Exopolysaccharide-Producing Microorganisms from Extreme Areas: Chemistry and Application

15

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15.1 Introduction

EPS-producing microorganisms have been isolated from different natural sources of both aquatic and terrestrial environments, like freshwater, marine water, wastewater, soils, biofilms and also extreme niches such as hot springs, cold environments, hypersaline and halophilic environments, salt lakes and salterns (Maugeri et al. 2002; Nichols et al. 2005a; Mata et al. 2006; Poli et al. 2007; Satpute et al. 2010; Poli et al. 2010; Andersson et al. 2011; Nicolaus et al. 2016).

Extreme environments, generally characterized by atypical temperatures, pH, pressure, salinity, toxicity and radiation levels, are inhabited by various microorganisms specifically adapted to these particular conditions.

These extreme environments have been identified as an important source of bacteria, archaea, algae and fungi with interesting applications, and the organisms, living there, have developed different strategies to cope with adverse living conditions, and the production of EPSs is a frequent survival strategy (Nicolaus et al. 2004; Nichols et al. 2005a). For example, bacteria living in extreme marine environments such as those found in the cold waters of polar regions, in ocean trenches or in deep-sea hydrothermal vents often use EPSs as an efficient protective barrier (Nichols et al. 2005a).

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The protection conferred by EPSs in these hostile environments is achieved by the formation of biofilms to withstand high pressure and/or temperature or by decreasing the freezing point of water in the vicinity of the bacteria (Nichols et al. 2005a). Similar strategies are used by thermophilic bacteria found in terrestrial habitats (Lin et al. 2011).

Polysaccharides produced by microbes can be generally classified by their biological functions into intracellular storage polysaccharides (glycogen), capsular polysaccharides which are closely linked to the cell surface (e.g. K30 O-Antigen) and extracellular bacterial polysaccharides (e.g. xanthan, sphingan, alginate, cellulose, etc.) that are important for biofilm formation and pathogenicity. This article will focus on the latter, also termed EPS, which are secreted to the surrounding environment and therefore can be efficiently harvested from cell-free culture supernatant in a continuous and cost-effective manufacturing process (Schmid et al. 2015).

The capability to synthesize exopolysaccharides has been observed for microorganisms belonging to both Archaea and *Bacteria* domains and in all kind of extremophilic microorganisms, by means of thermophiles, halophiles, psychrophiles, acidophiles, anaerobes and so on (Poli et al. 2007; VanFossen et al. 2008; Michel et al. 2009; Radchenkova et al. 2013; Casillo et al. 2017; Poli et al. 2017).

Due to their many interesting physicochemical and rheological properties, these biopolymers possess novel functionality that is generally superior to petrochemical-derived polymers in aspects that embrace biodegradability and environmental and human compatibility. Consequently, biopolymers of extremophiles are widely used in foods, cosmetics, pharmaceutical products, textiles, detergents, adhesives, oil recovery from wells, brewing and waste treatment processes (Poli et al. 2009).

15.2 Ecological and Physiological Roles

The EPS production process resulted to be a physiological mechanism for some microbial genera, such as *Xanthomonas*, *Leuconostoc*, *Pseudomonas* and *Alcaligenes*, which synthesized xanthan, dextran, gellan and curdlan (Finore et al. 2014), but also a response to biotic and abiotic stress factors (Donot et al. 2012).

These biomolecules carried out an ecological role, allowing the bacteria to proliferate in stressful environmental conditions; by means of high or low values of temperature, high salt concentration and extreme of pHs; and in the presence of more stress factors simultaneously (Nicolaus et al. 2010; Finore et al. 2015; Poli et al. 2017).

Microorganisms synthesized and released out of the cell polymers for their survival. Therefore, the role of energy reservoir and defensive agent has been attributed to the EPS; in addition their production can influence the cell functioning, the osmotic regulation and the symbiosis and sustain the microorganism in all vital function, from the adaptation to the cell reproduction (Steinbüchel 2001; Vijayendra and Shamala 2014).

The EPS production process necessitated a conspicuous energy expenditure for the microorganism, up to 70% of carbon investment. Evidently, the advantage coming from the EPS production was much more higher with respect to their survival (Wolfaardt et al. 1999).

Hot niches hosted a wide variety of prokaryotic microorganisms. They represented an interesting source of many bioactive compounds, including exopolysaccharides. Thermophilic microorganisms proliferated in a wide range of temperature, from 122 °C of hyperthermophile *Methanopyrus* species (Takai et al. 2008) up to 50–60 °C of thermotolerant microorganisms. Thermophiles producing EPS have been isolated from both *Bacteria* (*Aeribacillus*, *Bacillus*, *Brevibacillus*, *Geobacillus*, *Thermotoga* and *Thermus*) and Archaea (*Sulfolobus* and *Thermococcus*) domains (Kambourova et al. 2016). The exopolymers surrounded the microbial cells by contributing to their survival: (a) the roles of protection against predators, (b) the energy and carbon source reservoir and (c) the regular nutrient uptake even in environments wherein they would tend to be dispersed. In particular, marine thermophiles, isolated from deep-sea hydrothermal vents, showed ability to grow in the presence of metal ions and toxic substances; this capability was derived from the presence of exopolysaccharides bound with high-affinity cations and trace metals (Loaëc et al. 1997).

Cold environments are distributed all over the world and are characterized by a low nutritive substance diffusion; psychrophiles and psychrotrophs are microbes that thrive in these places, and need or tolerate low temperature values, respectively. Their capability to proliferate in freezing niches is related to different cellular mechanisms, from membrane lipid compositions to the cold-stable RNA conformation up to exopolysaccharide synthesis (Poli et al. 2017). The high amount of polyhydroxyl groups of EPS decreased both the freezing point of water and the ice nucleation temperature (De Maayer et al. 2014). The EPSs assumed a gelatinous aspect in nature, playing a cryoprotection role, because they modified the immediate surrounding environment of the cell (McLean 1918; Ewert and Deming 2013).

Abundant amount of exopolysaccharides have been found both in Antarctic and Arctic marine bacteria and in all cold environment (Poli et al. 2017). These polymers altered the chemical parameters around the microbial organisms, contributing to the adhesion of cells to surfaces with water and nutrient sequester, improving their uptake. In addition, the EPS can preserve the extracellular enzymes against the freezing temperatures, avoiding their denaturation (D'Amico et al. 2006). The EPSs protected the cells from viral attacks and influence the osmosis (Deming and Young 2017).

The obligately marine and psychrophilic γ-proteobacterium, *Colwellia psychrerythraea* strain 34H, is reported as an EPS-producing bacterium. The production of EPS did not change over growth-permissive temperatures of ~10 to −4 °C, but from −8 to −14 °C when samples froze, EPS production rose dramatically. Moreover, in salinity tests at 10‰–100‰ (and −1 and 5 °C), EPS production also increased at the freshest salinity tested, and the strain 34H recovered best from deep-freezing to −80 °C if first supplemented with a preparation of its own EPS, rather than other cryoprotectants like glycerol. These results suggested that the EPS represented a

survival strategy of microorganisms in a harsh environment and an interesting compound with potentially properties for biotechnological application (Marx et al. 2009). In a following paper, the detailed molecular primary and secondary structures of capsular polysaccharide from *C. psychrerythraea* 34H cells were reported. The polysaccharide consisted of a tetrasaccharidic repeating unit containing two amino sugars and two uronic acids bearing threonine as substituent. The structural features of this EPS resemble those present in antifreeze proteins and glycoproteins. These results suggested a possible correlation between the capsule structure and the ability of *C. psychrerythraea* to colonize subfreezing marine environments and, more, confirmed the potential properties of this polymer (Carillo et al. 2015).

