Review article

Articulating the exuberant intricacies of bacterial exopolysaccharides to purge environmental pollutants

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A B S T R A C T

Microbial exopolysaccharide (EPS) is composed of a mixture of macromolecules such as proteins, polysaccharides, humic-like compounds, and nucleic acids, which encase microbial cells in a three-dimensional matrix. The literature shows that the EPS possess significant properties such as renewable, biodegradable, eco-friendly, non-toxic, and economically valued product, representing it as a green alternative to the synthetic polymer. The cost-effective and green synthesis of the EPS must be encouraged by using agro-waste as a raw material. The main objective of the manuscript is to provide a comprehensive update on the various aspects pertaining to EPS, including the economic aspects of EPS production, provide an insight into the latest tools and techniques used for detailed structural EPS characterization along with updates in the integration of CRISPR/Cas9 technology for engineering the modification in EPS production, the role of newly discovered EPR3 as a signalling molecule in plant growth-promoting properties (PGP) or agricultural microbiology. Furthermore, the EPS achieved prospective interest prevailing potential environmental issues which can be subject to EPS treatment including, landfill leachate treatment, decolourization of dye from the effluent or waste generated by an industry, removal of radionuclides, heavy metals and toxic compounds from the various environments (aquatic and terrestrial), industry effluents, waste waters etc. are comprehensively discussed.

1. Introduction

Microorganisms produce a variety of biopolymers with different functionalities. Biopolymer's development is a general feature of microorganisms, inhabitants of a natural environment, including eukaryotes and prokaryotes. Bacterial biopolymers are classified into 4 groups: polysaccharides, polyesters, polyamides and inorganic polyanhydrides [1]. Based on their relative location, the biopolymers can either be intracellular or extracellular. The former is synthesized and accumulated only within the cell, while the latter is synthesized inside the cell and transported out by enzyme machinery or synthesized extracellularly with the aid of enzymes present on the cell wall. The polysaccharides account for most of the extracellular polymers' components [2]. Bacterial polysaccharides are high molecular-weight carbohydrate polymers grouped as capsular polysaccharides (CPS), lipopolysaccharides (LPS) and exopolysaccharides (EPS). Bacterial exopolysaccharide (EPS) acts as a matrix for biofilm development, providing a cohesive force for their attachment [3]. Different forms of exopolysaccharide structures exist, which include

capsule, slime and sheath. The capsule is defined as a discrete structure with definite outlines firmly attached to the cell's surface through non-covalent interactions or maybe covalently bound [4, 5]. Sheath is a linear EPS containing structure analogous to a capsule found in non-filamentous organisms surrounding the chain of cells. Slime is defined as a less organized EPS structure attached loosely to the cell surface and easily extracted into the surrounding environment [4].

Microbial EPS shows notable diversity in their structure and compositions. Homopolysaccharide and heteropolysaccharides are the two recognized groups based on the monomer present in EPS composition [6]. The critical components of EPS are carbohydrates, proteins, humic substances and nucleic acids [7]. The presence of various components in EPS attributes the properties such as adsorption, biodegradability, hydrophobicity/hydrophilicity [7]. In a biofilm matrix, EPS has an important role, as it promotes the formation of synergistic micro-consortia and cell-cell communication by immobilizing cells in proximity. EPS is vital in providing structural support and integrity in the development of biofilm. EPS plays a critical role in mass transfer; biofilm

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mediated adsorption of metals, defensive agent, signalling molecules, cell-cell interaction, biofilm formation, cryoprotectant, organic and inorganic compounds and; also acts as a source of nutrients [7]. Natural polysaccharides are found in a wide variety of living species, including bacteria, fungus, plants, and algae; However, bacterial and fungal EPS differs from plant and algal-based EPS in terms of purity and environmental safe. The extensive layer of loosely packed extracellular polymer "slime" produced by actively decomposing fungal cells is called "fungal extracellular polymeric substance matrix" [8, 9]. It highly resembles to the EPS produced by the bacteria. Nonetheless, both bacterial and fungal EPS are polysaccharides, glycolipids, glycoproteins etc. [10]. The difference in the incubation time, fermentation condition, production media are noted in the EPS production of bacteria and fungi.

An exceptional biodegradability, biocompatibility, cost-effective, reproducible physicochemical properties, higher adsorption ability, hydrophobicity/hydrophilicity, non-toxic nature for both humans and the environment make microbes derived EPS a potential alternative to conventional chemical polymers [7]. Ecological niche and natural environment influence the physiological role of EPS. Bacterial EPS encompass a wide range of composition, structure biosynthetic pathways, and extensively studied unique functional properties. Bacterial EPS have immense applications in the food processing industry, paper and textile, beverages industry, medical areas, biotechnology, agricultural sector, paint, cosmetic, petroleum industries [3]. EPS producing lactic acid bacteria (LAB) are widely used in the food industry as a starter culture to prepare fermented dairy products and as a coadjutant. Xanthan and curdlan are also used as food additives due to their thickening and gelling properties. EPS such as hyaluronic acid, levan, xanthan, bacterial cellulose, gellan gum are used to prepare cosmetics. Due to stabilising properties, better cross-linking ability with fabrics, bacterial cellulose and alginate can be used in the textile industry [11]. Dextran and hyaluronic acid are used in various medical applications such as blood plasma extenders and drug delivery respectively. Microbial EPSs with biomedical applications also include cellulose, dextran, xanthan gum, hyaluronic acid, kefiran, gellan, levan, and curdlan [12]. However, of all the bacterial EPS reported, only a few found commercial significance and produced industrially. The major limitation of EPS is the economic feasibility assigned to its industrial production [13]. The difference in the incubation time, fermentation condition, production media are noted in the EPS production of bacteria and fungi.

The main objective of the manuscript is to provide a comprehensive update on the various aspects pertaining to EPS including the economic aspects of EPS production, provide an insight into the latest tools and techniques used for detailed structural EPS characterization along with updates in the integration of CRISPR/Cas9 technology for EPS production, the role of newly discovered EPR3 as a signalling molecule in PGP or agricultural microbiology and environmental applications of EPSs.

2. Fermentation processes for microbial EPS production

Microbes are extensively researched and sought after for their metabolic products produced through fermentation, which are in high demand in the market. Fermentation is an exceptionally resourceful technology to produce highly valuable materials such as biopolymers to cope with the demands. The expansion of fermentation industries has resulted from the exploitation of microbial diversity. Despite having needs for biopolymers, efficiency in the production and minimizing the production cost is crucial to mark its place as a commodity product in large markets [14]. Fermentation factors highly influence the feasibility and cost of a fermentation process. As a result, optimizing a critical parameter is a precondition for designing a fermentation process [15].

