Chapter 10
Exopolysaccharide Productions from Extremophiles: The Chemical Structures and Their Bioactivities

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What Will You Learn from This Chapter

This chapter will discuss some remarkable examples of extremophilic bacteria, isolated from different ecosystems, which produce exopolysaccharides (EPSs). The chapter will also cover the properties of EPSs produced by extremophiles and their possible commercial applications ranging from pharmaceutical to food processing, detoxification and bioremediation. This chapter will also cover the techniques that are used to purify, analyse and structurally characterise the bacterial EPSs; the state of the art in the field of bacterial EPSs research, with mention to the main examples of well-studied and commercially exploited EPSs; an overview of the main EPSs' producing extremophiles that have been isolated from both aquatic and terrestrial environments; the description of the main biosynthetic routes leading to the EPSs production in archaeal and bacterial extremophiles, with reference to the enzymes involved and to the genetic manipulations for biosynthesis's tailoring; the analysis of the most interesting biological properties of extremophiles' EPSs (that can act as anti-inflammatory, immunomodulating and antiviral agents) and of their biotechnological applications in drug delivery systems; and the compendium of the most used techniques to perform the purification, the analysis of chemical composition, the

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identification of glycoside linkage position and substitution pattern, the determination of the molecular-weight distribution and the structural analysis of EPSs.

10.1 Introduction

The exopolysaccharides (EPSs) are high-molecular-weight heterogeneous polymers composed by some most recurring monomers like hexoses, pentoses, uronic acids and amino sugars that are often substituted either by organic non-carbohydrate or inorganic groups. Some remarkable examples of well-studied and commercially exploited EPSs include xanthan gum (produced by *Xanthomonas* species), gellan (produced by bacteria belonging to *Sphingomonas* genus), alginate (found in bacteria from the genera *Azotobater* and *Pseudomonas*), hyaluronan (produced by different bacterial strains, e.g. *Pseudomonas aeruginosa*) and levan (produced by species of the genera *Bacillus*, *Rahnella*, *Aerobacter*, *Erwinia*, *Streptococcus*, *Pseudomonas* and *Zymomonas* and recently found also in a species of *Halomonas* genus) (Freitas et al. 2011). Several studies have described the production of large quantities of these polymers also by bacterial species living in extreme environments, for example, Antarctic marine environments or hydrothermal vents, where they are thought to complex with various metal ions and contribute to their mobility and entry into the food web (Mancuso Nichols et al. 2004 and references therein). Bacterial EPSs usually are found as biofilm, a complex mixture constituted by proteins, nucleic acids and lipids besides the polysaccharides. The bacterial species that are able to produce EPSs include several extremophiles, a group of microorganisms that have developed various adaptation strategies, enabling them to compensate for the deleterious effects of harsh environmental conditions such as high temperatures and salt concentrations, low pH or temperature and high radiation. Among these strategies, the EPS biosynthesis is one of the most common protective mechanisms. Exopolysaccharides can be found as in capsular material or as dispersed slime in the surrounding environment. Considerable progress has been made in discovering and developing new microbial EPSs that possess novel industrial significance. In recent years the increased demand for natural polymers for pharmaceutical, food and other industrial applications has led to a remarkable interest in polysaccharides produced by microorganisms (Poli et al. 2011; Finore et al. 2014; Nicolaus et al. 2010).

This chapter will give a short review of extremophilic microorganisms as a source of EPSs with particular attention to their production, structural characterisation methods and biological activities.

10.2 Examples of Archaeal and Bacterial EPS Producers and Their Biosynthetic Pathway

EPS-producing microorganisms have been isolated from different natural sources of both aquatic and terrestrial environments, as well as in extreme niches such as hot springs, cold, hypersaline and halophilic environments, such as salt lakes and salterns (Poli et al. 2009, 2010; Yasar Yildiz et al. 2014).

EPSs produced by extremophiles embody promising biotechnological applications: their rheological properties, biological activities, metal-binding capabilities and new sugar composition make these biopolymers suitable for many applications. Indeed their potential biotechnological spectrum of application is very wide, ranging from antiviral and thickening agents to bioflocculanting and viscosifying agents and from biosurfactant and bioemulsifier to vaccine adjuvants. Moreover, for their metal-binding and pollutant bioadsorption activities, EPSs could be employed in the bioremediation processes (Finore et al. 2014).

Microorganisms belonging to *Bacteria* and *Archaea* domains have been described as EPS producers: some representative examples are listed in Table 10.1. In the *Archaea* domain, *Sulfolobus solfataricus* strain MT4, *Haloferax mediterranei*, *Haloferax gibbonsii* and *Haloarcula japonica* strain T5 represent some example of species able to produce EPS for which the participation in biofilm formation and solid surface adhesion has been suggested (Poli et al. 2011). In the range of thermophilic EPS producers (Table 10.1), species like *Thermus aquaticus* strain YT-1, *Thermotoga maritima* and several species belonging to the *Geobacillus* genus (*G. thermantarcticus* strain M1, *G. tepidimans* strain V264, *G. thermodenitrificans* strain B3-72, *Geobacillus* sp. strain 4004) showed the EPS production. Moreover, species of *Bacillus* genus (such as *B. licheniformis* strain T4 isolated from a shallow hydrothermal vent of Panarea Island, Italy) and *Aeribacillus* genus (*Aeribacillus pallidus strain* 418 isolated from the Bulgarian Rupi hydrothermal springs) have been reported as responsible for the synthesis of novel EPSs, as shown in Table 10.1 (Kambourova et al. 2009; Nicolaus et al. 2010; Spanò et al. 2013 and references therein).