In literature have been reported many examples of halophilic microorganisms able to synthesize exopolysaccharides and this property has been linked to a specific regulation role in the presence of salts. The polymers around the microbial cell attenuated the physical stress due to the salinity. Many halophilic microorganisms possessed exopolysaccharides around the cell, for protecting membrane integrity (DasSarma and DasSarma 2001; Poli et al. 2010; Qurashi and Sabri 2012; Oren 2013). Halophilic Archaea producing EPS are *Haloarcula*, *Halococcus*, *Haloferax* and *Natronococcus* (Nicolaus et al. 2010). Also halophilic *Bacteria* are good producers of EPSs, for example, *Halomonas maura* produced mauran, an exopolysaccharide deeply investigated and with a wide commercial use (Arias et al. 2003).

15.3 Microbial Exopolysaccharides' Isolation, Purification and Structure Definition

Exopolysaccharides are produced as exocellular polymers that generally account for about 40% to 95% of the extracellular polymeric substances (Flemming and Wingender 2001). Exopolysaccharides can be dispersed in the biofilm matrix surrounding the cell, or they can be found as a discrete layer enveloping the cell: usually the polysaccharides belonging to the cell envelope of the bacteria are also referred to as capsular polysaccharides (CPSs) and lipopolysaccharides (LPSs), the latter being present only in Gram-negative bacteria. In general, the term EPS indicates the extracellular polysaccharide molecules that are not tightly bound to the cell surface but sloughed off to form slime, although the release of polysaccharides from the cell surface is not an absolute criterion to distinguish EPSs from the other carbohydrate capsular components (Roberts 1996).

Isolation of EPSs is a challenging task since these polymers are found embedded in a complex matrix also containing proteins and other biomolecules, i.e. the biofilm matrix. Therefore, the quantitative recovery of an EPS is very difficult to achieve because usually a fraction can remain bound to the cell and because the sample can be contaminated from intracellular materials released during isolation procedures after cell disruption. There is no single isolation and purification protocol generally efficient for EPS recovery; indeed the isolation procedure can change depending on the microbial source of EPSs.

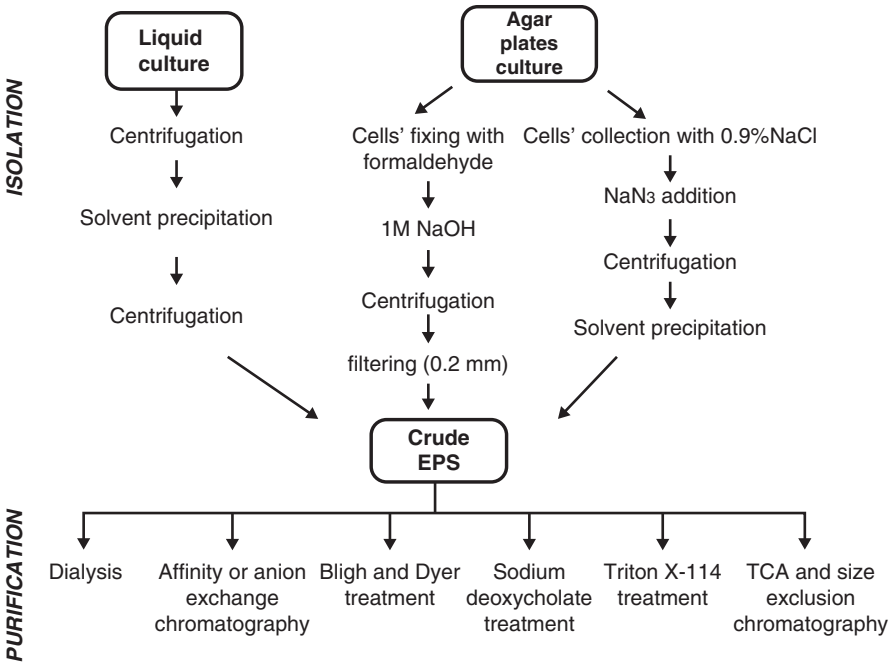

Fig. 15.1 General scheme of water-soluble EPSs' isolation and purification. (Partially adapted from Di Donato et al. (2016))

Isolation of water-soluble EPSs is usually implemented by cold ethanol precipitation, although also other solvents can be used, for example, acetone, isopropyl alcohol or methanol. The protocols to be adopted strongly depends on the bacterial growth method: indeed, in the case of static mode (seeding on agar plates), a previous step of cell's fixing or removal is required in order to avoid EPS contamination from endocellular molecules; then washing with an alkali solution and centrifugation will afford a crude EPS sample (Bales et al. 2013). Another strategy is represented by washing with NaCl solution and adding a bacteriostatic agent in order to preserve cell integrity; after cell removal by centrifugation, addition of solvent will precipitate the EPS for further purification. If a liquid culture has been implemented, the solvent precipitation has to be forerun by centrifugation to remove intact cells (Fig. 15.1) (Di Donato et al. 2016).

Dialysis is the classical method of EPSs' purification, although in order to remove contaminants, for example, LPSs, other methods can be used including chromatography; dissolution in 0.01 M EDTA followed by extraction with chloroform/methanol (Bligh and Dyer treatment); suspension in 0.05 M Tris HCl and addition of sodium deoxycholate followed by acidification with acetic acid (20%) and centrifugation to remove LPSs; dissolution in water or 0.01 M EDTA in the presence of Triton X-114, followed by addition of NaCl 2% w/v and cold ethanol to

precipitate the purified EPS (Du et al. 2017); or finally trichloroacetic acid (TCA) addition to remove nucleic acids followed by ethanol precipitation and then gel filtration chromatography for EPS final purification (Bales et al. 2013).

In the case of water-insoluble EPSs, for example, cellulose, isolation is carried out in harsh conditions, such as treatment with acetic acid and nitric acid at 95 °C or with NaOH at 80 °C, followed by washing with distilled water and neutralization acetic acid, thus achieving a purified polysaccharide (Rangaswamy et al. 2015).

The complete structural definition of the EPSs is carried out by means of chemical, analytical and spectral techniques. First of all, the gross chemical composition of a purified EPS is assessed by determination of the total carbohydrate content (DuBois et al. 1956), of the total protein content (Bradford 1976), of the nucleic acids and of the uronic acids (Spanò et al. 2013). The monosaccharide composition and the determination of linkage positions are carried out by hydrolysis of the polymer followed by liquid or gas chromatography. The hydrolysis is usually carried out by treatment in trifluoroacetic acid (TFA) at 110–120 °C followed by analysis of the resulting mixture by means of TLC or of high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD). The hydrolysed polymer can also be subjected to derivatization (by per-acetylation or silylation treatments), and in these cases, the identification of monomer sugars is carried out by means of gas chromatography (GC) analysis. The determination of linkage positions of sugars in the EPS is accomplished by methylation analysis (MA), i.e. treatment with methyl iodide followed by acidic hydrolysis, reduction and acetylation/silylation: the so-obtained volatile alditol acetates or methylsilanes are then identified by GC-MS. The chemical analysis is completed by determination of functional and substituting groups that is commonly implemented by means of Fourier-transform infrared spectroscopy (FTIR) or nuclear magnetic resonance (NMR) (Mishra and Jha 2013). The spectral NMR and FTIR techniques are useful to confirm the chemical composition of the EPSs (determination of the number and the type of monomer sugar residues identified by chemical degradation), but they also allow to gain other fundamental information like the anomeric configurations of the monosaccharides and their sequence in the polymer backbone.