Further, the production cost of EPS can be minimized by (i) replacing a pure substrate with a cheaper and natural one, (ii) optimized condition for enhanced yield, (iii) development of potential strain through genetic manipulation or mutagenesis and, (iv) proper downstream processing [14]. Fermentation parameters such as temperature, pH, oxygen

concentration, agitation, bioreactor design, medium composition, strain of bacteria greatly influence the fermentative bacterial EPS production [16]. The factors in medium composition such as carbon sources, nitrogen source, C/N ratio, incubation time, rate of aeration, optimum pH, and trace elements are also considered as it is reported to affect the productivity of EPS [3]. During fermentative EPS production, the fermentative broth turns into a highly viscous broth. An increase in the viscosity of broth may affect the production parameters as it blocks oxygen transfer and heat exchange and hinders the blending of media ingredients [15].

2.1. Low-cost natural substrate for EPS production

EPS production is reasonably expensive, making it unfavourable in the polymer market instead of having novel properties. Media composition enhances the cost of EPS production in fermentation processes and becomes economically unattractive. Thus, developing a cost-effective method for fermentative production of EPS is most desirable to compete with the synthetic polymer market in terms of economic and performance. Earlier, defined media were used for EPS production and replaced low-cost natural alternatives to increase cost-effectiveness. Since EPS is an accumulation of carbohydrates and the growth of the cell is dependent on the bioavailability of carbon source, the selection of appropriate carbon source (including sugar) is an essential component of the medium since it accounts for more than 70% of the total cost of EPS production [17]. Recent research has concentrated on the quest for alternate and less expensive carbon supplies, and food and agro-waste have proven to be suitable for producing efficient and cost-effective exopolysaccharides. EPS production can be economically favourable by using a low-cost natural substrate such as agro-industrial waste such as whey, molasses and palm oil mill effluents, olive oil wastewater, syrup, fruit juice, fruit, vegetable, agricultural waste emerges as a conventional and emerging approach for waste management and the production of bioactive compounds.
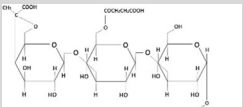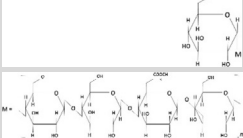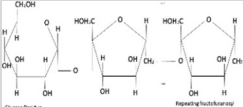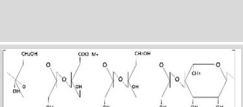
Table 1 shows different types of EPS and its potential producer cultivated in fermentation media using different raw materials with its sugar content, the optimum concentration required, incubation time and maximum EPS production.

3. Structural characterization

The nature and properties of exopolysaccharides are augmented by their physical, chemical and biological characteristics. Estimating potential applications and their behaviour in a different environment is possible by evaluating the properties of exopolysaccharides. The building block of polysaccharides show struc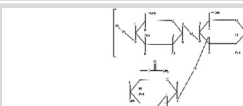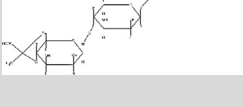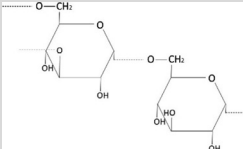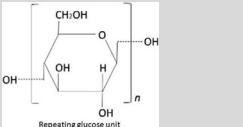tural similarities but can differentiate through their linkage pattern. Characterization of exopolysaccharides concerns identification of monosaccharide composition and its configuration, molecular weight, sequence and glycosidic linkage pattern, branch structure and chain group constituents [13]. Structural complexity makes exopolysaccharides challenging to characterize by a single technique. Thus, consolidation of techniques is paramount for the thorough structural characterization of EPS. (See Figure 1)
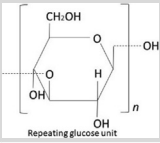
The first step in determining EPS composition is acid hydrolysis, which causes breakage of the glycosidic link between the monosaccharide residues in the polysaccharide chain. The acids used are sulfuric acid, hydrochloric acid, trichloroacetic acid and trifluoroacetic acid. In acid hydrolysis of exopolysaccharide, the critical factors are acid concentration, temperature, and time duration. A high concentration of acid promotes monosaccharide degradation, and low temperature leads to incomplete polymer hydrolysis [41]. Conversion of less volatile sugar monomer to the volatile and stable derivative is an essential step in Gas Chromatography (GC) analysis. A monosaccharide is either derivatized into trimethylsilyl ether derivatives in nonaqueous organic solvents such as pyridine or dimethyl sulfoxide or alditol acetate derivatives such as trifluoroacetate alditol derivatives, trifluoroacetic acid alditol

Table 1. Bacterial EPS production from different natural waste resources.