Pseudoalteromonas strain 721 isolated from deep-sea hydrothermal vents produced an EPS that exhibited gel formation and viscoelastic behaviour at increasing temperature. In the Antarctic and Arctic ecosystems (see also Table 10.1), *Pseudoalteromonas* sp. SM9913, *Pseudoalteromonas* strain CAM025 and CAM036 and *Colwellia psychrerythraea* strain 34H have been described as source of EPS from cold marine environments: in the case of *Pseudoalteromonas* CAM 025 and CAM036, sulphated heteropolysaccharides with high levels of uronic acids and acetyl groups have been described, and cryoprotection activities have been suggested (Mancuso Nichols et al. 2004 and references therein).

Table 10.1 Most remarkable examples of microbial species producing bioactive or biotechnologically useful EPSs

Group	Microbial species	Activities/biotechnological applications	References
Archaeabacteria	*Sulfolobus solfataricus* strain MT4 *Haloferax mediterranei* *Haloferax gibbonsii* *Haloarcula japonica* strain T5	EPS-producer strains Participation in biofilm formation and solid surface adhesion suggested	Poli et al. (2011) and references therein
Thermophiles	*Thermotoga maritima* *Geobacillus* genus*:* *G. thermantarcticus* strain M1 *G. tepidimans* strain V264 *Geobacillus* sp. strain 4004	Responsible for the synthesis of novel EPSs	Kambourova et al. (2009), Nicolaus et al. (2010), Spanò et al. (2013) and references therein, Mastascusa et al. (2014)
	Thermus aquaticus strain YT-1	TA-1 Immunomodulatory activity TA-1 stimulated macrophages cells to produce the cytokines TNF-α and IL-6	Lin et al. (2011)
	Geobacillus thermodenitrificans strain B3-72	EPS-2 stimulate the cytokine production (IL-12, IFN-γ, TNF-α and IL-18) in PBMC cells in a concentration-dependent manner EPS-2 treatment in PBMC affected on HSV-2 replication, increasing the inflammatory response	Arena et al. (2009)
Hydrothermal springs/vents	*Bacillus* genus (*Bacillus licheniformis* strain T4)	EPS-1, containing tetrasaccharide-repeating units formed by sugars with a mannano-pyranosidic configuration Immunomodulatory effect: production of IFN-α, IL-12, IFN-γ, TNF-α, IL-18 in vitro Inhibition of HSV-2 replication in PBMC (human peripheral blood mononuclear cells) by upregulating	Spanò et al. (2013), Arena et al. (2006)

(continued)

Table 10.1 (continued)

Group	Microbial species	Activities/biotechnological applications	References
		the expression of pro-inflammatory cytokines	
	Aeribacillus pallidus strain 418	EPSs with high molecular weight and high thermostabiliy	Kambourova et al. (2009), Nicolaus et al. (2010) and references therein
	Pseudoalteromonas strain 721	EPS with gelling properties and viscoelastic behaviour at increasing temperature	Poli et al. (2010) and references therein
Antarctic and Arctic ecosystems	*Pseudoalteromonas* sp. SM9913 *Colwellia psychrerythraea* strain 34H	Source of EPS from cold marine environments	Mancuso Nichols et al. (2004) and references therein
	Pseudoalteromonas strain CAM025 *Pseudoalteromonas* strain CAM036	Sulphated heteropolysaccharides with high levels of uronic acids and acetyl groups Cryoprotective agent	
Halophiles	*Halomonas maura* strain S-30 *Halomonas anticariensis* strain FP35 *Halomonas ventosae* strain A112 *Halomonas eurihalina* strain F2-7	Highly sulphated EPSs Formation of stable emulsions	Poli et al. (2009) and references therein
	Halomonas smyrnensis	First examples of levan producer *Halomonas* species Cytoprotective activity against toxic agents: Brine Shrimp Test Drugs delivery system: nano-carrier system for peptide and protein	Poli et al. (2009) and references therein Sezer et al. (2011)
	Halomonas alkaliantarctica	Fructo-glucan polymer with high viscosity at low pH values/high NaCl concentration Proposed as viscosity control agent	Poli et al. (2010) and references therein
	Salipiger mucosus A3T	EPS rich of fucose. Proposed as source of fucose	Llamas et al. (2010)
	Zunongwangia profunda SM-A87	First marine bacterium that produces EPS with the high yield of 8.90 g/L	Qin et al. (2010)