NMR spectroscopy is a useful tool for the determination of EPSs' backbone composition and conformation; indeed thanks to 1D and 2D ^{1}H- and ^{13}C-NMR techniques, coupled with the use of relevant databases such as Carb-Bank, SUGABASE or CASPER, it is possible to estimate the number of sugar residues present in an EPS and their mode of linking. In particular, the 2D heteronuclear techniques like HSQC, HMQC or HMBC are useful for the determination of the anomeric configuration of monomer sugars, the homonuclear TOCSY or DQF-COSY is useful for the identification of the single monosaccharides, and finally NOESY and HMBC techniques allow the determination of glycosidic linkages sequence along the polysaccharide backbone (Duus et al. 2000). FTIR is also a valuable tool in the structural definition of EPSs since it allows to recognize the presence of the peculiar functional groups characterizing either monosaccharides or probable substituents in the polymer backbone (Wiercigroch et al. 2017). Molecular weight's determination is another important issue to be addressed for a complete structural characterization of

an EPS. Several classical techniques are available for such a study, for example, light scattering, analytical ultracentrifugation, viscosity determination and size-exclusion chromatography (SEC). More recently some innovative techniques like high-performance size-exclusion chromatography (HPSEC) coupled with refractive index (RI) detection or multi-angle laser light scatter (MALLS) detection have gained increasing attention. Such techniques require smaller quantities of samples, compared to the other methods, and enable faster analyses: in particular RI is a useful tool for the determination of molecular weight distribution, while MALLS detector allows to evaluate the absolute molecular weight with higher accuracy (Gómez-Ordóñez et al. 2012).

15.4 Examples of Polysaccharide-Producing Extremophilic Microorganisms

The demand of biomolecules is growing quickly because of their advantageous application in a wide variety of market segments (e.g. biotechnology, biomedicine, cosmetics, pharmaceutical industry, food processing, etc.).

Polysaccharides represent one of the more interesting classes of biomolecules for biotechnological application, due to their wide range of functional properties which make them able to form gels, films and membranes. In particular, carbohydrate polymers from natural sources have the significant advantage to be biocompatible, biodegradable, bioadhesive and nontoxic.

Moreover, the research of polysaccharides from microbial origin is very interesting, in particular those produced by extremophilic microorganisms. Extremophiles are able to thrive in a wide variety of harsh habitats because of their capability to counterbalance extreme physical or chemical parameters by means of different strategies. One of these mechanisms is the synthesis of special biomolecules with unique proprieties. Extreme biomolecules have the important advantage of resisting and be effective even in the harsh environmental conditions in which the extremophilic microorganisms live (such as extreme temperature, pH, salt concentration and hydrostatic pressure). These parameters are very close to those of biotechnological processes; therefore this kind of biomolecules can be considered an important source of special compounds for industrial application. In addition, the unique properties of these substances make them possible to carry biotechnological processes at high temperatures or high saline concentrations; thus the risk of contamination is reduced to a minimum (Raddadi et al. 2015).

In this paragraph we report the different polysaccharides isolated from extremophilic microorganisms from Eurasia of which the chemical characterization has been completely or partially performed.

Thermophilic bacteria are the largest group of polysaccharides extracted from extremophilic microorganisms. In Table 15.1 are reported some examples.

Concerning psychrophiles, most of polysaccharide-producing microorganisms have been isolated from Antarctic regions. In literature, there is actually only one example of polysaccharide isolated from psychrophilic bacteria from Eurasia. Ko

Table 15.1 Examples of polysaccharides from thermophilic bacteria

Producer	Sampling site	Chemical composition	EPS/yields	Reference(s)
Thermotoga maritima Cocultivation with *Methanococcus jannaschii*	Geothermal-heated marine sediment at Vulcano, Italy	Glc/Rib/Man (1:0.06:0.03)	5% of the dry mass	Rinker and Kelly (2000), Johnson et al. (2005), and VanFossen et al. (2008)
Bacillus licheniformis B3-15	Shallow marine hot spring, Vulcano, Italy	Man/Glc (1:0.3)	165 mg/l	Maugeri et al. (2002) and Arena et al. (2006)
Bacillus licheniformis T14	Shallow hydrothermal vent, Panarea Island, Italy	Fru/Fuc/Glc/GalN/Man (1.0:0.75:0.28:tr:tr)	Maximum EPS yield of 366 mg/l with sucrose as C-source	Spanò et al. (2013) and Gugliandolo et al. (2014)
Geobacillus sp. 4001	Shallow hydrothermal vents and marine hot springs, Flegrean Region, Italy	Man/Glc/Gal/ManN (1:0.1:tr:tr)	Maximum EPS yield of 60 μg/l with trehalose as C-source	Nicolaus et al. (2002, 2003, 2004)
Geobacillus sp. 4004	Shallow hydrothermal vents and marine hot springs, Flegrean Region, Italy	Gal/Man/GlcN/Ara (1:0.8:0.4:0.2)	50 μg/l	Nicolaus et al. (2002, 2004)
Geobacillus thermodenitrificans B3-72	Shallow hydrothermal vent, Vulcano island, Italy	Man/Glc (0.3:1)	Maximum EPS yield of 70 mg/l with glucose/sucrose as C-sources	Nicolaus et al. (2000) and Arena et al. (2009)
Geobacillus tepidamans V264	Hot spring, Velingrad, Bulgaria	Glc/Gal/Fuc/Fru (1:0.07:0.04:0.02)	Maximum EPS yield of 111.4 mg/l with maltose as C-source	Kambourova et al. (2009)
Aeribacillus pallidus 418	Hot spring, Rupi basin, Bulgaria	EPS1: Man/Glc/GalN/GlcN/Gal/Rib(1:0.16:0.09:0.08:0.07:0.04)	130 mg/l	Radchenkova et al. (2013, 2014)
		EPS2: Man/Gal/Glc/GalN/GlcN/Rib/Ara (1:0.5:0.46:0.35:0.24:0.16:0.14)		
Brevibacillus thermoruber strain 423	Hot spring, Blagoevgrad region, Bulgaria	Glc/Gal/GalN/Man/Man (1:0.3:0.25:0.16:0.04)	Maximum EPS yield of 863 mg/l with maltose as C-source	Yasar Yildiz et al. (2014)
Rhodothermus marinus DSM 4252^T	Submarine hot spring, Iceland	Main components: Xyl, Ara and Glc	8.8 mg/g cell dry weight	Sardari et al. (2017)
Rhodothermus marinus MAT 493	Submarine hot spring, Iceland	Main components: Glc, Ara, Xyl and Man	13.7 mg/g cell dry weight	Sardari et al. (2017)

Monosaccharide codes: D-arabinose (Ara), D-fructose (Fru), D-fucose (Fuc), D-galactose (Gal), D-galactosamine (GalN), D-glucose (Glc), D-glucosamine (GlcN), D-mannose (Man), D-mannosamine (ManN), D-ribose (Rib), D-xylose (Xyl)

et al. (2000) isolated an extracellular polysaccharide (molecular mass over 2×10^6 Da) from the marine isolate *Hahella chejuensis* (Ko et al. 2000). The monosaccharidic composition of the carbohydrate polymer was partially characterized and consisted of galactose, glucose, xylose and ribose.