No	EPS	Producer	Chemical structure	Carbon source	Carbohydrate concentration in carbon source (% total sugar)	Substrate concentration (g/L)	Maximum Yield obtains (g/L)	Time taken for EPS production (hours)	References
1	Succinoglycan	*Agrobacterium radiobacter*		Sugar beet molasses	51.2	10*	22.7	103	[18]
2	Succinoglycan	*Agrobacterium radiobacter* NBRC 12665 (Immobilized cell)		Sugarcane molasses	56.4	75	14.1	192	[19]
3	Levan	*Halomonas* sp.		Sugar beet molasses	55	30	12.4	210	[20]
4	Levan	*Bacillus polymyxa* (NRRL B- 18475)		Sugar beet molasses	56.4	150	3.6	120	[21]
5	Levan	*Bacillus polymyxa* (NRRL B- 18475)		Sugarcane syrup	19.5	150	6.5	120	[21]
6	Levan	*Zymomonas mobilis*		Sugarcane molasses	60–65	250	2.533	24	[22]
7	Levan	*Zymomonas mobilis*		Sugarcane syrup	—	250	15.5	24	[22]
8	Gellan	*Sphingomonas paucimobilis* ATCC-31461		Cane molasses	41	112.5	13.814	48	[23]
9	Xanthan	*Xanthomonas campestris* PTCC1473		Cheese whey	3.91	65.2	16.4	48	[24]
10	Xanthan	*Xanthomonas pelargonii* PTCC1474		Cheese whey	3.91	80	12.8	48	[24]
11	Xanthan	*Xanthomonas campestris* PTCC1473		Date extract	71.58	40	11.2	72	[25]
12	Xanthan	*Xanthomonas campestris* PTCC 1473		Broomcorn stem	65.34	40	8.9	0.75	[26]
13	Xanthan	*Xanthomonas campestris pv maniothis*		Apple juice	15.34 (pectin)	700	45	60	[27]
14	Dextran	*Leuconostoc mesenteroides* NRRL B512(f)		Carob pod and cheese whey	45	20 (sucrose initial concentration in CPE) + 5 g/L (lactose from cheese whey)	7.23	12	[28]
15	Dextran	*Leuconostoc mesenteroides* strain VKM V-2317D		Sugar beet molasses	50	175	49	96	[29]
16	Dextran	*Leuconostoc mesenteroides* NRRL B-512		Sugarcane molasses	47.2	200	9.44	17	[30]
17	Dextran	*Leuconostoc mesenteroides* NRRL B512(f)		Carob pod extract	45	20 (sucrose initial concentration in CPE)	8.56	12	[28]
18	Bacterial cellulose	*Gluconacetobacter xylinus* PTCC 1734		Date syrup	73.1	1000**	43.5	336	[31]
19	Bacterial cellulose	*Gluconacetobacter intermedius* SNT-1		Sugarcane molasses	8.4 (H_2SO_4-heat pretreated molasses)	45.8 (1:4 dilution)	12.6	168	[32]
20	Bacterial cellulose	*Gluconacetobacter persimmonis* GH-2		Muskmelon	7	20	8.08	336	[33]

(continued on next page)

Table 1 (*continued*)

No	EPS	Producer	Chemical structure	Carbon source	Carbohydrate concentration in carbon source (% total sugar)	Substrate concentration (g/L)	Maximum Yield obtains (g/L)	Time taken for EPS production (hours)	References
21	Bacterial cellulose	*Gluconacetobacter persimmonis* GH-2		Orange juice	6.9	20	6.18	336	[33]
22	Curdlan	*Rhizobium radiobacter* ATCC 6466	Repeating glucose unit	Date juice	86.65	120	22.83	51	[34]
23	EPS	*Enterobacter* A47	—	Cheese whey	78.4	70	6.40	76.8	[35]
24	EPS	*Bacillus* sp. ZBP4	—	Beet molasses	46.3	—	60	24	[36]
25	EPS	*Pseudomonas fluorescens*	—	Sugarcane molasses	48.3	—	2.843	48	[37]
26	EPS	*Bacillus* strain CMG1403	—	sugar beet	20	100	5.52	240	[38]
27	EPS	*Bacillus* strain CMG1403	—	cane molasses	50	100	4.85	240	[38]
28	EPS	*Lactobacillus confusus*	—	Coconut water	1.78	1000	12.9	24	[39]
29	EPS	*Lactobacillus confusus*	—	Sugarcane syrup	10	1000	8.8	24	[39]
30	EPS	*Bacillus sphaericus* 7055	—	Sugar beet molasses	48.6	25	0.847	48	[40]

— = Not available.
* = 10° Brix solution (1°Brix solution = 1 g of sugar in 100 g of solution).
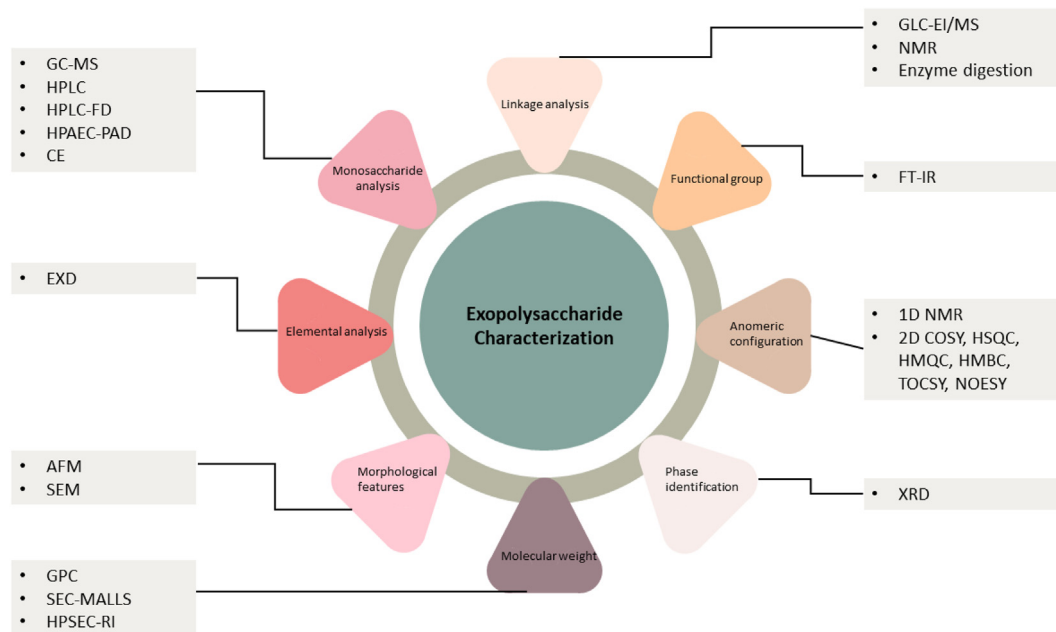** = value in mL.

Figure 1. Exopolysaccharide characterization techniques.

derivatives, acetic acid oxime derivatives and acetic acid nitrile derivatives, formed in solvents such as pyridine, butylene oxide or methyl imidazole. Hexamethyldisilane (HMDS), trimethylchlorosilane (TMCS), trimethylsilyl (TMS) are used as trimethylsilating agents. Preparation of alditol acetate of monosaccharide includes a reduction step, which is further derivatized by acetylation in the presence of solvent and then subject to assay by gas chromatography. Gas Chromatography coupled with Flame Ionization Detection (FID) is also an extensively used method for analysis because of its higher sensitivity and selectivity [42].

High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) was also used to investigate monosaccharide compositions of exopolysaccharide. Compared to GC analysis, this technique is more straightforward, as it does not require the derivatization of sugar [13]. Hence HPAEC-PAD is considered as an

alternative to Gas Chromatography. HPLC with UV detector or pulsed amperometric detection is also used for monomer analysis [43]. HPLC-RI and HPAEC-PAD are liquid chromatographic methods with differences in sensitivity and efficiency and do not require any derivatization step [44]. Paper chromatography (PC) and thin layer chromatography (TLC) are also used at a basic level monomer compositional analysis [45]. Capillary electrophoresis is also used to quantify monosaccharides in EPS without sugar modification after acid hydrolysis [13].