Saline soils collected from salterns and sediments from salt lakes represent the extreme niches in which halophiles EPSs producers have been isolated: some main examples are reported in Table 10.1. High sulphate content EPSs have been described for the EPSs produced by *Halomonas maura* strain S-30, *Halomonas anticariensis* strain FP35, *Halomonas ventosae* strain A112 and *Halomonas eurihalina* strain F2-7: for these two latters, the formation of stable emulsions has been also recorded (Poli et al. 2010 and references therein). *Halomonas smyrnensis*, isolated from a saltern area in Turkey, represents the first examples of levan producer *Halomonas* species (Poli et al. 2009). This microorganism could be considered an alternative cheap source of levan polymer for which bioflocculant properties in the treatment of industrial wastewaters have been described (Table 10.1). Levan from *H. smyrnensis* has been tried as a nano-carrier system for peptide and protein drugs delivery. *Halomonas alkaliantarctica* isolated from a salt lake in Antarctica produced a fructo-glucan polymer that presented a high viscosity at low pH values and at high NaCl concentration, resulting as a viscosity control agent in proposed biotechnological applications (Poli et al. 2010). *Salipiger mucosus* A3T (Table 10.1), a halophilic species belonging to the *Alphaproteobacteria* genus and isolated from the Spanish Mediterranean seaboard, produced an EPS rich of fucose that was synthesised essentially during the exponential phase growth (Llamas et al. 2010): the authors suggested its use as source of fucose in spite of fucose-rich polysaccharides usually extracted from brown algae with more laborious and expensive procedures. *Zunongwangia profunda* SM-A87 isolated from deep-sea sediment in southern Okinawa Trough is the first marine bacterium that produces EPS with the high yield of 8.90 g/L (Qin et al. 2010).

Bacterial polysaccharides are comprised of repeating units of sugar moieties, which are synthesised by a group of enzymes named 'glycosyltransferases'. In some cases the EPS biosynthetic pathway is known, and the genetic manipulation has been employed in order to not only optimise the yield of EPS production but also to tailor the EPS chemical composition and structure. In fact, in addition to elucidation of biosynthesis mechanisms, it is possible to improve the microbial productivity via strain improvement strategies or to modify physicochemical and/or rheological properties of the biopolymer by changing its composition, length or degree of branching (Nicolaus et al. 2010). There are three main biosynthetic routes for microbial EPSs. The synthesis of some glucan- or fructan-type homopolysaccharides is carried out in the extracellular environment through the action of specifically secreted sucrase enzymes (Nicolaus et al. 2010). These glucansucrases and fructansucrases are glycoside hydrolases that act on sucrose and catalyse the transglycosylation reactions forming the polymer chain. Biosynthetic pathways of microbial heteropolysaccharides and of some homopolysaccharides are more complex and involve five distinct steps: (1) uptake of sugar subunits and their activation with a high-energy bond through their conversion into sugar nucleotides, (2) assembly of the repeating monosaccharide unit on an isoprenoid lipid carrier by sequential transfer of monosaccharides from sugar nucleotides by glycosyltransferases, (3) addition of any acyl groups, (4) polymerisation of the repeating unit and (5) secretion of the polysaccharide from the cell membrane into the extracellular environment. Despite the structural diversity of EPSs, there are only three

mechanisms for the polymerisation, namely, ABC-transporter-dependent, synthase-dependent and the most commonly used wzy-dependent pathways (Nicolaus et al. 2010).

Analysis of the flanking regions of a mini-Tn5 insertion site in an EPS-deficient mutant of *Halomonas maura* strain TK71 led to the identification of five ORFs (epsABCDJ), which form part of a gene cluster (*eps*) with the same structural organisation as others involved in the biosynthesis of group 1 capsules and some EPSs. The possibility that mauran, the polysaccharide produced by *H. maura*, might be synthesised via a Wzy-like biosynthesis system is reported by Arco et al. (2005).

The genomes of three model extreme thermophiles, an archaeon, *Pyrococcus furiosus* (*T*opt of 98 °C), and two bacteria, *Thermotoga maritima* (*T*opt of 80 °C) and *Caldicellulosiruptor saccharolyticus* (*T*opt of 70 °C), encode numerous carbohydrate-active enzymes, many of which have been biochemically characterised in their native or recombinant forms. In *Streptococcus thermophilus* Sfi6 the biosynthesis of exopolysaccharides is regulated by eps gene cluster of 14.5-kb region comprised of 13 genes, namely, epsA to epsM (Stingele et al. 1996). Gene epsA located at the beginning of the cluster is involved in the regulation of EPS expression, and the central region (epsE, epsF, epsG, epsH and epsI) of the gene cluster is involved in the biosynthesis of the tetramer repeating unit. Gene epsE encodes the galactosyltransferase, catalysing the first step of biosynthesis of the repeating unit. Genes upstream (epsC and epsD) and downstream (epsJ and epsK) of the central region regulate polymerisation and export of the EPS (Stingele et al. 1996). In the case of *Zunongwangia profunda* SM-A87, its genome was the first sequenced in the phylum of *Bacteroidetes* (Qin et al. 2010). It possesses two polysaccharide biosynthesis gene clusters, and the genome analysis reveals its adaptation to the deep-sea environment and ecological role in sedimentary organic nitrogen degradation.

For a systems-based approach to the EPS biosynthetic pathways, microbial genome sequence is considered as a starting point, and from this point of view, next-generation sequencing (NGS) technologies play a significant role by enabling high-throughput genomic data at very high speed with a relatively low cost. When the whole-genome sequence of the EPS-producer microorganism is not available, sequence data of a taxonomically close species could also be used for systems-based studies. This approach has been used for the improvement of levan production by the halophilic strain *Halomonas smyrnensis* AAD6^T, where first, the available whole-genome sequence of a taxonomically close microorganism, *Chromohalobacter salexigens* DSM 3043, was used to construct a comprehensive genome-scale metabolic model, and then this model was recruited and adopted to the producer strain via integration of the available biochemical, physiological and phenotypic features of *H. smyrnensis* AAD6^T. With metabolic system analysis of this generic metabolic model, significant improvement in levan yields was obtained (Ateş et al. 2011, 2013).

