). Recently, *Aeribacillus pallidus* YM-1 was reported to produce a novel bioemulsifier consisting of lipids (47.6 %), carbohydrates (41.1 %), and proteins (11.3 %) (Zheng et al. 2012). Carbohydrate fraction consisted of glucose (36.6 %), altrose (30.9 %), mannose (24.4 %) and galactose (8.1 %).

A presence of pyruvate and sulphate for EPS produced by *Bacillus thermantarticus* was reported (Nicolaus et al. 2004). Lin et al. (2011) characterized the primary structure of TA-1, the major EPS secreted by *Thermus aquaticus*. The polymer consists of tetrasaccharide-repeating units of galactofuranose, galactopyranose, and N-acetylgalactosamine and lacks acidic sugars. Five per cent of the dry mass in the aggregates of *Thermotoga maritima* was polysaccharide consisting of 91.2 % glucose, 5.2 % ribose and 2.7 % mannose (Johnson et al. 2005). *Sulfolobus* strains produce extracellular polysaccharides containing mannose, glucose, galactose, and N-acetylglucosamine (Koerdt et al. 2010).

Another feature of EPSs from all reported thermophiles seems to be their high molecular weight (beginning from several hundred kDa). A molecular weight of 380, 400, 600 and 1000 kDa was reported for EPSs from thermophilic bacilli (Nicolaus et al. 2003). The molecular weight was determined to be about 700 and 1000 kDa for EPSs from *Aeribacillus pallidus* 418 (Radchenkova et al. 2013). EPS from another thermophile, *Geobacillus thermoantarcticus*, had a molecular weight approximately 300 kDa (Manca et al. 1996). The molecular weight of the polymer from *Geobacillus tepidamans* V264 was higher than 1000 kDa (Kambourova et al. 2009). According to Kumar et al. (2007) mesophilic bacteria synthesize polysaccharides with molecular weight of 10–30 kDa. These values are significantly lower than the established ones for thermophilic bacteria. High molecular weight was announced for some EPSs from mesophiles, like EPSs excreted by *Alteromonas macleodii* (300 and 1500 kDa) (Raguénès et al. 1996, 2003); alginates produced by *Pseudomonas* species (34–500 kDa) (Conti et al. 1994), gellan (250 and 490 kDa) (Milas et al. 1990). EPSs from lactobacilli are also characterized by a different molecular weight, from 10 to 1000 kDa according to Patel et al. (2010) and from 1000 to 5000 kDa according to Kralj et al. (2004).

Table 4.2 Properties of exopolysaccharide synthesized by thermophilic microorganisms

Domain	Microorganism	Growth temperature (°C)	Carbohydrate and protein content	Molecular weight (Da)	EPS composition	Reference
Bacteria	*Thermus aquaticus* YT-1	60	n.d.	500,000	Galactofuranose/ galactopyranose/N-acetylgalactosamine (1:1:2)	Lin et al. (2011)
	Thermotoga maritima (Co-cultivation with *Methanococcus jannaschii*)	80	5 % carbohydrate	n.d.	Glucose/ribose/mannose (1:0.06:0.03)	Johnson et al. (2005)
	Geobacillus thermoantarcticus	65	96 % carbohydrate 0.2 % protein	EPS1: 300,000 EPS2: 300,000	EPS1: mannose/glucose (1.0:0.7); tEPS2: mannose/glucose (1:tr)	Manca et al. (1996; Nicolaus et al. (2004)
	Bacillus licheniformis B3-15	45	66 % carbohydrate 5 % protein	600,000	Mannose/glucose (1:3)	Maugeri et al. (2002)
	Bacillus licheniformis T14	50	99 % carbohydrate 1.2 % protein	1,000,000	Fructose/fucose/glucose/galactosamine/ mannose (1.0:0.75:0.28:tr:tr)	Spanò et al. (2013);
	Geobacillus sp. 4001	65	81 % carbohydrate 7 % protein	380,000	Mannose/glucose/galactose/ mannosamine (1:0.1:tr:tr)	Nicolaus et al. (2002); Nicolaus et al. (2004)
	Geobacillus sp. 4004	65	65 % carbohydrate 2.6 % protein	>1,000,000	Galactose/mannose/glucosamine/ arabinose (1:0.8:0.4:0.2)	Nicolaus et al. (2002); Nicolaus et al. (2004)
	Geobacillus thermodenitrificans B3-72	65	80 % carbohydrate 3 % protein	EPS2: 400,000	EPS1: mannose/glucose (0.3:1) EPS2: mannose/glucose (1:0.2)	Nicolaus et al. (2000)
	Geobacillus tepidamans V264	60	100 % carbohydrate	>1,000,000	Glucose/galactose/fucose/fructose (1:0.07:0.04:0.02)	Kambourova et al. (2009)