Linkage analysis is a resourceful approach to determine the linkage position. The position of glycosidic linkage are analyzed through three methods: (i) enzymatic degradation, (ii) methylation, and (iii) NMR spectroscopy. Analysis of sequence using exoglycosidase digestion plays a crucial role in the structural determination of N- and O- glycan [46]. Methylation analysis is widely used to study substitution patterns like the position of glycosidic linkage, terminal unit, branching point, and determination of ring size of monosaccharide in the glycan chain. In methylation, the free hydroxyl group of exopolysaccharides was per-methylated, depolymerized, reduced, and acetylated. The resultant partially methylated alditol acetates (PMAAs) were usually analyzed by Gas-Liquid Chromatography (GLC), and the peaks are described and determined by their retention time and their electron impact mass spectrometry (EI-MS) fragmentation patterns [47]. Uronic acid derivatives cannot be analyzed by methylation procedure because of hydrolysis of their methyl ester group [46].

Information regarding the monomer unit in EPS, its anomeric configuration, type of linkage, branching pattern, non-carbohydrate substitutes is obtained from the spectra of NMR spectroscopy. ^{1}H and ^{13}C spectra, determines the anomeric configuration (α or β) of a single monomeric subunit, number and proportion [48]. 2D NMR technologies are also used to analyse the structure of exopolysaccharide. It includes correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), ^{1}H–^{13}C heteronuclear single-quantum coherence (HSQC), ^{1}H–^{13}C heteronuclear multiple quantum coherence (HMQC), ^{1}H–^{13}C heteronuclear multiple-bond correlation (HMBC), nuclear overhauser effect spectroscopy (NOESY) and rotating frame overhauser enhancement spectroscopy (ROESY) [49].

Molecular weight is one of the important fundamental parameters in the overall characterization of EPS that often affects its physicochemical and biological properties. The molecular weight of EPS varies with the species and monomeric composition. Various techniques have been employed to elucidate information regarding the average molecular weight, which includes light scattering, analytical ultracentrifugation, intrinsic viscosity, gel permeation chromatography, size exclusion chromatography (SEC), High-performance size-exclusion chromatography with RI detector or multi-angle laser light scattering (MALLS) [50].

Initially, Gel Permeation Chromatography (GPC) was the most adopted method to determine the molecular weight of polysaccharides. Size exclusion chromatography (SEC) coupled with a multi-angle laser light scattering (MALLS) is absolute and favourable for determining the molecular weight of EPS. Another approach used for molecular weight determination is size exclusion chromatography, and the result is further analyzed by Matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The calibration curve of unknown EPS is compared with that of standard obtain by SEC and confirmed by MALDI-TOF [51]. High-performance size-exclusion chromatography with online detectors like refractive index (RI) and multi-angle laser light scatter detection (MALLS) is considered an advanced SEC development. The coupling of RI with HPSEC gives a signal directly proportional to the polymer concentration; thus, it offers information on the molecular weight of a specific polysaccharide. High-performance size-exclusion chromatography with multi-angle laser light scatters detection (HPSEC-MALLS) was also employed to assess absolute molecular weight by calculating the amount of scattered light at each angle [52]. As compared to gel permeation chromatography, better resolution is the significant advantage of this method.

Fourier-transform infrared (FT-IR) spectroscopy is also applied to analyze glycosidic bond and functional group present in EPS. Attenuated total reflectance (ATR) combined with Fourier transform infrared (FTIR) spectrometers is a rapid and direct method. Compared to traditional FTIR transmittance spectroscopy, the cumbersome procedure of sample preparation as a KBr pallet is eliminated, and also it requires only a minimal amount of sample for analysis [54].

Besides, energy dispersive x-ray spectroscopy (EDS or EDX) is used for the elemental analysis of EPS. Identification of phase of exopolysaccharide, i.e., amorphous, or crystalline is widely detected by the X-ray powder diffraction (XRD) technique [55]. Atomic force microscopy (AFM), confocal laser scanning microscopy (CLSM), infrared spectroscopy, nuclear magnetic resonance imaging (NMRI), Raman spectroscopy (RM), and scanning electron microscopy (SEM) provides good opportunity to investigate polysaccharide [53]. Advancement in microscopic methods and spectroscopic techniques provides a broader scope for the detailed study of exopolysaccharide [13]. Thermogravimetric analysis (TGA) measures the amount and frequency of weight variation concerning time and temperature. The EPS application also depends on thermal stability; thus, it is an important parameter to investigate. Besides TGA, Thermal analysis also includes techniques such as differential scanning calorimetry (DSC), differential thermal analysis (DTA) [56].

4. Biological activities of bacterial EPSs

In addition to structural characterization, characterization by biological activities is also an essential consideration in the characterization of the EPS. The biological characterization of EPS provides insight into the properties of EPS and also its further application. The most comprehensive research among various biological activities of EPS is antioxidant activity.

4.1. Antioxidant activity

Reactive oxygen species (ROS) are produced as a consequence of different cellular processes, inflammation, and stress in biological systems. ROS interacts directly with the cellular membrane and components, causing negative repercussions. As a result, antioxidants are utilized to interact with free radicals and reduce their adverse effects. The antioxidant properties of EPS have attracted more attention than those of other metabolites because of their substantial biological behavior, structural architecture, and surface characteristics [57]. The *in vitro* assay of antioxidant activities includes reducing power, metal chelating ability, lipid peroxidation inhibition assay, superoxide, hydroxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals scavenging activity. The antioxidant activity of EPS is strongly influenced by its source, monosaccharide composition, functional group, molecular weight, linking pattern, uronic acid, sulfate, and protein concentration [43].

Exopolysaccharides appear to be more easily manipulated chemically, resulting in functional alterations, than other naturally occurring antioxidants. Sulfonation, carboxymethylation, selenylation, acetylation, and phosphorylation considerably enhance its physicochemical properties and increase antioxidant activity by amplifying protons. After acetylation, carboxymethylation, sulfation, and phosphorylation, significant changes in the structure, physicochemical characteristics, and antioxidant properties of the exopolysaccharide have been observed [58]. Bacterial EPSs show prominent free radical scavenging and metal chelating ability; however, variations in the factors influencing antioxidant activity may occur between *in vivo* and *in vitro* analyses [59]. The EPS produced by *Leuconostoc mesenteroides* has a substantial immunomodulatory effect, inducing the generation of anti-inflammatory cytokine IL and a high antioxidant capacity under *in vitro* conditions [60].