Considering that the microbial biodiversity of marine and terrestrial ecosystems is relatively unexplored, it is reasonable to hypothesise that the isolation and identification of new microorganisms will provide wide opportunities for new industrial fields.

10.3 Most Remarkable Examples of Bioactive EPS

Natural bioactive polysaccharides isolated from several sources have attracted much attention in the field of biochemistry and pharmacology: indeed several polysaccharides or their glycoconjugates have been shown to exhibit multiple biological activities including anti-inflammatory, immunostimulating, antiviral, antioxidant, etc.

Moreover, polysaccharides are biodegradable materials expressing biocompatibility; thus, they could represent as versatile tools for application in biomedical fields such as tissue engineering, drug delivery, prostheses and medical devices. İn the following sections, some examples of bioactive EPS, also listed in Table 10.1, are discussed in detail.

10.3.1 Anti-inflammatory/Immunomodulating

Inflammation is a complex and well-coordinated response of the innate and adaptive immune system following infection or injury. This process is characterised by a vascular response and recruitment of circulating leukocytes, defined initially by polymorphonuclear granulocytes followed by monocytes, which differentiate locally into macrophages. Host-defence mechanisms are divided into two distinct, but inextricably linked, pathways: the innate and the adaptive immune responses. The first one mounts a rapid response to injury by means of phagocytosis, production of reactive oxygen species (ROS) and release of cytokines, autacoids and lipid mediators that coordinate and amplify the local inflammatory response. On the contrary, the adaptive immune response mounts more slowly and furnishes a more focused response mechanism that requires the identification of specific molecular structures and depends on the generation of large numbers of antigen receptors (i.e. T-cell receptors and immunoglobulins). The resolution of inflammation occurs mainly via clearance of apoptotic cells by phagocytosis and by the production of anti-inflammatory mediators, such as IL-10 and TGF-β, in the lesion by tissue macrophages that phagocyted apoptotic cells.

An exopolysaccharide named EPS-1, containing tetrasaccharide-repeating units formed by sugars with a mannano-pyranosidic configuration, was isolated from a halophilic and thermotolerant *Bacillus licheniformis* strain (Table 10.1). EPS-1 was tested for its immunomodulatory effect by means of the production of different cytokines (IFN-α, IL-12; IFN-γ, TNF-α, IL-18) involved in the immune response in human peripheral blood mononuclear cells (PBMC) during the HSV-2 virus infection. Results showed that EPS-1 affected on cytokines production in a dose-dependent manner stimulating IFN-α production. Moreover, EPS-1 induced the production of IL-12 at considerable levels; IFN-γ and TNF-α were also detected (Arena et al. 2006). Later, Arena et al. (2009) (Table 10.1) reported the immunomodulatory and antiviral effects of an exopolysaccharide EPS-2, isolated from a strain of *Geobacillus thermodenitrificans*. EPS-2 also stimulated the cytokine

production in PBMC cells in a concentration-dependent manner, like EPS-1. A high level of IL-12, IFN-γ, TNF-α and IL-18 was revealed after EPS-2 treatment (Arena et al. 2009).

Bacteria produce a high quantity of exopolysaccharides when they form biofilm, which is a consortium of microorganisms immobilised and penned within EPS that is able to limit the diffusion of substance and antimicrobial compounds. From the biofilm of the thermophilic bacteria *Thermus aquaticus* YT-1, a novel EPS named TA-1 with interesting immunomodulatory effects was isolated (Table 10.1). TA-1 stimulated macrophages cells to produce the cytokines TNF-α and IL-6 (Lin et al. 2011).

10.3.2 Antiviral

Pathogenesis is the process by which virus infection leads to disease. Pathogenic mechanisms include implantation of the virus at a body site, replication at that site and then spread to and multiplication within sites (target organs) where disease or shedding of virus into the environment occurs. Viruses cannot synthesise their genetic and structural components, and so they rely almost exclusively on the host cell for these functions. Pathogenesis at the cellular level can be viewed as a process that occurs in progressive stages leading to cellular disease. An essential aspect of viral pathogenesis at the cellular level is the competition between the synthetic needs of the virus and those of the host cell.

The antiviral activity linked to the immunoregulatory effect of bacterial polysaccharides was reported by Arena et al. (2006, 2009). In the first paper, the antiviral effect of a novel exopolysaccharide EPS-1 produced by a strain of thermotolerant *Bacillus licheniformis* (Table 10.1), isolated from a hot spring of Vulcano Island (Italy), has been reported. EPS-1 inhibited HSV-2 replication in PBMC (human peripheral blood mononuclear cells) by upregulating the expression of pro-inflammatory cytokines. In a later paper, an antiviral exopolysaccharide EPS-2, produced by a strain of *Geobacillus thermodenitrificans*, is reported. EPS-2 treatment in PBMC, at a concentration of 200 and 300 μg/mL, is affected on HSV-2 replication, in a concentration-dependent way, increasing the inflammatory response (Arena et al. 2009).