The isolation of polysaccharides from halophilic bacteria is reported for only six strains, from which six carbohydrate polymers have been chemically characterized in total. The examples of polysaccharides from *Halophiles*, together with the one isolated from the psychrophile *Hahella chejuensis*, are reported in Table 15.2.

The last group is represented by microorganisms belonging to the Archaea domain. In literature eight polysaccharide-producing Archaea have been chemically characterized (Table 15.3). It is interesting to notice that glucose and mannose are almost always present, often as main monosaccharides.

The research on polysaccharides from Eurasian extremophilic microorganisms has mainly developed over the last 20 years. In fact, analysing all the tables, it is possible to notice that most of the studies are dated after 2000. The growing interest in this topic has essentially two reasons: first, it is important to deeply investigate the physiological mechanisms at the basis of the polysaccharide production, in order to better understand the ecological role of these biomolecules in extremophiles; in addition, the unique proprieties of extremophilic carbohydrate polymers make them highly attractive to biotechnological industry. These subjects will be deeply investigated and discussed in the following paragraphs.

15.5 Application and Biological Activities

In an extreme environment, the synthesis of exopolysaccharides (EPSs) is in response to adaption to prohibitive conditions. Therefore, these biomolecules produced by extremophiles showed unique features for adapting to extreme conditions. EPS-producing microorganisms, in particular those from extreme habitats, have become the natural source of polysaccharides of growing interest for their bioactivities and physicochemical properties; therefore they represent very promising compounds for biotechnological applications. Herein, we report the most representative examples of EPSs isolated from extremophiles having great potential in application in numerous industrial sectors such as tissue engineering, drug delivery and cosmetic (Table 15.4).

15.5.1 Halophiles

Among halophiles microorganisms, *Halomonas* represents the most common genera producing EPSs. Mauran is a highly polyanionic sulphated exopolysaccharide produced by a moderately halophilic bacterium *Halomonas maura*. For its unique physicochemical properties, mauran has been successfully employed in the nanoparticle synthesis and application for sustained drug delivery, cancer chemotherapy and bioimaging and for its antioxidant defence mechanism along with

Table 15.2 Examples of polysaccharides from psychrophilic and halophilic bacteria

Producer	Sampling site	Chemical composition	EPS/yields	Reference(s)
Psychrophiles				
Hahella chejuensis	Marado, Jeju Island, Republic of Korea	Gal/Glc/Xyl/Rib	9.23 g/l	Ko et al. (2000) and Lee et al. (2001)
Halophiles				
Halomonas smyrnensis	Çamaltı Saltern area, Sasalı, İzmir province, Aegean Region of Turkey	Levan. Repeating unit of β-(2,6)-d-fructofuranosyl residues	Maximum EPS yield of 1.844 g/l with sucrose as C-source	Poli et al. (2009)
Halomonas anticariensis strain FP35	Fuente de Piedra saline wetland, province of Málaga, southern Spain	Man/GalA/Glc (1:0.82:0.33)	296.5 mg/l	Mata et al. (2006)
Halomonas anticariensis strain FP36	Fuente de Piedra saline wetland, province of Málaga, southern Spain	Man/GalA/Glc (1:0.92:0.4)	499.5 mg/l	Mata et al. (2006)
Halomonas ventosae strain Al12	Saline soils in Jaén, southeastern Spain	Man/Glc/Gal (1:0.43:0.25)	283.5 mg/l	Mata et al. (2006)
Halomonas ventosae strain Al16	Saline soils in Jaén, southeastern Spain	Man/Glc/Gal (1:0.42:0.22)	289.5 mg/l	Mata et al. (2006)
Halomonas eurihalina strain H212	Saline soil in Alicante, Southern Spain	High sulphate content and significant amounts of uronic acid	1.6 g/l	Béjar et al. (1998)

Monosaccharide codes: D-fructose (Fru), D-galactose (Gal), D-galacturonic acid (GalA), D-glucose (Glc), D-mannose (Man), D-ribose (Rib), D-xylose (Xyl)

Table 15.3 Examples of polysaccharides from Archaea

Producer	Sampling site	Chemical composition	EPS/yields	Reference(s)
Archaea				
Thermococcus litoralis	Shallow marine thermal spring, Naples, Italy	Mannan: Man (only monosaccharidic constituent)		Rinker and Kelly (1996, 2000)
Sulfolobus solfataricus MT4	Hot acidic spring, Agnano, Italy	Glc/Man/GlcN/Gal (1:0.8:0.15:0.11)	Exopolysaccharide yield of 8.4 mg/l (fermentor culture)	Nicolaus et al. (1993)
Sulfolobus solfataricus MT3	Hot acidic spring, Agnano, Italy	Glc/Man/GlcN/Gal (1:0.8:0.64:0.61)	Exopolysaccharide yield of 7.0 mg/l (fermentor culture)	Nicolaus et al. (1993)
Sulfolobus tokodaii	Beppu hot springs, Japan	Man/Glc/Gal/GlcNAc		Koerdt et al. (2010)
Haloarcula hispanica ATCC33960	Solar saltern, Alicante, Spain	Acidic exopolysaccharide: Man/Gal/Glc (1:0.77:0.02)	EPS yield of 30 mg/l (in AS-168 medium)	Lü et al. (2017)
Haloterrigena turkmenica	Sulphate saline soil in Turkmenistan (Central Asia)	Sulphated heteropolysaccharide: Glc/GlcN/GlcA/Gal/GalN (1:0.65:0.24:0.22:0.02)	Maximum EPS yield of 206.8 mg/l in HTR complex medium +1% Glc (w/v)	Squillaci et al. (2016)
Haloferax gibbonsii	Saltern, Spain	Man/Glc/Gal/Rha (0.6:0.3:1:0.3)		Paramonov et al. (1998)
Haloferax mediterranei	Salt ponds, Alicante, Spain	Man/Glc/Gal/amino sugars/uronic acids, Man as major component	Maximum EPS yield of 2.6 g/l	Antón et al. (1988) and Parolis et al. (1996)

Monosaccharide codes: D-galactose (Gal), D-galactosamine (GalN), D-glucose (Glc), D-glucuronic acid (GlcA), D-glucosamine (GlcN), N-acetyl-D-glucosamine (GlcNAc), D-mannose (Man), D-rhamnose (Rha)

Table 15.4 Examples of the most relevant EPSs from extremophiles with potential application in different industrial sectors