(continued)

Table 4.2 (continued)

Domain	Microorganism	Growth temperature (°C)	Carbohydrate and protein content	Molecular weight (Da)	EPS composition	Reference
	Aeribacillus pallidus 418	55	80.5 % carbohydrate 19 % protein	EPS1: 700,000 EPS2: 1,000,000	EPS1: mannose/glucose/galactosamine/ glucosamine/galactose/ribose (1:0.16:0 .1:0.09:0.07:0.06:0.04) EPS2: mannose/galactose/glucose galactosamine/glucosamine/ribose/ arabinose (1:0.5:0.46:0.35:0.24:0.16:0. 14)	Radchenkova et al. (2013)
	Brevibacillus thermoruber 423	55	92 % carbohydrate 6 % protein	n.d.	Glucose galactose galactosamine mannose mannosamine (1:0.3:0.25:0.16:0.04)	Yasar Yildiz et al. (2014)
Archaea	*Thermococcus litoralis*	88	n.d.	41,000	Mannose	Rinker and Kelly (1996)
	Sulfolobus solfataricus MT4	88	75 % carbohydrate 0.1 % protein	n.d.	Glucose/mannose/glucosamine/ galactose (1.2:1.0:0.18:0.13)	Nicolaus et al. (1993)
	Sulfolobus solfataricus MT3	75	75 % carbohydrate 0.1 % protein	n.d.	Glucose/mannose/glucosamine/ galactose (1.2:1.0:0.77:0.73)	Nicolaus et al. (1993)
	Sulfolobus tokodaii	76	n.d.	n.d.	Mannose/glucose/ galactose/N-acetylglucosamine	Koerdt et al. (2010)
	Sulfolobus acidocaldarius	76	n.d.	n.d.	Mannose/glucose/ galactose/N-acetylglucosamine	Koerdt et al. (2010)

n.d. no data

EPSs from thermophilic bacteria were thermostable, with the highest thermostability reported for the polymers from *Geobacillus tepidamans* V264 (280 °C) (Kambourova et al. 2009), *Geobacillus thermodenitrificans* strain B3-72 (240 °C) (Arena et al. 2006) and *Bacillus licheniformis* (240 °C) (Spanò et al. 2013). The information concerning thermostability of exopolysaccharides from mesophilic bacteria is very scared. A moderately halophilic bacterium was described to produce highly thermostable exopolysaccharide (melting point at 207 °C) (Cojoc et al. 2009).

5 Specific Conditions for EPS Synthesis by Thermophiles

Increasing in the yield of bacterial EPSs traditionally is achieved by strain selection and/or optimization of cultivation conditions, however each given bacterium has physiological limits that could be difficult to overcome (Freitas et al. 2011a). Regulation of biosynthesis of bacterial EPSs is a complex process as a large number of enzymes and regulatory proteins are involved (Jaiswal et al. 2014). Despite the structural diversity of EPSs, four mechanisms are known in bacteria for the polymerization, namely, extracellular biosynthesis, synthase dependent biosynthesis, ABC-transporter dependent and the most commonly used wzx/wzy- dependent pathways. The mechanism of EPS biosynthesis involving sugar nucleotide synthesis, repeating unit synthesis, and polymerization of the repeating units was well studied in mesophilic microorganisms (De Vuyst et al. 2001; Freitas et al. 2011b). Recently a hypothetical mechanism for sugar uptake in EPS biosynthesis similar to those involved in the synthesis of polysaccharides from mesophiles was suggested for the thermophile *Brevibacillus thermoruber* 423 (Yildiz et al. 2015). Essential genes associated with EPS biosynthesis were detected by genome annotation and the biosynthesis of NDP-sugars was shown. Genome information revealed the presence of ABC-transporter dependent pathway (Bth.peg.2228, Bth.peg.4273, Bth.peg.3612, Bth.peg.3618, Bth.peg.4275) in EPS biosynthesis by *Brevibacillus thermoruber* 423. In addition, an exopolysaccharide synthesis pathway in the hyperthermophilic bacterium *Thermotoga maritima* was identified (Johnson et al. 2005). Transcriptional analysis of exopolysaccharide formation by this microorganism in a co-culture with *Methanococcus jannaschii* showed a strong upregulation of a gene, encoding a polypeptide. This polypeptide contains a motif found in peptide-signalling molecules in mesophilic bacteria. Characterization of the complete 15 kb St Sfi6 eps gene cluster of *Streptococcus thermophilus* revealed high degree of similarities among the products and known glycosyltransferases and their potential role in the synthesis of the repeating monomer was suggested (Delcour et al. 2000). The genes which encode the proteins or enzyme required for the biosynthesis of EPS are located on chromosomes in those which are thermopiles (Yildiz et al. 2015) and on plasmid in most lactic acid bacteria (Laws et al. 2001). In contrast, a lack of plasmids encoding components required for slime production was observed for thermophilic LAB *Streptococcus thermophilus* (Harutoshi 2013) and a location of