10.3.3 Application in Drug Delivery Systems

EPSs have the capacity to establish polymeric matrices. This ability enables their in vitro manipulation to create novel structures in which bioactive compounds are encapsulated, developing new applications in drug delivery systems.

Halomonas smyrnensis strain AAD6 (JCM 15723) (Table 10.1) was reported as a producer of high levels of levan exopolysaccharide. Studies on biocompatibility

were performed, and the results exhibited that this levan did not affect cellular viability and proliferation of osteoblasts and murine macrophages. Moreover, the toxicity test carried out by Brine Shrimp Test showed a protective effect of levan against a toxic agent (Poli et al. 2009). Because of the amphiphilic nature of levan, it is able to form nanoparticles by self-assembly in water. The levan EPS isolated from strain AAD6 was studied for its potential use as a biopolymer in nanoparticle drug delivery system. Levan nanoparticles encapsulated with bovine serum albumin (BSA) were prepared and used as a model to investigate their different properties. The size of nanoparticles varied between 200 nm and 537 nm, and their encapsulation capacity also varied (ranged between 49.3% and 71.3%) depending on the levan concentration used. Moreover, the increasing in vitro release of BSA from the nanoparticles was displayed to be controlled release of proteins (Sezer et al. 2011).

10.4 Chemical and Structural Characterisation

The complete characterisation of an unknown EPS requires the definition of the polymer's chemical composition and its structural analysis. The chemical composition is defined on the basis of the total carbohydrate content and of other molecules, such as proteins, and on the identification of the monosaccharide unit type of their relative molar ratio and of the presence of substituting groups. The structural analysis is defined by determining the sequence of glycosidic bonds and the possible tridimensional distribution of monomer components, i.e. the polysaccharide conformation. These tasks are accomplished by combining different analytical and instrumental techniques, i.e. chromatography, spectrometry and spectroscopy, which are used along all the steps of exopolysaccharide characterisation. The different methods and techniques required for the complete EPSs characterisation are listed in Table 10.2 and will be discussed in detail in the following sections.

10.4.1 Purification

The purification of EPSs is the first step of the study: usually intact cells are removed by heating the bacterial culture broth at 90–95 °C (in order to denature enzymes that hydrolyse the polysaccharide) followed by centrifugation. The EPS can be separated from the broth by means of precipitation (by adding alcohol or complexing metal ions) or ultrafiltration. The crude EPS obtained is then purified by combining different chromatography techniques, for example, gel filtration chromatography and anion-exchange chromatography. Usually the purified EPS is freeze-dried to be stored for long times in order to perform the chemical and structural characterisation.

Table 10.2 Summary of main methods required for EPSs characterisation and relative information obtained

Technique/method	Type of information
Dubois method	Total carbohydrate content
Bradford method	Total protein content
• TFA hydrolysis at 110–120 °C/ per acetylation or silylation/ GC analysis • TFA hydrolysis at 110–120 °C/HPAE-PAD analysis	Monosaccharide composition
Methylation analysis (MA)/ GC-MS analysis of alditol acetates	Determination of linkage positions
• Size-exclusion chromatography (SEC) • HPSEC with refractive index (RI) detection • HPSEC with multi-angle laser light scatter (MALLS) detection	Molecular-weight distribution
Sedimentation analysis	Intrinsic viscosity η
Nuclear magnetic resonance: • 1D NMR (^{1}H NMR/^{13}C NMR) • 2D homo- and heteronuclear correlation (COSY, HSQC, HMQC or HMBC, TOCSY, NOESY)	• Presence and nature of substituting groups • Number/type of monomer sugar residues • Anomer configurations • Monosaccharide sequence in the polymer backbone
Fourier transform-infrared spectroscopy (FT-IR)	Presence of functional and substituting groups

10.4.2 Compositional Analysis

The gross chemical composition of an EPS is determined by measuring the total carbohydrate content, the protein and nucleic acid contents: the total carbohydrate content can be assessed by means of the Dubois method based on the use of phenol-sulphuric acid reagent; the total protein content is estimated using the Bradford method; finally the nucleic acids' content is determined spectrophotometrically by measuring absorbance at λ 260 nm. Uronic acid content is another important chemical feature of EPS that can be determined according to the method described by Blumenkrantz and Asboe-Hansen (Spanò et al. 2013).

10.4.3 Determination of Monosaccharide Composition, Glycoside Linkage Position and Substituting Groups

The most common sugars that can be found as constituents of bacterial EPS include hexoses (D-glucose, D-galactose, D-mannose, D-allose, L-fucose, L-rhamnose), pentoses (D-ribose, D-arabinose, D-xylose), uronic acids (D-glucuronic, D-mannuronic and D-galacturonic acids) and amino sugars (D-glucosamine, D-galactosamine). The nature and the molar ratio of the monosaccharides that constitute an EPS can be