EPS-producing microorganisms	EPS *(as reported in literature)*	Biological activities/application	Reference(s)
Halophiles			
Halomonas maura	Mauran	High viscosity and pseudoplasticity, metal-binding capacity; antioxidant, haemocompatibility/water treatment, drug delivery, cancer chemotherapy, bioimaging, nanotechnology	Bouchotroch et al. (2001), Arias et al. (2003), and Raveendran et al. (2013a, b)
Halomonas eurihalina H96	EPS H96	Gelificant ability/biodetoxification and water treatment	Béjar et al. (1998)
Halomonas ventosae strains Al12^T and Al16	/	Emulsifying activity/surfactant	Mata et al. (2006)
Halomonas anticariensis strains FP35^T and FP36	/	Emulsifying activity/surfactant	Mata et al. (2006)
Halomonas smyrnensis strain AAD6^T	Levan	Anti-cytotoxic, high biocompatibility/cosmetic, food and medical sectors	Poli et al. (2009), Sam et al. (2011), and Sezer et al. (2011)
Salipiger mucosus	/	Emulsifying activity/surfactant	Llamas et al. (2010)
Thermophiles			
Alteromonas infernus	EPS GY785/GY785 DROS (after depolymerization and sulphation)	Activation of normal human serum (NHS), bone and skin regeneration/drug delivery, tissue engineering	Raguénès et al. (1997a, b), Zanchetta et al. (2003a, b), and Poli et al. (2017)
Vibrio diabolicus	EPS HE800/HE800 DROS (after depolymerization and sulphation)	Activation of normal human serum (NHS), bone and skin regeneration/drug delivery, tissue engineering	Raguénès et al. (1997a, b), Zanchetta et al. (2003a, b), and Poli et al. (2017)
Bacillus licheniformis strain B3-15	EPS2-B3-15	Immunomodulatory, antiviral/drug delivery	Nicolaus et al. (2000), Maugeri et al. (2002), Arena et al. (2006), Spanò et al. (2013), and Gugliandolo et al. (2015)
Geobacillus thermodenitrificans strain B3-72	EPS2-B3-72	Immunomodulatory, antiviral/drug delivery	Nicolaus et al. (2000), Maugeri et al. (2002), Arena et al. (2006), Spanò et al. (2013), and Gugliandolo et al. (2015)

Bacillus licheniformis strain T14	EPS1-T14	Immunomodulatory, antiviral/drug delivery	Nicolaus et al. (2000), Maugeri et al. (2002), Arena et al. (2006), Spanò et al. (2013), and Gugliandolo et al. (2015)
Thermus aquaticus YT-1	EPS TA-1	Immunomodulatory	(Lin et al. 2011)
Geobacillus tepidamans V264	/	Anti-cytotoxic/drug delivery	Kambourova et al. (2009)
Aeribacillus pallidus 418	/	Emulsifying activity/surfactant	Radchenkova et al. (2013, 2014)
Brevibacillus thermoruber strain 423	EPS-FT; EPS-P	High biocompatibility/tissue engineering, drug delivery	Nwodo et al. (2012) and Yasar Yildiz et al. (2014)
Psychrophiles			
Colwellia psychrerythraea strain 34H	/	Cryoprotective/food, pharmaceutical and cosmetic industries	Carillo et al. (2015)
Pseudomonas sp. ID1	/	Cryoprotective/food, pharmaceutical and cosmetic industries	Carrión et al. (2015)
Pseudoalteromonas sp. SM9913	EPS SM9913	Good flocculating agent, good adsorptive effect/biodetoxification and water treatment	Qin et al. (2007) and Li et al. (2008)
Archaea			
Haloterrigena turkmenica	/	Emulsifying, antioxidant/food, pharmaceutical and cosmetic industries	Squillaci et al. (2016)

haemocompatibility under in vitro conditions using L929 (mouse fibroblast cell line) and mice liver homogenate (Bouchotroch et al. 2001; Arias et al. 2003; Raveendran et al. 2013a, b). Other halophilic EPS producers belonging to the genus *Halomonas* are *H. eurihalina*, *H. ventosae* and *H. anticariensis*. Nineteen strains belonging to *H. eurihalina* were studied for their ability to produce EPS in two different culture media. Results showed that the chemical composition of the polysaccharides was affected by the strain and by the culture medium. All EPS exhibited an unusually high sulphate content. Moreover, the EPS from strain H96 contained significant amounts of uronic acid. EPS from strain H96, cultivated in defined NH medium (minimal medium), showed an interesting rheological feature reaching a viscosity value of 30,000 cP at pH 3.0. This gelificant ability, probably due to its high uronic acid content, is attractive for industrial application, for example, in biodetoxification and water treatment (Béjar et al. 1998).

H. ventosae strains Al12^T and Al16 produced polymers showing a molecular mass of about 50 kDa, and their main components were glucose, mannose and galactose. Moreover, they exhibited emulsifying activity on several hydrophobic substrates. *H. anticariensis* strains FP35^T and FP36 also excreted polymers having a molecular mass of about 20 and 46 kDa, respectively, and were composed mainly of glucose, mannose and galacturonic acid. All EPSs produced solutions of low viscosity and pseudoplastic features. Furthermore, they also exhibited a high affinity for binding cations and incorporated considerable quantities of sulphates, just as do those produced by *H. maura* and *H. eurihalina*, which is very uncommon in bacterial polysaccharides, but represents an advantageous feature for biotechnological application. Both bacteria formed biofilms both in polystyrene wells and borosilicate test tubes. In particular, *H. ventosae* strain Al16 gave the best results in biofilm formation assays, possibly due to the high emulsifying activity of its polysaccharide (Mata et al. 2006).

Halomonas smyrnensis strain AAD6^T, isolated from soil samples taken from Çamaltı Saltern area in Turkey, was found to produce high levels of levan (Poli et al. 2009, 2013). This EPS did not affect cellular viability and proliferation in two different cellular systems tested, osteoblasts and murine macrophages, demonstrating its high biocompatibility. Besides, it displayed a protective effect against the toxic activity of avarol implying its additional use as an anti-cytotoxic agent. The potential applications of levan as an industrial gum, a blood plasma extender, a sweetener, an emulsifier, a formulation aid, a stabilizer, a thickener, a surface-finishing agent, an encapsulating agent and a carrier for flavour and fragrances are known (Shih et al. 2005; Beine et al. 2008). Then, *Halomonas* sp. AAD6 represented an alternative cheap source of levan polymer when grown on defined media hypothesizing its larger employment in industrial application being a non-pathogenic microorganism (Sam et al. 2011; Sezer et al. 2011).

A species of halophilic, EPS-producing bacterium belonging to the *Alphaproteobacteria*, is the type strain (A3^T) of *Salipiger mucosus*, isolated on the Mediterranean seaboard. The EPS produced by *S. mucosus* was able to emulsify high percentages of pure hydrocarbons (tetradecane, octane, kerosene, xylene and crude oil) more than other chemical surfactants used in comparison. This ability

could be ascribed to the presence of acetyl groups which render the EPS somewhat hydrophobic. Furthermore, the EPS was also able to bind cations and to incorporate high quantities of sulphates, which represent a very unusual feature in bacterial polysaccharides (Llamas et al. 2010).

15.5.2 Thermophiles

EPS producers were also found among thermophiles isolated from different thermophilic habitats. Remarkable antiviral and immunomodulatory activities against herpes simplex virus type 2 (HSV-2) were showed by EPSs produced by *Bacillus licheniformis* strain B3-15, *Geobacillus thermodenitrificans* strain B3-72 and *B. licheniformis* strain T14, three thermophilic and thermotolerant bacilli isolated from Aeolian Islands shallow vents. All EPSs were not cytotoxic towards peripheral blood mononuclear cells (PBMC) at the concentration of 300 μg·mL^{-1}. They were able to interfere HSV-2 replication in PBMC. This ability, expressed as logarithm, was higher for EPS2-B3-15 (0.82) compared with EPS2-B3-72 (0.49) and EPS1-T14 (0.63). Further investigations showed a correlation between the antiviral effect of EPSs and the immune response involved in the controlling viral replication. Indeed, EPS treatment caused high production of Th1 cytokines (IFN-γ, IFN-α, TNF-α, IL-12 and IL-18) by PBMC, which means the inhibition of viral replication by induction of antiviral state in neighbouring cells (i.e. IFNs) or the destruction of virus-infected cells (i.e. TNF-α and IL-18). EPS2-B3-15 exhibited the best antiviral potential compared with the other EPSs assayed (Nicolaus et al. 2000; Maugeri et al. 2002; Arena et al. 2006; Spanò et al. 2013; Gugliandolo et al. 2015; Marino-Merlo et al. 2017).

