Chapter 2
Microbial Production of Extracellular Polysaccharides from Biomass

Ebru Toksoy Öner

Abstract The interest in polysaccharides has increased considerably in recent years, as they are candidates for many commercial applications in different industrial sectors like food, petroleum, and pharmaceuticals. Because of their costly production processes, industrial microbial polysaccharides like xanthan, dextran, curdlan, gellan, and pullulan constitute only a minor fraction of the current polymer market. Therefore, much effort has been devoted to the development of cost-effective and environmentally friendly production processes by switching to cheaper fermentation substrates. In this chapter, various microbial polysaccharide production processes utilizing cheap biomass resources like syrups and molasses, olive mill wastewater, cheese whey, various vegetable and fruit pomace, pulp and kernels as well as carbon dioxide and lignocellulosic biomass like rice hull and bran, sawdust, and fibers are discussed with a special focus on the employed pretreatment methods.

Keywords EPS · Microbial exopolysaccharides · Polysaccharides · Biomass · Fermentation

2.1 Introduction

Since the beginning of twentieth century, technologies related to microbial production of biomolecules like enzymes, antibiotics, metabolites, and polymers have matured to a great extend. Currently, microbes are used for commercial production of a wide variety of products such as pesticides, fertilizers, and feed additives in agrochemical sector, biopharmaceuticals and therapeutics in the healthcare sector, biopolymers and biofuels in the energy and environment sectors. According to recent market reports, growing environmental concerns and increasing demands from end-use sectors are expected to increase the global market for microbial products to about 250 billion US dollars by 2016 [1].

E. T. Öner (✉)
IBSB—Industrial Biotechnology and Systems Biology Research Group,
Bioengineering Department, Marmara University,
Goztepe Campus, 34722 Istanbul, Turkey
e-mail: ebru.toksoy@marmara.edu.tr

Polysaccharides are natural, non-toxic, and biodegradable polymers that cover the surface of most cells and play important roles in various biological mechanisms such as immune response, adhesion, infection, and signal transduction [2, 3]. Investigations on the alternative treatments applied by different cultures throughout the history revealed the fact that the utilized plants and fungi were rich in bioactive polysaccharides with proven immunomodulatory activity and health promoting effects in the treatment of inflammatory diseases and cancer. Hence considerable research has been directed on elucidating the biological activity mechanism of these polysaccharides by structure-function analysis [4].

Besides the interest on their applications in the health and bionanotechnology sectors, polysaccharides are also used as thickeners, bioadhesives, stabilizers, probiotic, and gelling agents in food and cosmetic industries [5–7] and as emulsifier, biosorbent, and bioflocculant in the environmental sector [8].

Polysaccharides are either extracted from biomass resources like algae and higher-order plants or recovered from the fermentation broth of bacterial or fungal cultures. For sustainable and economical production of bioactive polysaccharides at industrial scale, rather than plants and algae, microbial sources are preferred since they enable fast and high yielding production processes under fully controlled fermentation conditions. Microbial production is achieved within days and weeks as opposed to plants where production takes 3–6 months and highly suffers from geographical or seasonal variations and ever increasing concerns about the sustainable use of agricultural lands. Moreover, production is not only independent of solar energy which is indispensible for production from microalgae but also suitable for utilizing different organic resources as fermentation substrates [5].

According to recent reports, the global hydrocolloid market dominated by algal and plant polysaccharides like starch, galactomannans, pectin, carrageenan, and alginate is expected to reach 3.9 billion US dollars by 2012 [9]. Superseding these traditionally used plant and algal gums by their microbial counterparts requires innovative approaches and considerable progress has been made in discovering and developing new microbial extracellular polysaccharides (exopolysaccharides, EPSs) that possess novel industrial significance [6, 7]. A recent review pointed out to four EPSs, namely, xanthan, pullulan, curdlan, and levan, as biopolymers with outstanding potential for various industrial sectors [5]. However, when compared with the synthetic polymers, natural origin polymers still represent only a small fraction of the current polymer market, mostly due to their costly production processes. Therefore, much effort has been devoted to the development of cost-effective and environmentally friendly production processes such as investigating the potential use of cheaper fermentation substrates.

In this chapter, after a brief description of microbial polysaccharides, various microbial production processes utilizing cheap biomass resources as fermentation substrates are discussed with a special focus on the employed pretreatment methods.