determined by chemolytic methods, i.e. total hydrolysis of EPS that is carried out at high temperature (up to 110–120 °C) in strong acidic conditions by using concentrated trifluoroacetic (TFA) or formic or sulphuric acids. The resulting hydrolysis mixture can be analysed after reduction followed by per-acetylation or silylation that converts the released monomer sugars in the respective volatile derivatives (alditol acetates or methylsilanes) that can be analysed by means of gas chromatography coupled with mass spectrometry (GC-MS). Alternatively, the hydrolysis mixture can be analysed without any derivatisation of monosaccharides, by means of liquid chromatography methods like the high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), that is, a well-established method that allows either qualitative and quantitative analysis of monomer sugars released by acid. Chemolytic methods, for example, the methylation analysis (MA), are also employed for the determination of linkage positions. Commonly MA requires the following steps: methylation, by addition of methyl iodide; cleavage of glycosidic bonds, by classic acidic hydrolysis or by methanolysis (with methanolic HCl); reduction with $NaBH_4$ followed by acetylation or silylation; and GC-MS analysis of the resulting mixture of volatile alditol acetates or methylsilanes. This treatment causes methylation of the free OH groups and acetylation or silylation only for the OH groups involved in the glycoside bonds: in such a way the position of glycosidic linkages will be identified as those corresponding to the non-methylated OH groups present in the alditol derivatives. Different organic and inorganic substituent groups can be present in the EPS that also significantly affect their chemical and biological properties. The most common organic substituents include ester-linked residues of acetate, succinate, propionate, glycerate and hydroxybutanoate, the ketal-linked pyruvate unit or amino acids like L-glutamate or serine. The most common inorganic substituents are sulphate and phosphate groups. The presence of the substituting groups can be assessed by means of Fourier transform-infrared spectroscopy (FT-IR) or nuclear magnetic resonance (NMR) (Mishra and Jha 2013).

10.4.4 Molecular-Weight Distribution

The molecular-weight (M_w) distribution of an EPS is an important structural feature that often also affects biological activity of this kind of polymer. Different techniques can be employed for this purpose, for example, light scattering, sedimentation analysis in analytical ultracentrifugation, intrinsic viscosity determination, size-exclusion chromatography (SEC) or the more modern high-performance size-exclusion chromatography (HPSEC) techniques with refractive index (RI) detection or multi-angle laser light scatter (MALLS) detection.

Light scattering allows to measure the absolute M_w using the relationship, stated by the Rayleigh theory, between the intensity of light scattered by a molecule and its molecular weight and size. Sedimentation analysis is based on the determination of sedimentation velocity of polysaccharides during ultracentrifugation: the value of

M_w is deduced from the changes in the refractive index of the polysaccharide solution when it is subjected to high force fields. The measure of the intrinsic viscosity η is used for M_w determination at infinite dilution. In such conditions, η is a function of the average molecular weight (M_w) and can be calculated by means of the Mark-Houwink-Sakurada equation $\eta = K M_w{}^{\alpha}$ where K and α are constants for a given polymer/solvent/temperature system.

Size-exclusion chromatography (SEC) is widely employed for the M_w characterisation of different kinds of polymers. This technique is based on the use of soft gels, for example, Sepharose CL-4B or porous polystyrene-divinylbenzene resins, and the M_w is calculated by comparison with external standard calibration curves derived by using pullulans and dextrans as standard polymers. Significant improvements in the use of this technique have been achieved by coupling with online detectors like light-scattering detectors and viscometers. More recent developments of SEC are represented by high-performance size-exclusion chromatography (HPSEC) technique using online detectors like refractive index (RI) or multi-angle laser light scatter (MALLS) detectors. Although widely used, such methods present some major drawbacks like longer time of analysis and need of higher amount of samples. For such reasons the use of HPSEC methods is emerging since they afford faster analyses and requires smaller quantities of samples. HPSEC columns are made of rigid matrixes of small porous silica microspheres as such or bonded with glycerylpropyl groups, of agarose or methacrylate polymers. The online detection is often performed with RI, a universal detector that is useful for the determination of M_w distribution since there is a linear correlation between the EPS concentration and its M_w. Nevertheless, higher accuracy can be achieved by using a MALLS detector that allows the absolute determination of M_w that is based on the calculation of the amount of scattered light at each angle detected, thus not requiring external calibration curves (Gómez-Ordonez et al. 2012).

10.4.5 Structural Analysis by NMR and FT-IR Techniques

NMR spectroscopy is a useful tool for the determination of both polysaccharides' composition and conformation. The structure elucidation of EPSs relies on the use of both 1D and 2D ^{1}H- and ^{13}C-NMR techniques and of relevant databases such as Carb-Bank, SUGABASE or CASPER. Indeed ^{1}H and ^{13}C chemical shift and coupling constant values are available in literature for many monosaccharides and can be used for assignment of polysaccharide resonances. 1D ^{1}H and ^{13}C techniques can afford a good estimation of the number of sugar residues present in an EPS: identification and integration of signals in the anomeric region of ^{1}H, corresponding to δ 4.4 – 5.5 ppm, can give such an information that is confirmed by the analysis of the anomeric region of 1D-^{13}C spectrum. Alternatively, this information can be gained by applying 2D heteronuclear techniques like HSQC, HMQC or HMBC that are more sensitive than single mono-dimensional analysis since they can resolve complex signal patterns that usually characterise the anomeric region of ^{1}H

spectrum. The following step is represented by the determination of the type of monosaccharides that constitute the EPS that can be identified by means of two-dimensional homonuclear and heteronuclear techniques since the typical chemical shift values and J-coupling patterns of most monosaccharides are listed in several databases. The homonuclear TOCSY or DQF-COSY spectra afford information on the spin system of single monosaccharides: such techniques allow resolution of the so-called bulk region of ^{1}H spectrum in which usually the resonance relatives to non-anomeric protons strongly overlap. The ^{13}C shift values relative to such protons are then easily deduced by comparison with HSQC or HMBC spectra. NMR can also be used for assignment of anomeric configuration since usually the β-anomers of pyranose sugars resonate at higher field than the corresponding α-isomers; in addition the calculation of direct coupling constant $^{1}H–^{13}C$ for the anomer proton ($J_{C1,H1}$) can also be useful, since usually its value is about 170 Hz for the α-anomeric sugars, while for the β forms it is ~ 160 Hz.