the EPS gene cluster in chromosomal DNA was reported for *Lactobacillus fermentum* TDS030603 (Dan et al. 2009).

Despite of the fact that the composition and the amount of microbial EPSs are genetically determined, they also depends of several factors, such as type of strain, carbon and nitrogen sources, mineral salts, trace elements, the medium component ratio, fermentation conditions (temperature, pH, agitation and aeration (Nicolaus et al. 2010). The synthesis of EPS by microbial cells basically depends on the carbon and nitrogen availability in the culture medium. EPS producing microorganisms utilize sugars as their carbon and energy source; ammonium salts and amino acids are their source of nitrogen (Gandhi et al. 1997; Czaczyk and Wojciechowska 2003). Usually lower cost sugars like glucose, maltose or sucrose were used as a carbon source despite the fact that in some cases higher production was observed in a presence of other sugars. The polymer production was lower in a medium containing glucose in comparison with maltose in the case of several thermophilic producers (Rinker and Kelly 2000; Kambourova et al. 2009; Radchenkova et al. 2013; Yasar Yildiz et al. 2014). The increased production in abundance of carbon source and minimal nitrogen was reported by several authors (Radchenkova et al. 2013; Yasar Yildiz et al. 2014). The addition of extra nitrogen favors the biomass production but diminishes EPS production. Production may or may not be growth associated. EPS production is growth associated for *Aeribacillus pallidus* 418 (Radchenkova et al. 2013), *Brevibacillus thermoruber* 423 (Yasar Yildiz et al. 2014), *Geobacillus tepidamans* (Kambourova et al. 2009) and differed from the reports for some mesophilic producers synthesizing EPS during the whole stationary phase (Conti et al. 1994; Raguénès et al. 1997).

Oxygen is a key substrate in aerobic bioprocesses for EPS production, whose continuous supply should be ensured because of its low solubility in broths. Product formation by aerobic thermophiles could be increased by optimization of agitation and aeration rates. The size of gas bubbles and their dispersion throughout the reactor volume are critical for its performance. The smaller is the bubble size the larger is the surface area for gas contact that could improve oxygen transfer rate. Low solubility of oxygen in the medium especially at enhanced temperatures of thermophilic processes often determines the oxygen (air) transfer as a rate-limiting step in the aerobic bioprocess. High temperature sharply decreases oxygen solubility, one of the most important parameter in massive EPS production in bioreactors. Oxygen transfer in bioreactors is an object of investigations by many authors, especially in recent years (Garcia-Ochoa and Gomez 2009). However, the information concerning optimization of agitation and aeration conditions in thermophilic processes for EPS production is still very scarce. Aeration and agitation were proven to be crucial for attaining maximum productivity in microbial aerobic processes by *Aeribacillus pallidus* 418 (Radchenkova et al. 2014). According to some authors (Rau et al. 1992; Radchenkova et al. 2014), dissolved oxygen limitation is a desirable condition for enhanced polymer production. It is well known that one of the physiological roles of EPSs is cell adaptation to unfavorable conditions; for extremophiles it could

serve as enhancer for bacterial survival at severe conditions of extreme niches (Nicolaus et al. 2010). A short oxygen limitation could provoke EPS synthesis as a cell response to this limitation. In stirred bioreactors, a high number of variables like stirrer speed, type and number of stirrers, gas flow rate influence mixing and mass transfer (Garcia-Ochoa and Gomez 2009). A number of impellers have been reported to ensure uniform distribution of substrates and high heat and mass transfer rates in polysaccharide-producing broths (Chhabra 2003). Radial flow turbines type Rushton are very popular in the equipment of the most laboratory scale stirred tank systems, changing a shear stress on the medium. Study on the influence of agitation and aeration revealed that both parameters influenced specific EPS production (Radchenkova et al. 2014) however agitation was much more effective than aeration.