4.2. Immunomodulatory activity

EPS modulate innate and adaptive immune response by regulating key molecules of the immune system. The presence of phosphate group in the chemical composition and high molecular weight of the EPS are the two major factors responsible for the vital immunomodulatory activity. The negative charge of the phosphate groups stimulates the immune system by stimulating lymphocytes and macrophages, whereas the high molecular weight of EPS acts as a suppressor. Cell surface pattern recognition receptors (PRR) (like Ctype lectins) that can also function as EPS receptors have the ability to change cell signaling and transcription factors, as well as trigger or increase cytokine production [61]. In inflammation condition, EPS to play a crucial role in maintaining immunological balance by promoting T cell differentiation into regulatory T cells and maintaining immune homeostasis [62]. Greater attention is received by EPS obtained from native LAB or its mutants as an immunomodulatory agent. Various models are used to study the immunomodulatory effects, such as macrophage (in vitro), mice and rat models (in vivo). EPS activates MAPK pathway which activates macrophage and enhances host immune response [63].

EPS produced by Lactobacillus paraplantarum shows potential anti-inflammatory effect in the wister rat subjected to the inflammation in the hind paw induced by the carrageenan injection. The intraperitoneal administration of EPS in dose-dependent manner not only reduce the secretion of pro inflammatory mediators also reduces pain and paw swelling in rat [64]. EPS produced by Bacillus subtilis protects mice from the intestinal pathogen Citrobacter rodentium, which causes acute colitis. It has the ability to block T cell activation in a wide sense, allowing it to regulate T cell-mediated immune responses in a variety of inflammatory disorders [65]. EPS of Lactobacillus helveticus act as a potential immunomodulatory agent by exhibiting strong effect on elevating the proliferation of macrophages and enhancing phagocytosis, acid phosphatase activity, nitric oxide production and cytokines production [66].

5. Engineering tactics for desirable microbial exopolysaccharide production

Metabolic engineering concerns the detailed analysis of the target gene to undergo various manipulation measures to achieve the decisive goal of enhancing and forming the desired product. Metabolic engineering of the genes involved in each stage of the biosynthetic pathways offers the maximization of EPS production and enhancement of the EPS characteristics. EPS have promising field application due to its exclusive gifts, but the bearing of diverse genes and complex biosynthetic pathway makes the metabolic engineering studies for EPS production more cumbersome. Also, it is a stumbling block to sequence the whole genome and the development of gene manipulation techniques for the EPS producer individually [67].

An inadequate amount of sugar nucleotide precursor was identified as one of the major factors limiting microbial EPS production and consequently established a linear correlation between the concentration of sugar nucleotide precursor and EPS yield [68]. Enzymes such as phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP glucose dehydrogenase, and UDP-galactose-4-epimerase plays a vital role in the synthesis of sugar precursor, which eventually increases EPS production. The mutant strain of Alcaligenes sp. NX-3-1 obtained through the Low-energy nitrogen ion beam implantation technique demonstrated higher specific activity of these enzymes than wild type strain [69]. Overexpression of these enzymes increases the level of sugar nucleotide, which eventually raises EPS production. Overexpression of UDP-glucose pyrophosphorylase (UGP) gene in Ganoderma lucidum, a medicinal mushroom, also reported high intracellular polysaccharide (IPS) content, extracellular polysaccharide (EPS) production as compared to the wild-type strain [70]. The available carbon is converted into EPS or cell mass, opting through alternative metabolic routes by EPS producing bacteria [71]. Thus, the strategy also applied to enhance the expression of the key enzymes, which plays an essential role in re-routing the carbon flux towards the synthesis of sugar precursors. High carbon flux towards EPS production is also reported in the Lb. delbrueckii subsp. bulgaricus due to a rise in the expression of UGP and UGE [72]. Single enzyme manipulation might not be sufficient to increase the production of desired EPS; thus, a simultaneous expression of multiple enzymes is required. However, overexpression of some enzymes to augment sugar nucleotide precursor pool does not always enhance EPS production [71, 73].

Along with these, glycosyltransferase genes also play a significant role. The overexpression of the glycosyltransferase gene results in the formation of a highly mucoid colony and EPS production by Brucella sp. [74]. An increase in the amount of EPS production by overexpression of the glycosyltransferase is also reported by [75]. Some reports also suggest that EPS production is not affected by increasing the expression of glycosyltransferases [76].

5.1. CRISPR/Cas9

CRISPR/Cas9 technology, a genome editing technique, is highly advanced, faster, easier, and accurate in genome manipulations than other conventional DNA manipulation techniques [77]. It shows the ease in the addition, removal, or alteration of the genome, which ultimately leads to the desired improvement in research in various fields like biology, medicine, biotechnology, microbiology, and the production of industrially important products biopolymers. Advance synthetic biology and metabolic engineering had transformed microbes into cellular factories for the generation of valuable products [78]. The complex metabolic circuits are effectively controlled and rewire by insertion of nonsense mutation using the CRISPR technique, which causes inactivation of the target gene. Besides, global transcription machinery engineering (gTME) can be re-added successfully to different host strain by changing its homologous transcription factor sequences by CRISPR/Cas9 to the desired one for achieving desired phenotypic properties. Site-directed mutagenesis is used to implement changes in the sequence, but CRISPR/Cas9 technology is advantageous and highly recommended when high accuracy is chosen. CRISPR/Cas system widens the coverage in metabolic engineering, as a complex pathways buildup of multiple genes and during engineering the coordinate behavior of genes is fundamental to achieve the desired phenotype [67].