The sequence of the monosaccharide residues within the EPS can also be achieved by means of 2D techniques like NOESY and HMBC. In particular, NOESY spectrum allows to identify NOEs (nuclear Overhauser effects) between signals belonging to directly linked monosaccharide residues, thus affording information on glycosidic linkages sequence along the polysaccharide backbone.

Finally, NMR can be used for identification of substituents since the signals of most organic groups, for example, acyl or methyl groups, do not overlap to carbohydrate resonances, being found in high field regions at about 0.2–0.5 ppm. On the other hand, the presence of inorganic groups like phosphates can be assessed by means of heteronuclear $^{31}P–^{1}H$ long-range coupling measurements. The interpretation of such complex data can be supported by the use of software programmes, for example, PRONTO freely available at this link: http://www.crc.dk/chem/pronto/welcome.html (Duus et al. 2000).

Fourier transform-infrared spectroscopy (FT-IR) is another spectral technique that can be applied to EPS structural investigation. Indeed some typical signals support polysaccharide recognition like the signals in the fingerprint region, i.e. below 1500 cm^{-1}, where broad stretching bands relative to C–O–C and C–O (at 1700 cm^{-1}) ring bonds and C–H bending (at 1410 cm^{-1}) are typically found; like the stretching peak of hydroxyl groups (usually at around 3300 cm^{-1}); or, finally, like the signals around 2900 cm^{-1} attributable to the C–H stretching of either methyl or methylene groups.

10.5 Conclusions and Future Perspectives

EPSs are extracellular biopolymers produced by many extremophilic microorganisms that use them as a valuable adaptation strategy to extreme environmental conditions. EPSs protect extremophiles from the deleterious effects of extreme pH, temperature, salt concentration or radiation by forming capsular materials or dispersed biofilm in the surrounding environment. Indeed, several extremophiles,

isolated either from aquatic or terrestrial environments, have been shown to be EPSs producers. Notably, these biopolymers represent a promising class of compounds for applications in several fields, i.e. pharmaceutical, food or biotechnology sectors since they possess interesting properties, for example, biological and biotechnological properties. Indeed several examples of bioactive EPSs acting as anti-inflammatory, immunomodulating or antiviral agents are described in literature. Other promising applications in the pharmaceutical fields are as dug delivers or as vaccine adjuvants. Moreover thanks to their features they can act as metal binders, thus affording a valuable tool for bioremediation processes, or as biosurfactant or bioemulsifier, thanks to their rheological properties like viscosity or thermostability. Many other possible applications can be found for extremophiles EPSs that in future could play a pivotal role in biotechnology.

Take-Home Message

- EPSs are produced by several extremophilic microorganisms, belonging to both *Archaea* and *Bacteria* domains that have been isolated from different ecosystems.
- The biosynthesis of EPSs can take place either into the cell or in the extracellular milieu; it follows three main routes, all regulated by a group of enzymes named 'glycosyltransferases'.
- The genetic manipulation of the EPSs' producing bacteria has been employed to optimise the yield of and for tailoring polymers' chemical composition and structure.
- The structural characterisation of EPSs requires a combination of analytical and instrumental techniques that include chromatography, IR spectroscopy, 1D and 2D NMR (^{1}H NMR/^{13}C NMR).
- The extremophiles' EPSs possess unique properties that can be exploited for a variety of biological and biotechnological applications.

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References

Arco Y, Llamas I, Martınez-Checa F, Argandona M, Quesada E, del Moral A (2005) epsABCJ genes are involved in the biosynthesis of the exopolysaccharide mauran produced by *Halomonas maura*. Microbiology 151(9):2841–2851

Arena A, Maugeri TL, Pavone B, Iannello D, Gugliandolo C, Bisignano G (2006) Antiviral and immunoregulatory effect of a novel exopolysaccharide from a marine thermotolerant *Bacillus licheniformis*. Int Immunopharmacol 6(1):8–13

Arena A, Gugliandolo C, Stassi G, Pavone B, Iannello D, Bisignano G, Maugeri TL (2009) An exopolysaccharide produced by *Geobacillus thermodenitrificans* strain B3-72: antiviral activity on immunocompetent cells. Immunol Lett 123(2):132–137

Ateş Ö, Toksoy Öner E, Arga KY (2011) Genome-scale reconstruction of metabolic network for a halophilic extremophile, *Chromohalobacter salexigens* DSM 3043. BMC Syst Biol 5:12

Ateş Ö, Arga KY, Toksoy Öner E (2013) The stimulatory effect of mannitol on levan biosynthesis: lessons from metabolic systems analysis of *Halomonas smyrnensis* AAD6(T). Biotechnol Prog 29:1386–1397