Anaerobic processes for EPS production by hyperthermophiles often do not need even in agitation as EPS is accumulated as a biofilm. Sufficient biofilm formation on nylon mesh by continuous cultures of *Thermotoga maritima* was obtained in anaerobic chemostat at $D = 0.25\ h^{-1}$ (Pysz et al. 2004). Batch and continuous cultures without agitation and at gas sparging were used to compare specific physiological features in EPS synthesis by the hyperthermophilic archaeon *Thermococcus litoralis* and hyperthermophilic bacterium *Thermotoga maritima* (Rinker and Kelly 2000) or to optimize growth of *Thermococcus hydrothermalis* (Postec et al. 2005). In other cases, low rate of agitation was reported as optimal, 100 rpm for *Thermotoga maritima* (Johnson et al. 2005), low mechanical agitation for two thermophilic archaea belonging to the genus *Sulfolobus* (Nicolaus et al. 1993).

Cultivation of thermophiles in bioreactors is characterized by some features. The need in good aeration and agitation is determined by the lower solubility of oxygen in higher temperature, although the oxygen transfer coefficient increases (Shih and Pan 2011; Kennes and Veiga 2013). Another disadvantage is the high investment cost required for the compressors and heat exchangers in thermo-reactor (Van Groenestijn et al. 2002). Higher temperature results in higher evaporation heat and this heat needs to be recovered. Sharp and Raven (1997) recommended bioreactors made of stainless steel with added nickel, molybdenum or chrome or with a teflon coating to prevent corrosion caused by high temperatures, salt concentrations and sulfide. In the case of anaerobic membrane bioreactor ceramic membranes are used at thermophilic conditions due to their thermal stability and long lifetime (Abeynayaka and Visvanathan 2011).

The observed levels of EPS production by thermophilic producers are lower than those reported for mesophilic producers and usually varied in the range 50–200 μg mL^{-1} (Kambourova et al. 2009; Manca et al. 1996; Nicolaus et al. 2003). A higher amount of EPS (366 μg/mL) has been reported for the facultative thermophile *Bacillus licheniformis* (Spanò et al. 2013) for 48 h cultivation at temperature of 50 °C in a complex medium. Highest production (863 μg/mL) in comparison with other thermophiles was reported for a thermophile *Brevibacillus thermoruber* 423 (Yasar Yildiz et al. 2014).

6 Biotechnological Potential of Exopolysaccharides from Thermophilic Microorganisms

Biotechnological applications of EPSs range from traditional areas as food, pharmaceutical and cosmetic industries to novel biomedicine areas. New microbial polysaccharides could conquer the traditional polysaccharide market in the case of using cheap substrates, development of low costly downstream processes, better functional or novel properties. According to Belsito et al. (2012), 19 microbial polysaccharides are currently used in cosmetic formulations. None of them is produced by a thermophilic microorganism.

About 12 reports on EPS production by thermophiles, polysaccharide composition and properties are currently known. EPS synthesis by thermophilic microorganisms suggests some advantages like:

- Short fermentation processes (often lasting several hours) (Kambourova et al. 2009; Radchenkova et al. 2013; Yasar Yildiz et al. 2014) due to the high growth rate at elevated temperature and lower concentration of nutrient components**.**
- Good mass transfer at high temperature for cultivation (Turner et al. 2007; Kumar et al. 2011).
- Viscosity of culture liquid is lower at high temperature that suggests lower energy consumption (Haki and Rakshit 2003).
- Performance of processes at high temperature reduces the risk of contamination (Turner et al. 2007; Kikani et al. 2010; Xiao et al. 2015).
- Non pathogenic products from thermophiles are applicable in food and cosmetic industry (Nicolaus et al. 2010).
- EPSs synthesized by thermophilic bacteria and archaea are suggested to keep their emulsifying and rheological properties at high temperature, in which many processes in food industry are performed (Sajna et al. 2013)
- Usually thermostable molecules can remain effective even at extreme conditions of pH, temperature, and salinity due to their more rigid molecule.
- They form stable oil/water emulsions needed for cosmetic industry (Radchenkova et al. 2014).

Among the disadvantages of exploring hot spring potential are the low biomass and respectively low EPS synthesis by thermophiles (Krebs et al. 2014). Additionally, the proportion of microorganisms reluctant to cultivation-based approaches is very high in extreme environments (Lorenz et al. 2002). These disadvantages could be overcome to some extent by exploration of better producers and optimizing of cultivation conditions. Exciting prospects for increasing production yield are found in genetic engineering. The genetic manipulation of bacteria is much easier than that for higher organisms (Morris and Harding 2014). Genetic manipulation could tailor chemical composition and structure of EPS, which further determine their specific explorations.