The extracellular polysaccharide TA-1 secreted by the thermophilic bacteria *Thermus aquaticus* YT-1 also showed immunomodulatory activity by stimulation of macrophage cells to produce the cytokines TNF-α and IL-6, which increases the immune response. The presence of D-galactofuranose residues in the EPS TA-1 could be probably responsible for observed immunoregulatory activity through Toll-like receptor 2 within macrophages, the first line of host defence against bacterial infection (Lin et al. 2011).

Geobacillus tepidamans V264, a thermophilic bacteria isolated from Velingrad hot spring, Bulgaria, secreted an extracellular polysaccharide exhibiting an anti-cytotoxic activity evaluated by means of brine shrimp test, towards avarol, a natural toxic sesquiterpene hydroquinone isolated from *Dysidea avara* sponge (Tommonaro et al. 2015). The biopolymer increased the value of LD_{50} of avarol, more than 12-fold, from 0.18 μg mL^{-1} up to 2.24 μg mL^{-1}. The activity exerted by EPS could be related to the adhesion of toxic compounds to the surface of the polysaccharide. Hence, this EPS could be used in pharmacy as anti-cytotoxic drugs (Kambourova et al. 2009).

From southwest of Bulgaria, in Rupi Basin hot springs, the strain *Aeribacillus pallidus* 418 producing an exopolysaccharide was isolated. The EPS exhibited good emulsifying properties, which could be improved using mixtures with

other biopolymers. In particular, the mixture of EPS from *A. pallidus* 418 with xanthan showed the best synergy in terms of stability of emulsion. Both these properties (good emulsifying properties and the enhanced synergistic activity) of EPS represented valuable features for its industrial exploration (Radchenkova et al. 2013, 2014).

In the same region of Bulgaria, from a hot spring close to the village Gradeshnitsa, Blagoevgrad region, a thermophilic microorganism, which belonged to the phylum *Firmicutes* and closely related with other strains from the species *Brevibacillus thermoruber*, *B. thermoruber* strain 423, was isolated. Its colonies exhibited high mucoidity, and it was a high-level exopolysaccharide (EPS)-producing thermophile. Chemical studies showed that the EPS was a heteropolymer composed of glucose as prevailing monomer unit. At first, it was purified in two fractions, as a flow through column (EPS-FT) and peak of salt elution (EPS-P), and next assayed for its biocompatibility with the monkey kidney fibroblast cell line Cos-7, considering that biocompatibility is one of key factors for medical applications. Results showed a no pathogenicity of the pure EPS fractions on cellular line used together with their high biocompatibility, and then this study suggested their potential use in biomedical applications, such as scaffolds or matrices in tissue engineering, drug delivery and wound dressing (Nwodo et al. 2012; Yasar Yildiz et al. 2014).

From deep-sea hydrothermal vent located in the Gulf of California, two EPS-producing bacteria have been isolated, *Vibrio diabolicus* and *Alteromonas infernus*. The EPS GY785 produced by *A. infernus* was a branched, sulphated polysaccharide and showed a high molecular weight (up to 10^6 Da), while EPS HE800 produced by *V. diabolicus* was a linear glycosaminoglycan and showed a molecular mass of about 8×10^5 Da. Both EPSs exhibited very interesting biological activity after depolymerization and, next, sulphation of the hydroxyl groups present on the low molecular weight (LMW) EPSs. The over-sulphated EPSs, named HE800 DROS and GY785 DROS, interacted with C1q protein of the complement pathway system by activation of normal human serum (NHS) incubated with various amounts of GY785 DR or HE800 DR, to restore the haemolytic activity of serum deficient in complement protein C1q. However, EPS HE800 already showed very interesting biological properties in regard to bone and skin regeneration (Raguénès et al. 1997a, b; Zanchetta et al. 2003a, b; Courtois et al. 2014; Poli et al. 2017).

15.5.3 Psychrophiles

Cold-adapted microorganisms (psychrophiles and psychrotolerant) are widespread in terrestrial environments and marine ecosystem. Despite the large number of psychrophilic microorganisms reported in literature, few of them are described as EPS-producing microorganisms. Psychrophilic γ-proteobacterium *Pseudomonas* sp. ID1 is a cold-adapted bacterium isolated from a marine sediment sample collected from South Shetland Islands (Antarctica). This microorganism produced an EPS mainly composed of glucose, galactose and fucose and had a molecular mass higher than 2×10^6 Da. This biopolymer exhibited emulsifying activity against different food

and cosmetic oils much higher than commercial gums (xanthan gum and Arabic gum), cryoprotective activity, pseudoplastic flow behaviour, low thixotropy and yield stress. All these properties of EPS of *Pseudomonas* sp. ID1 suggested its significant cryoprotection role for the strain and make it a promising alternative to commercial polysaccharides as emulsifier and cryoprotectant agent for food, pharmaceutical and cosmetic industries (Carrión et al. 2015).

Psychrotolerant bacterium *Pseudoalteromonas* sp. SM9913 secreted large quantities of EPSs. The yield of EPS increased as the temperature decreased in the tested range, indicating that the EPS production of strain SM9913 had cold adaptation. Under optimal growth conditions (15 °C, 52 h), the yield of EPS reached 5.25 g l21 (dry weight), which was higher than that reported for the EPSs produced by other psychrotolerant microorganisms (Nichols et al. 2005b). Structural analysis of EPS SM9913 showed that it consisted mainly of glucose, with arabinose, xylose and a minor peak for mannose. This biopolymer enhanced the thermostability of protease MCP-01 (the main protease secreted by strain SM9913) at 40 °C, by preventing its autolysis. In the presence of EPS (1% w/v), the protease activity of MCP-01 showed no evident change after 150 min incubation. In contrast, the protease activity in the absence of EPS was quickly lost, with 90% of the activity lost after 135 min incubation at 40 °C. In addition, the flocculation experiment showed that the EPS could make colloidal and suspended particles in solution conglomerate, suggesting that the EPS was a very good flocculating agent and had a good adsorptive effect. Therefore, it might play an important role for strain SM9913 in enriching nutrient particles in the deep-sea environment (Qin et al. 2007; Li et al. 2008).

15.5.4 Archaea

The most valuable example of biotechnologically interesting EPS produced by an Archaea is that reported by Squillaci et al. (2016). In that paper the isolation and the chemical characterization of the EPS secreted by *Haloterrigena turkmenica* together with its applicative properties are discussed. The microorganism produced the EPS mainly in the middle exponential growth phase and reached the maximal production (20.68 mg EPS per 100 ml of culture medium) in the stationary phase. Results obtained by means of anion-exchange chromatography and SEC-TDA Viscotek indicated that the EPS was composed of two main fractions of 801.7 and 206.0 kDa. It was a sulphated heteropolysaccharide containing glucose, galactose, glucosamine, galactosamine and glucuronic acid. EPS exhibited interesting emulsifying activity towards *n*-hexane while was capable of producing stable emulsions with vegetable oils. EPSs supplied with emulsifying ability could be employed in the food industry as emulsifier and stabilizer agents (Duboc and Mollet 2001). Moreover, EPS displayed also a moderate antioxidant activity evaluated by means of DPPH, FRAP and TAC assays. In DPPH assay, at a concentration of 10 mg/ml, the radical scavenging activity of the EPS was 68.2% ± 1.1 with IC50 value of 6.03 mg/ml, whereas hyaluronic acid (standard used) did not show scavenging capacity. In TAC and FRAP assays also, the EPS showed the ability to react with both Mo^{6+} and Fe^{3+}

ions showing a linear dose-dependent antioxidant activity. All these features make the EPS produced by *H. turkmenica* a possible candidate for wide applications in several industrial sectors (Squillaci et al. 2016).