Many research groups had productively exploited the Crisper/cas9 system for desired EPS production and PHA productions. The CRISPR/cas9 genome editing approach significantly contributed to the generation of EPS variants in Bacillus polymyxa [79]. In identified EPS gene cluster, five different genes, including 3 GTs (pepF, pepJ, pepC) and 2 genes involved in precursor synthesis (ugdH1, manC), are deleted separately. Along with this, to generate EPS deficient mutant, an 18kb fragment (clu) is also deleted. The EPS variant produced by mutant exhibit different chemical composition and rheological properties compared to wild type [80] carried out using CRISPRi system to reduce dissimilation of carbon through central metabolism to intensify hyaluronic acid production in Bacillus subtilis by repression of some genes. CRISPRi system causes the reduction in the expression of pfkA or zwf in base strain individually, subsequently shows improvement in the hyaluronic acid production with a decrease in molecular weight. However, on multiplexed repression of the expression of both pfkA and zwf, it resulted in the increment of hyaluronic acid titer up to 108% and the molecular weight compared to base strain. Moreover, the CRISPR-Cas system also applied Streptococcus mutans, specifically targeting glucosyltransferases gene for inhibiting biofilm formation, which causes dental plague biofilm. It successfully edited gtfB and gtfBgtfC, which results in an immense decrease in EPS production, and biofilm-forming ability also ceases [81].

5.2. A new perception: EPR3

EPR3 is a structurally distinctive and novel carbohydrate-binding receptor found across the plant kingdom and has a potential role in

developing new strategies. *Epr3* consists of ten exons and encodes a protein of approximately 70 kDa [82]. The compact structure of EPR3 shows a cloverleaf shape arrangement, with three linked modules (M1, M2 & LysM3) stabilized by 3 internal disulfide bridges. EPR3 is a member of a broad and well-studied group of plant receptors that can detect EPS from a variety of bacteria. one α-helix and three elongated β-strands in the form of βαββ arrangement are found in M1 module. Similarly, M2 domain contains βαβ fold with no defined α-helix. βααβ fold of LysM proteins, with a root-mean-square deviation (RMSD) of 1.2 Å are present in LysM3 [83]. According to Protein Data Bank (PDB), M1 in EPR3 has no structural homologues, making it a unique fold among carbohydrate-binding proteins; however, M2 correlates with LysM and LysM3 is a typical LysM motif [84]. Bacterial EPS plays a significant role in biofilm development, protection against environmental stress, attachment of bacteria to the root surface, formation of infection threads, and restricting undesirable strain to infect the root [85]. Microscale thermophoresis reveals the ability of EPR3 to differentiate different compositions and structures of EPS. According to the phenotypic characterization of epr3 mutants and EPS-addition studies, monitoring of bacterial EPS is exerted during colonization and infection of epidermal cells, regardless of the mode of infection. The epidermal and cortical tissue infection in Lotus and Medicago root was stimulated by EPS perception and EPR signalling [82, 86]. Although this family of receptors is found across the plant kingdom, none has functionally characterized in planta, except for *Lotus* and *Medicago* EPR3, which opens up a new avenue of receptor exploration [83].

6. Potential environmental applications

6.1. Landfill leachate treatment

Municipal Solid Waste (MSW) is considered a threat due to the tremendous increase in population, urbanization, industrialization in the past several decades. When MSW is dumped in a landfill, there is an increase in demand for landfill owing to a constant trash supply, which makes its treatment expensive. Recycling and reuse of organic waste from MSW decreases the volume of generated waste. Therefore, the most efficient, considerable and competent method is composting [87]. However, water drainage through the waste creates a substantial amount of composting leachate throughout the composting process. Composting leachate encompasses various toxic and hazardous chemicals, making it a severe contaminant that risks human health, hygiene, and the environment [88]. For the treatment of composting leachates, several methods are available with more or fewer limitations.

The coagulation and flocculation process is an uncomplicated, cost-effective, and productive method for removing organic material and heavy metals from the leachate. However, chemical coagulants in the coagulation and flocculation process cause severe health and environmental issues; thus, bacterial EPS is explored as a viable replacement [89]. However, this process is also used to treat old and stabilized landfill leachates [90]. Young landfill leachate is high in organic content offers raw material for EPS-producing organisms to thrive [7].

EPS of *Rhizomonas* sp. causes 45% reduction in COD from landfill leachate. EPS at a concentration of 20 mg/L at pH 7–7.5 results in more than 85% removal of humic acid. EPS is influential compared to chemical coagulants due to its low dosage concentration and maximum humic acid removal. Furthermore, the removal efficiency between optimum and low effective concentration is 20%. In COD removal, EPS at 50 mg/L shows a similar result with alum at 500 mg/L. Furthermore, bioflocculant delivers an optimal result at the pH of leachates, requiring no pH modifications. As a result, it is demonstrated that EPS is more beneficial [91].

The major issues concern with landfill sites is the infiltration of leachates and aquifers. Here, EPS producer can overcome this problem as EPS contains essential properties that can hinder liquid flow, diminish the pore size to reduce liquid flow, and reduce hydraulic conductivity. Thus, the unique properties of EPS make it suitable to use as a barrier to block groundwater contamination [7]. Environmental applications of bacterial exopolysaccharide are depicted in Figure 2.

6.2. Dye decolorization

Synthetic dyes are widely used for product colouring in textile, paper & pulp, printing, leather processing, cosmetics, plastics, pharmaceuticals, and food processing. The effluents from these industries are highly supplemented with toxic compounds, heavy metals, and components of synthetic dyes. The release of this untreated wastewater on land or water bodies will have a deleterious effect on aquatic biota, humans, and the surrounding environment [92]. Biosorption arises as a prospective technology for the evacuation of dye and treatment of wastewater effluents [93]. EPS secreted by microorganisms contain adsorption properties and can eradicate organic and inorganic materials from the wastewater [94]. The biosorption capacity of EPS is credited to the presence of different functional groups like amino, carboxyl, hydroxyl, phosphoric, sulfhydryl, phenolic, which build attractive force between dye and EPS [94, 95].