Another disadvantage is some cases could be the high cost of the received product resulting from the used substrate. Substrate utilization is lower in thermophilic

processes than in mesophilic ones as the biomass yield is comparatively low. Use of agricultural waste or dairy waste could lead to reduction in EPS cost. The inherent costs of large-scale fermenters are significantly higher in comparison with simple extraction processes for plant polysaccharides however biotechnological advances in bacterial large-scale processes result in large EPS quantities and correspondingly lowering the cost of the product.

Comparatively low levels of EPS synthesis by thermophilic microorganisms and correspondingly higher production cost determine the interest for the development of microbial EPSs used in high-value market niches, where the desired properties or the degree of purity could not be suggested by the traditional polymers (Kumar et al. 2007). Novel biopolymers produced by thermophilic bacteria could suggest different chemical structure and correspondingly different physicochemical properties valuable for medical and pharmaceutical applications, especially for drug delivery, tissue engineering, as immunostimulatory, immunomodulatory, antitumor, antiviral, anti-inflammatory and antioxidant agents (Arena et al. 2009; Sam et al. 2011; Lee and Mooney 2012; Freitas et al. 2014).

Over the past several years, anti-tumour activity of microbial exopolysaccharides was reported by several authors (Khalikova et al. 2006; Nwodo et al. 2012). Marine bacterium isolated from hydrothermal vent was shown to produce new EPS (Courtois et al. 2014). In its native or over-sulphated form it modulated the complement system suggesting an effective treatment of diseases caused by deregulation of the immune system and overactivation of the complement system. A dose-dependent immunomodulatory and antiviral effects of extracellular polysaccharides, produced by *Bacillus licheniformis* strain T14 and *Geobacillus thermodenitrificans* strain B3-72 were proven and a partial restoration of immunological disorders after treatment with this EPS was observed (Arena et al. 2006, 2009). The novel EPS1-T14 was able to act as immunomodulator inhibiting herpes simplex virus type 2 (HSV-2) replication by triggering the production of Th1-type cytokines (Gugliandolo et al. 2014). Interesting chemical and rheological characteristics were reported for a new fucose containing EPS from *Bacillus licheniformis* strain T14 isolated from Panarea Island (Spanò et al. 2013).

A novel extracellular polysaccharide TA-1 from *Thermus aquaticus* YT-1, stimulated macrophage cells to produce the cytokines, which increases the immune response (Lin et al. 2011). D-galactofuranose residues in the novel TA-1 are probably responsible for TA-1 immunoregulatory activity within macrophages, the first line of host defense against bacterial infection. EPS from *Aeribacillus pallidus* 418 has a potential application in cosmetic industry due to its good emulsifying properties (Radchenkova et al. 2014). Properties of EPSs can be changed dramatically using mixtures with other biopolymers. A stable emulsion, especially valuable for cosmetic industry was received as a result of synergistic action of EPS from *Aeribacillus pallidus* 418 with xanthan (Radchenkova et al. 2014).

As many of the food production processes run at elevated temperature thermostability of exopolysaccharides synthesized by thermophiles is an important characteristic for their industrial applications (Sajna et al. 2013). High temperature of destruction of these EPSs suggests easy preparation of emulsions for different food

and cosmetic creams at higher temperature where viscosity is lower and mixing is easier. It is also a preposition for long term preservation of the received products even at room temperature. The solutions of the thermostable EPSs are able to maintain high viscosity at high temperature of oil drilling fluids. They could have a great potential as flocculating agents in the thermophilic processes of municipal and wastewater treatment.

Investigations on exopolysaccharides from thermophilic microorganisms revealed their interesting properties like high molecular weight suggesting good viscosity; stability of the molecules in harsh industrial conditions, good emulsifying properties and good synergism, biological activity against cytotoxic compounds, antiviral and immunomodulating activities suggesting the potential of EPSs from thermophilic microorganisms in several biotechnological and biomedical processes.

Current review gathered the information on EPS synthesis by thermophilic microorganisms, industrially valuable properties of the novel biopolymers and their biological activities in trends for possible potential future applications.

Conflict of Interest Margarita Kambourova, Nadja Radchenkova, Iva Tomova, and Ivanka Bojadjieva declare that they have no conflict of interest.

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