15.6 Biosynthesis of EPSs and Genetic Strategy for Their Hyperproduction

Bacteria exopolysaccharides are synthesized via different biosynthesis pathways, and the genes responsible for the synthesis are often clustered within the genome. The knowledges related to EPS biosynthetic processes and the genetic regulation are essential to produce tailor-made biopolymers. Intracellular synthesis of homo- and heteropolysaccharides is a complex process that proceeds via intracellular assembly of sugar nucleotide precursors. In EPS biosynthesis, different enzymes and regulatory molecules are involved in several metabolic pathways. It begins with the entry of the sugars in the cell, which are catabolized by periplasmic oxidation or intracellular phosphorylation: sugars that do not take part in the central metabolic pathways act as a raw material for EPS manufacture (Freitas et al. 2011). The intracellular EPS-synthetic machinery requires charged and energy-rich precursor monosaccharides: these letters are in the form of nucleotide diphosphate/monophosphate sugars (NDP-/NMP-sugar). This is a crucial step of biosynthesis: sugars often are in the form of sugar-1P and rarely in the form of sugar-2P or sugar- 6P and serve as activated primary residues (Madhuri and Prabhakar 2014). Some intermediates like fructose-6P or glucose-1P, in majority of cases, lead to synthesis of uridine diphosphate-N-acetyl glucosamine (UDP-GlcNAc), uridine diphosphate-N-acetyl galactosamine (UDP- GalNAc) and dTDP-rhamnose, precursor molecules for EPS synthesis (Boels et al. 2001). In subsequent step, phosphoglucomutase enzyme catalyses the conversion of sugar-6P to sugar-1P, and UDP-glucose pyrophosphorylase and dTDP-glucose pyrophosphorylase catalyse the conversion of sugar-1P to UDP-glucose and dTDP-glucose. The conversion of UDP-GlcNAc to UDP-GalNAc in *Lactobacillus rhamnosus* has been found to be catalysed by UDP-N-acetylglucosamine 4-epimerase (Boels et al. 2001). At present four general mechanisms are known in bacteria: (a) the Wzx-/Wzy-dependent pathway, (b) the ATP-binding cassette (ABC) transporter-dependent pathway, (c) the synthase-dependent pathway and (d) the extracellular synthesis by the use of a single sucrase protein. In the first three biosynthesis pathways, the precursor molecules, which are necessary for the stepwise elongation of the polymer strands, are realized inside the cell, while for the extracellular production, the polymer strand is elongated by direct addition of monosaccharides obtained by cleavage of di- or trisaccharides (Fig. 15.2).

(a) In the Wzx-/Wzy-dependent pathway the repeating units, linked to an undecaprenol diphosphate anchor (C55) at level of the inner membrane, are assembled by different glycosyltransferases (GTs) and translocated across the cytoplasmic membrane by a Wzx protein (flippase protein). The Wzy polymerase and the polysaccharide co-polymerase (PCP) protein are responsible for polymerization

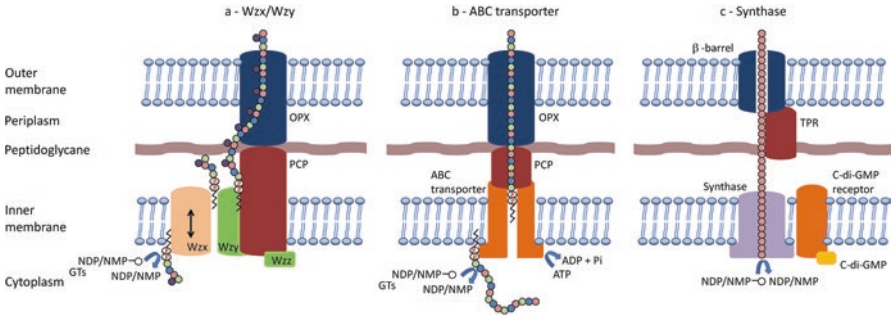

Fig. 15.2 Schematic representation of EPS biosynthesis pathways

process. The polysaccharide export (OPX) protein families and the PCP carry the transport across the membranes. (b) In the ABC transporter-dependent pathway, the EPS chain is assembled on a lipid carrier situated in the inner leaflet of the inner membrane before the transportation across the inner membrane by an ABC transporter. The EPS is then exported through the periplasm and across the outer membrane by the OPX and the PCP families of proteins. (c) In the synthase-dependent pathway, a complete polymer chain is polymerized and secreted across the inner membrane by inner-membrane synthase proteins. The activity of the polysaccharide synthase is post-translationally regulated by an inner-membrane c-di-GMP receptor. The EPS is then exported across the outer membrane by TRP-containing protein and an integral outer-membrane beta-barrel.

In the first mechanism, the repeating units, which are linked to an undecaprenol diphosphate anchor (C55) at level of the inner membrane, are assembled by different glycosyltransferases (GTs) and translocated across the cytoplasmic membrane by a Wzx protein, also known as flippase. Then, at level of the periplasmic space, the repeating units are polymerized by the Wzy protein before they will be exported to the cell surface (Islam and Lam 2014). Subsequently, the transport of polymers from the periplasm to the cell surface is due to the polysaccharide co-polymerase (PCP) and the outer-membrane polysaccharide export (OPX; formerly OMA) protein families (Cuthbertson et al. 2009; Morona et al. 2009). EPSs assembled by the Wzx/Wzy pathway are heteropolymers (e.g. xanthan) and possess different sugar patterns, up to four or five types of sugars in their chemical structure. All strains using this pathway carry the genes for the flippase (Wzx) and the polymerase (Wzy) within their extracellular polysaccharide operons.

In the synthase-dependent pathway, complete polymer strands are secreted across the membranes and the cell wall: this pathway is independent of a flippase for the translocation of the repeating units. Both polymerization and translocation processes are performed by a single synthase protein. In the case of alginate and cellulose, for example, the synthase protein is a single subunit of an envelope-spanning multiprotein complex (Rehm 2010; Whitney and Howell 2013). This pathway is often utilized for the synthesis of homopolymers: this is the case of curdlan

biosynthesis, for example, in which only β-(1–3)-linked glucose is present or in the case of bacterial cellulose, consisting only of β-(1–4)-linked glucose units.

In the extracellular synthesis pathway, the biosynthesis of polymers, such as dextran or levan, occurs via GTs, which are secreted and covalently linked to the cell surface. In this case the responsible enzymes involved in the process transfer the activated precursor monosaccharides from substrate to growing polysaccharide that assembles in a final structure by the formation of linkage pattern and degree of branching. The Wzx-/Wzy-dependent pathway is responsible for the biosynthesis of diutan, gellan, welan, xanthan and colanic acid also known as the M antigen (an EPS with no commercial application but is of high interest due to pathogenicity in enterobacteria studies). Alginate, cellulose, curdlan and hyaluronic acid are examples of EPSs in which the synthase-dependent mechanism is responsible for their synthesis; dextran, levan and mutan are the common examples that require dextran sucrase and levan sucrase as enzymes and sucrose as a substrate, respectively (extracellular pathway) (Boels et al. 2001).