Various aspects are responsible for the effectiveness of EPS in decolorization. The decolorization efficiency depends on the type of dye, structure, pH, temperature, ionic strength, EPS concentration, and contact duration [96]. EPS produced by *Ochrobactrum sp.* is proved to be potential with a removal efficiency of 89.4% for Remazol Blue dye at 72 h incubation and 30 °C temperature [97]. EPS synthesized by *Acinetobacter baumannii* is effectively used in dye removal. After 48 h of decolourization experiment, decolorization efficiency of 98.62% for congo red dye are observed by EPS of *Acinetobacter baumannii* [98]. The novel bioflocculant produced by *Alteromonas sp.* possesses dye removal efficiency of 98.5%, 97.9%, 72.3% for congo red, direct black, and methylene blue, respectively [99]. Moderate decolorization capacity for cationic dyes is shown by EPS of *Paenibacillus elgii*, with a decolorization rate of 65% for methylene blue and 72% for Red X-GRL [100]. EPS synthesized by *Klebsiella* sp. shows a removal efficiency of 67.82% for sulfamethoxazole in an aqueous solution under all optimum conditions. Whereas in domestic wastewater, the removal efficiency is reduced to 53.27% [101]. According to studies, EPS produced by bacterial strains such as *Bacillus, Exiguobacterium, Klebsiella, Pseudomonas,* and *Staphylococcus* is excellent at removing dyes from wastewater. EPS are efficacious in removing dyes
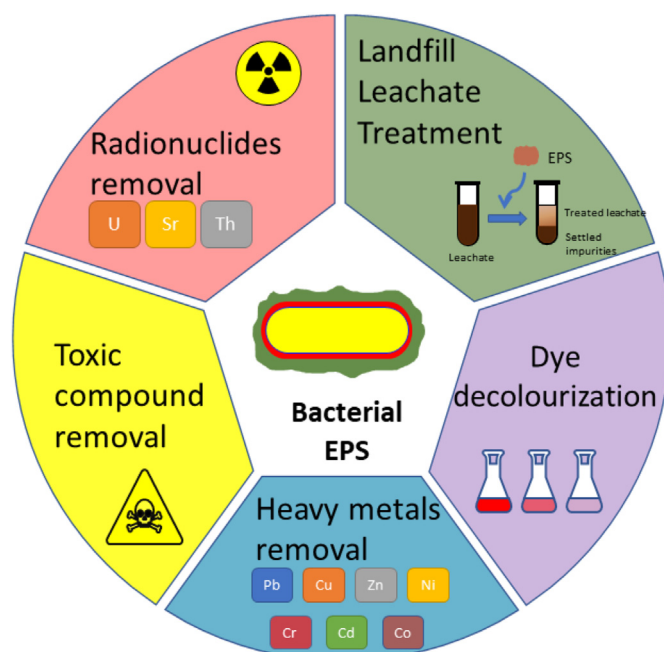

Figure 2. Environmental applications of bacterial exopolysaccharide.

such as whale dye, medi-blue, fawn dye, and mixed dyes, with its decolorization efficiency ranging between 20-99.9% [95].

The consortium of bacteria dominates in dye removal efficiency as compare to single bacteria. The EPS produced from the consortium of Staphylococcus sp. and *Pseudomonas* sp. are potentially used to treat indigotin printing and dyeing wastewater and show decolorization efficiency of 86.5% [102]. *Bacillus cereus* B-11 cultivated on molasses wastewater produces EPS, an effective flocculating agent in removing reactive dyes from the aqueous solutions. The efficiency of decolorization exhibit by 25 mL of EPS for Light-Yellow K-4G and Turquoise Blue KN-G are f 97.5 and 94.7%, respectively, in 500 mL of 100 mg•L-1 dye solution [103]. Thus, EPS unveils its potential for the treatment of wastewater as well as dye-containing solutions.

6.3. Heavy metal removal

Heavy metals are introduced into aquatic bodies by industrial operations such as mining, fossil fuel combustion, and metallurgical processes. Heavy metals are toxic and carcinogenic, posing a severe threat to aquatic life, human health, and the environment [104]. Therefore, heavy metal removal or remediation and biosorption are most widely studied. In metal remediation, the most significantly applied method for metal adsorption is the use of bacterial EPS. The use of biopolymer is more advantageous, practical, readily available and safe in use, and also being non-living material, it avoids the issue of pathogenicity [105]. The presence of a functional group in EPS provides binding sites for the chelation of metal in biosorption [7]. In addition, EPS shows supreme metal-binding properties with different specificity and selectivity.

Several experimental conditions influence metal uptake, such as pH, temperature, initial concentration of metal, concentration of EPS, stirring rate, charge density [106]. EPS extracted from *Azotobacter beijreinckii* and *Bacillus subtilis* reported 26% and 48% chromium removal rate from aqueous solution with its initial concentration of 10 ppm [107]. In recent studies, EPS obtain from *Bacillus mucilaginosus* depicted remarkable iron ions removal from aqueous solution under optimum conditions. The optimum biosorption condition contains pH 5, 200–800 mg/L initial ion concentration, 60 mg/L EPS dosage, and under these conditions, the absorption rate obtains above 90% [108]. According to the most recent study, the EPS produced by *Bacillus licheniformis* strain KX657843 isolated from the earthworm's gut proves to be a productive agent in heavy metal remediation. The EPS mediated metal remediation is pH-dependent. EPS reported the metal removal potential of 54% and 42% at pH 5, which increases considerably to 86% and 81% at pH 8 for Cu (II) and Zn (II), respectively, at a metal concentration 25 mg/L in solution. The overall metal removal efficiency of 94.8% for both metals are observed at 100 mg/L EPS concentration [109]. Generally, through electrostatic interactions, the carbohydrate and protein moieties in EPS are furnished with their metal-binding properties and binds with cations. Chromium possesses an adverse effect on human health; thus, it is necessary to pre-treat it before discharge. the EPS of *Pseudochrobactrum saccharolyticum* LY10 confirms greater efficiency for Cr (VI) removal. An intracellular and extracellular fraction is studied for Cr (VI) reduction. Among them, within 16 h experiment, a maximum Cr (VI) reduction rate of 81.5% is observed in EPS, and the least reduction rate is found in cell wall with 30.1%. The order of reduction ability of different fractions of cell are EPS (81.5%) > soluble fraction of cell (69.2%)> cell membrane (49.3%) > cell wall (30.1%) [110].

Along with the pure culture, EPS produced by the consortia of bacterial culture also shows tremendous success in heavy metal removal. The absorption of 87.12% of Cd, 19.82% of Zn, and 37.64% of Cu in the polymeric matrix is shown by the EPS secreted from the bacterial consortium from a hydrocarbon-contaminated water body [111]. The compound extracellular biopolymer-based bioflocculant produced by the consortium of *R. radiobacter* F2 and *B. sphaeicus* F6 shows significant cadmium uptake aqueous solution in 60 min at its optimum pH 7 [112]. EPS of gram-negative bacterial consortia are proven to be efficient in the

removal of various metal from the aqueous solution with its removal efficiency of 77.15% for Zn, 78.18% for Pb, 74.48% for Cr, 66.63% for Ni, 71% for Cu, 72.71% for Cd and 76.12% for Co [113]. The bio removal efficiency for Ni, Cu, and Cr by EPS produced by mixed culture with an initial heavy metal concentration of 50 mg/L. A remarkable difference had been seen in metal removal efficiency in the media containing molasses and control that lack molasses. The removal rate is 32%, 75.7% and 51.1%, respectively, in molasses containing media significantly higher than control media [114].