Duus JO, Gotfredsen CH, Bock K (2000) Carbohydrate structural determination by NMR spectroscopy: modern methods and limitations. Chem Rev 100:4589–4614

Finore I, Di Donato P, Mastascusa V, Nicolaus B, Poli A (2014) Fermentation technologies for the optimization of marine microbial exopolysaccharide production. Mar Drugs 12:3005–3024. https://doi.org/10.3390/md12053005

Freitas F, Alves VD, Reis M (2011) Advances in bacterial exopolysaccharides: from production to biotechnological applications. Trends Biotechnol 29(8):388–398. https://doi.org/10.1016/j.tibtech.2011.03.008

Gómez-Ordonez E, Jiménez-Escrig A, Rupérez P (2012) Molecular weight distribution of polysaccharides from edible seaweeds by high-performance size-exclusion chromatography (HPSEC). Talanta 93:153–159

Kambourova M, Mandeva R, Dimova D, Poli A, Nicolaus B, Tommonaro G (2009) Production and characterization of a microbial glucan, synthesized by *Geobacillus tepidamans* V264 isolated from Bulgarian hot spring. Carbohydr Polym 77(2):338–343

Lin MH, Yang YL, Chen YP, Hua KF, Lu CP, Sheu F, Lin GH, Tsay SS, Liang SM, Wu SH (2011) A novel exopolysaccharide from the biofilm of *Thermus aquaticus* YT-1 induces the immune response through Toll-like receptor 2. J Biol Chem 286(20):17736–17745. https://doi.org/10.1074/jbc.M110.200113

Llamas L, Mata JA, Tallon R, Bressollier P, Urdaci MC, Quesada E, Bejar V (2010) Characterization of the exopolysaccharide produced by *Salipiger mucosus* A3T, a halophilic species belonging to the Alphaproteobacteria, isolated on the Spanish Mediterranean seaboard. Mar Drugs 8(8):2240–2251

Mancuso Nichols CA, Garon S, Bowman JP, Raguénès G, Guézennec J (2004) Production of exopolysaccharides by Antarctic marine bacterial isolates. J Appl Microbiol 96:1057–1066. https://doi.org/10.1111/j.1365-2672.2004.02216.x

Mastascusa V, Romano I, Di Donato P, Poli A, Della Corte V, Rotundi A, Bussoletti E, Quarto M, Pugliese M, Nicolaus B (2014) Extremophiles survival to simulated space conditions: an astrobiology model study. Orig Life Evol Biosph 44(3):231–237

Mishra A, Jha B (2013) Microbial exopolysaccharides. In: Rosenberg E et al (eds) The prokaryotes – applied bacteriology and biotechnology. Springer, Berlin, pp 179–192. https://doi.org/10.1007/978-3-642-31331-8_25

Nicolaus B, Kambourova M, Toksoy Oner E (2010) Exopolysaccharides from extremophiles: From fundamentals to biotechnology. Environ Technol "Special Issue Extremophiles" 31 (10):1145–1158

Poli A, Kazak H, Gurleyendag B, Tommonaro G, Pieretti G, Toksoy Oner E, Nicolaus B (2009) High level synthesis of levan by a novel *Halomonas* species growing on defined media. Carbohydr Polym 78(4):651–657

Poli A, Anzelmo A, Nicolaus B (2010) Bacterial exopolysaccharides from extreme marine habitats: production, characterization and biological activities. Mar Drugs 8(6):1779–1802

Poli A, Di Donato P, Abbamondi GR, Nicolaus B (2011) Synthesis, production, and biotechnological applications of exopolysaccharides and polyhydroxyalkanoates by archaea. Archaea 2011:693253. https://doi.org/10.1155/2011/693253

Qin QL, Zhang XY, Wang XM, Liu GM, Chen XL, Xi BB, Dang HY, Zhou BC, Yu J, Zhang YZ (2010) The complete genome of *Zunongwangia profunda* SM-A87 reveals its adaptation to the deep-sea environment and ecological role in sedimentary organic nitrogen degradation. Genomics 11:247. https://doi.org/10.1186/1471-2164-11-247

Sezer AD, Kazak H, Toksoy E, Akbuga J (2011) Levan-based nanocarrier system for peptide and protein drug delivery: optimization and influence of experimental parameters on the nanoparticle characteristics. Carbohydr Polym 84(1):358–363

Spanò A, Gugliandolo C, Lentini V, Maugeri TL, Anzelmo G, Poli A, Nicolaus B (2013) A novel EPS-producing strain of *Bacillus licheniformis* isolated from a shallow vent off Panarea Island (Italy). Curr Microbiol 67:21–29. https://doi.org/10.1007/s00284-013-0327-4

Stingele F, Neeser JR, Mollet B (1996) Identification and characterization of the eps (exopolysaccharide) gene cluster from *Streptococcus thermophilus* Sfi6. J Bacteriol 178:1680–1690

Yasar Yildiz S, Anzelmo A, Ozer T, Radchenkova N, Genc S, Di Donato P, Nicolaus B, Toksoy Oner E, Kambourova M (2014) *Brevibacillus themoruber*: a promising microbial cell factory for exopolysaccharide production. J Appl Microbiol 116:314–324. https://doi.org/10.1111/jam.12362