Bacterial polysaccharides have interesting and unique properties for industrial applications and are used as emulsifiers, viscosifiers, stabilizers or gelling agents. Due to these valuable properties, several studies were performed to genetically engineer the producing organisms in order to improve the yield of production or to generate new polysaccharide variants. Putative targets for engineering are the molecular weight, addition of substituents, composition and sequence of sugar components. Recently, intensive research focused on mechanisms underlying EPS biosynthesis pathways, genome sequencing, protein structure analysis and new bioinformatics tools aid to understand the principles of EPS formation. Engineering strategies can be subdivided into different categories. One is an increased volumetric productivity: these studies were mostly aiming at increasing sugar nucleotide precursors to enhance the carbon flux towards the final polymer, and the genes of precursor biosynthesis were overexpressed (Schmid et al. 2015). Overexpression of genes involved in the EPS assembly such as GTs, Wzx and Wzy, both as single genes and whole cluster, resulted in enhanced yields and precursor conversion rates, while in other cases, it had a negative effect presumably due to distorting the multidomain protein complex involved in polymerization and secretion (van Kranenburg et al. 1999). Another approach is to increase the EPS productivity by increasing transcription of the operons, which encode the EPS biosynthesis proteins. Single-gene knockouts were also described to enhance yield and alter EPS chemical structure, as shown in *Azotobacter vinelandii* (Gaytán et al. 2012). However, the strategy to enhance productivity based on genetic engineering might be interesting for EPS with reduced viscosifying properties, for example, due to lower molecular weight. The optimization of manufacturing process parameters might be more promising than engineering EPS biosynthesis for many established industrial EPS producers. The highest titres of highly viscous EPS such as xanthan are around 30–50 g/L and represent the current maximum amount, which is manageable by existing bioprocess units (Hublik 2012).

Another strategy of engineering EPS biosynthesis is to alter the molecular structure and therefore the chemical characteristics and behaviour of the final

biopolymer. These modifications can be based on deleting substituents or monomeric sugars from the side chain or binding new substituents: in these cases a change of the ratio of decoration, such as the degree of acetylation and pyruvylation, occurred (Donati and Paoletti 2009). The degree of acetylation and pyruvylation has opposite effects on viscosity. A high degree of pyruvylation resulted in higher viscosity, whereas the presence of more acetyl groups decreased viscosity of EPS. This finding is a general rule for polysaccharides and can be used in tailoring the EPS viscosity.

Other engineering approaches with respect to the production of xanthan variants included the length of the side chain. A truncated tetramer xanthan version, obtained by deletion of the terminal mannose via inactivation of the glycosyltransferase (GT GumI), resulted in a much lower viscosity. The further removal of the glucuronic acid from the side chain by inactivation of GT GumK resulted in enhanced viscosity compared to the wild type (Schmid et al. 2015). The molecular weight of xanthan was synthetically adjusted by controlling the expression level of the Wzy polymerase Gum E (Galván et al. 2013), while for alginate a similar effect was observed by an overexpression of alginate polymerase *alg8/alg44* in *Azotobacter vinelandii* producing a high molecular weight alginate type (Díaz-Barrera et al. 2012).

In some EPS, overexpression or mutation of genes involved in the polymerization/degradation process (e.g. synthase, Wzy, PCP/lyases, glucosidases) represented another possibility to change the rheological properties of the polymers (Rehm 2010; Galván et al. 2013).

As concerning the haloarchaeal EPSs, although the chemical structures have been solved, little is known about their biosynthesis. The EPS from *Haloferax mediterranei* ATCC 33500 was identified to be a heteropolysaccharide containing mannose as the major component. The repeating unit of EPS in *H. mediterranei* contains one mannosyl and two N-acetyl-glucosaminuronyl moieties, and one N-acetyl-glucosaminuronyl group is modified by a sulfonic group. Based on the complete genome sequence of *H. mediterranei*, a gene cluster involved in EPS biosynthesis in *H. mediterranei* was identified. Deletion of the gene cluster eliminated EPS synthesis. The mutant strain deficient of EPS biosynthesis showed a remarkable decrease in viscosity and foaming propensity of culture broth and increase in content of dissolved oxygen and enhanced the production of polyhydroxyalkanoate (Zhao et al. 2013). Lü et al. (2017) purified an acidic exopolysaccharide from an extremely halophilic archaeon *Haloarcula hispanica* ATCC 33960, which mainly composed of mannose and galactose with a small amount of glucose in a molar ratio of 55.9:43.2:0.9. The authors reported the identification of two glycosyltransferase genes (HAH_1662 and HAH_1667), responsible for the synthesis of EPS. Deletion of either HAH_1662 or HAH_1667 led to loss of the EPS production. In addition, the mutants of *Haloarcula hispanica* displayed a different cell surface morphology, retarded growth in low salty environment, an increased adhesion and swimming ability, suggesting that its biosynthesis might act as an adaptable mechanism to protect the cells against harsh environments.

In conclusion, as emerged from this overview, the genes involved in the different biosynthesis pathways encode various types of GTs, polymerizing and branching

enzymes, but also enzymes responsible for addition of substituents or modifications of sugar chain. However, several steps are currently understood, and sometimes the differences between the pathways become less defined. Genome or plasmids contain the genes encoding these enzymes in most of the EPS-producing bacteria (Rehm 2010). The clustering of several GTs and polymerizing as well as secreting enzymes facilitate the identification of EPS operons. Even if many gene clusters involved in the EPS biosynthesis have been known for several years, the function and mode of action of most of the genes and proteins are not completely clarified.

Moreover, the identification of novel EPS clusters by next-generation sequencing approaches will enhance our understanding of EPS synthesis pathway variation and modification. By using different tools, such as bioinformatics, structural information of proteins and EPSs, it will enable the implementation of synthetic biology approaches for tailoring microbial EPS. The insights in Wzx and Wzy topology and mechanism might open up the opportunity for incorporation of desired sugars or sugar derivatives resulting in modified EPS structures with hitherto unknown material properties (Rehm 2015). Recently, an innovative bi-enzymatic process was reported, stating that from sucrose, the production of short-chain fructooligosaccharides and oligolevans was obtained. This system was based on an immobilized levansucrase and an endo-inulinase, resulting in a highly efficient synthesis system with a yield of more than 65% and a productivity of 96 g/L/h (Tian et al. 2014). The utilization and combination of several carbohydrate-modifying enzymes create the potential for industrial production of different low molecular weight oligo- or polysaccharides with applications as food additives (prebiotics) or in medicine.

15.7 Conclusion and Future Perspectives

Microbial EPSs are ubiquitous in the extreme environments where they are crucial for microbial survival. Most of the functions attributed to EPSs are related to a protective role, which are dependent on the ecological niches in which the producer microorganisms live. They could support the microbial communities to tolerate extremes of temperature, salt concentration and nutrient supply, building an interface between the bacterial cell and its surrounding environment. Several EPSs produced by microorganisms from extreme habitats show biotechnological promise. By examining their structure and chemical/physical characteristics, it is possible to gain insight into their commercial application; they are employed in several fields ranging from food-processing to pharmaceutical industries, through to the bioremediation ability of polluted areas. Considering that most of extreme ecosystems and, therefore, the respective microbial communities are still unexplored, it is reasonable to hypothesize that the isolation of new microorganisms, together with their biomolecules, in particular exopolysaccharides, will provide interesting perspectives for new industrial processes in several fields.

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