6.4. Toxic compound removal

Polycyclic aromatic hydrocarbons (PAHs) persist in the soil for longer due to their hydrophobic nature, thermodynamic stability, and aggregation properties. The use of EPS accomplishes PAH biodegradation. Extraction of PAH from the contaminated soil executed by EPS produced by bacteria is also well reported [115]. EPS secreted by bacteria plays a dominant role in the establishment of effective cell attachment and biofilm development. The biodegradation of phenanthrene (PHE) and pyrene (PYR) was carried out using the bacterial strain *Micrococcus* sp. PHE9 and *Mycobacterium* sp. NJS-P with EPS and without EPS. Bacterial species with EPS develop potential biofilm on the humin. EPS content in the biofilm shows a striking correlation with the efficiency of phenanthrene and pyrene degradation [115]. Non-polar groups from protein, aromatic lipids, and polysaccharides of EPS provide a binding site for PHE and PYR and thus cause their accumulation in the EPS layer. Also, EPS acts as a sorbent, favouring mass transfer of PHE and PYR from an aqueous phase. EPS in biofilm accelerates PHE and PYR biodegradation by increasing its bioavailability by expanding the interfacial area between cell and substrate. The biodegradation rate by biofilm is 35.18 mg L^{-1} day^{-1} for phenanthrene and 5.08 mg L^{-1} day^{-1} for pyrene. EPS and phenanthrene exhibit a spontaneous and exothermic interaction. Hydrophobic interactions play an essential role in binding phenanthrene to EPS [116]. The enhanced solubility of phenanthrene in bulk water, triggered by polysaccharide, increases its mass transfer. *Pseudomonas aeroginosa* EPS exhibits significant binding with 25.1 g mL^{-1} phenanthrene and 14.9 g mL^{-1} pyrene [117]. However, all the reported applications for EPS on PAH removal are applied on a laboratory scale.

6.5. Radionuclides removal

Various anthropogenic activities release radioactive compounds into the environment. Metallurgical mining, nuclear power test, nuclear reactor discharges are foremost responsible for liberating radionuclides in the surrounding. Furthermore, the toxicity of radioactive composes poses highly detrimental effects on humans and the environment. Biosorption is the most applicable technology for the removal of radionuclide from the aqueous environment. Over conventional methods, biosorption is more cost-effective, significantly precise, and efficient method. EPS shows significant effectiveness and potentially high biosorption capacity to abolish toxic material.

Uranium isotopes are considered the most hazardous and researched radioactive metal due to their excessive toxicity and radioactivity [118]. Effect of pH, temperature, EPS dosage, initial uranium concentration, time of incubation plays a dominant role in metal uptake. EPS extracted from *Pseudomonas* sp. disclose accumulation of uranium. A maximum accumulation of 96 μg/mg of uranium is observed in the EPS. Uranium uptake by EPS depends on the pH range, whereas it is independent of temperature changes. Uranium accumulation decreases at pH below 5 [119]. EPS extracted by anaerobic activated sludge shows significant uranium uptake from wastewater. EPS mediated uranium removal efficiency is 98.3% at pH 6, with 20 mg/L initial uranium concentration and 56.1 g/L EPS concentration; however, the maximum adsorption capacity of uranium is 0.891 mg/g EPS [120]. EPS extracted from aerobic activated sludge shows uranium removal efficiency of 93% at an optimum pH of 6 and other optimum conditions. According to Langmuir fitting, the

maximum adsorption capacity of uranium to EPS is 333.3 mg/g [71]. EPS from *Shewanella* sp. HRCR-1 biofilms show uranium (VI) immobilization through its sorption and reduction [121]. The EPS of *P. aeroginosa is* potentially used as a biosorbent in uranium removal. A maximum uranium extraction capacity of 985 mg U g^{-1} EPS is observed [122]. However, iron and thorium strongly inhibit uranium sorption by EPS. EPS extracted from *Pseudomonas* sp. plays a crucial role in the redox cycling of plutonium and affects mobility in the environment. Cells with intact EPS show more efficient plutonium (V) sorption than those without EPS [123]. High thorium tolerance is reported by *Ochrobactrum intermedium. O. intermedium* is a potential biofilm former, and EPS in media does not hinder the presence of thorium. EPS recovered from thorium supplemented EPS medium is subject to FT-IR spectroscopy. In the FT-IR spectrum, absorbance rises at 3000–3200, 1600–1700 and 1000–1200 cm, specifically indicating hydroxyl, amino, and carboxyl groups EPS, respectively. Thus, it authenticates the role of EPS extracted from *O. intermedium* in the absorption of thorium [124]. Thus, EPS arose as a potential biosorbent in radionuclide treatment.

7. Conclusion and perspective

The review shows that bacterial exopolysaccharide can be a preferred polymer for environmental applications compared to other conventional synthetic polymers and can serve many fields of interest. Extensive research is required prior to its deployment in the field. The major drawback of EPS manufacturing is the high production costs because most synthetic production media contain sugar as a carbon source, which eventually results in a price hike. Thus, raw materials such as kitchen waste, agro-industrial waste, industrial effluents, sludge, and other solid and liquid waste need to be explored to produce EPS. Research and development in molecular engineering of the genes that influence EPS production are also restricted. Research should be conducted to design the desired mutant strain with novel properties, using different tools and techniques to increase productivity. CRISPR/Cas9 is still in its nascent stage to being used to engineer desired EPS; however, reports present on its use in PHA (Polyhydroxyalkanoates) production and similar studies are needed to be performed for engineering EPS. More dedicated research is recommended to optimise large-scale industrial processes, including improved upstream and downstream processes using the natural substrate as a raw material. Research on solid-state fermentation using the natural substrate for EPS production is still limited. The unique properties of EPS make it an effective tool for remediation methods that require further clarification. Concerning EPR3, the future challenges of ligand specificity, molecular processes, and downstream implications of carbohydrate signalling via *LysM* proteins, which are found in all forms of life, from bacteria to humans, may provide new views on carbohydrate signalling.

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