2.2 Microbial Polysaccharides

In nature, biopolymers often play important roles in maintaining cell viability by conserving genetic information, by storing carbon-based macromolecules, by producing either energy or reducing power, and by defending an organism against attack from hazardous environmental factors [10]. Microbial polysaccharides are high molecular weight carbohydrate polymers present either at the outer membrane as lipopolysaccharides (LPS) that mainly determine the immunogenic properties or secreted as capsular polysaccharides (CPS) forming a discrete surface layer (capsule) associated with the cell surface or excreted as EPS that are only loosely connected with the cell surface [11]. Whereas CPSs are assigned with functions directly related with pathogenicity like resistance to specific and nonspecific host immunity and adherence [12], EPSs fulfill a variety of diverse functions including adhesion, cell to-cell interactions, biofilm formation [13], and cell protection against environmental extremes [2].

Polysaccharides show considerable diversity in their composition and structure. They are generally classified as homopolysaccharides and heteropolysaccharides based on their monomeric composition [14]. Homopolysaccharides are composed of one type of monosaccharide repeating unit where sugar monomers are bound to form either linear chains (pullulan, levan, curdlan or bacterial cellulose) or ramified chains (dextran). Heteropolysaccharides are composed of two or more types of monosaccharides and are usually present as multiple copies of oligosaccharides, containing three to eight residues (gellan or xanthan) [15, 16]. Table 2.1 summarizes the chemical characteristics of major bacterial and fungal polysaccharides.

The microorganisms used as industrial or technical producers of EPS are chiefly the bacteria. Species of *Xanthomonas*, *Leuconostoc*, *Sphingomonas*, and *Alcaligenes* which produce xanthan, dextran, gellan, and curdlan are the best known and most industrially used (Table 2.1).

Dextran (synthesized by certain lactic-acid bacteria such as *L. mesenteroides*) was the first microbial polysaccharide to be commercialized and to receive approval for food use [22]. Xanthan gum is the EPS from the plant pathogen *X. campestris* bacterium and due to its exceptional rheological properties; xanthan has a considerable market [28]. Gellan produced by *S. paucimobilis* is gaining increasing attention due to its novel property of forming thermo-reversible gels and hold great commercial potential for food, pharmaceuticals, and predominantly environmental bioremediation [23]. On the other hand, because of its very high immunocompatibility and water-binding and retention capacity, hyaluronan is widely used in regenerative medicine and cosmetic applications [16]. Curdlan produced by the alkaline tolerant mesophilic pathogen *Alcaligenes faecalis* [20] can form aqueous suspensions which can form high-set gels upon heating and curdlan is also produced by *Cellulomonas flauigena* as an extracellular storage polymer [21]. Species of *Pseudomonas* and *Azotobacter* are the only microbial sources for alginate that is widely used as a thickening, stabilizing, and gelifying agent in food, textile, paper, and pharmaceutical industries [17, 18]. Contrary to plant cellulose, bacterial cellulose is produced and excreted to

Table 2.1 Principal bacterial and fungal polysaccharides

EPS	Monomers	Charge	Characteristics of chemical structure	Organism	Reference
Bacteria					
Alginate	Guluronic acid, Mannuronic acid	Anionic	Blocks of β-1,4-linked D-mannuronic residues, blocks of α-1,4-linked L-guluronic acid residues, and blocks with these uronic acids in random or alternating order	*Pseudomonas aeruginosa*, *Azotobacter vinelandii*	[17], [18]
Cellulose	Glucose	Neutral	β-1,4-D-glucan	*Gluconacetobacter xylinus*	[19]
Curdlan	Glucose	Neutral	β-1,3-D-glucan	*Alcaligenes faecalis*, *Cellulomonas flavigena*	[20], [21]
Dextran	Glucose	Neutral	α-D-glucan linked by α-1,6-glycosidic bonds; some 1,2-, 1,3-, or 1,4-bonds are also present in some dextrans	*Leuconostoc mesenteroides*	[22]
Gellan	Glucose, Rhamnose, Glucuronic acid	Anionic	Partially O-acetylated polymer of D-glucose-1,4-β-D-glucuronic acid-1,4-β-D-glucose-1,4-β-L-rhamnose tetrasaccharide units connected by α-1,3-glycosidic bonds	*Sphingomonas paucimobilis*	[23]
Hyaluronan	Glucuronic acid, Acetylglucosmine	Anionic	Repeating units of β-1,4-linked disaccharides of β-D-N-Acetylglucosmine-β-1,3-D-Glucuronic acid	*Pseudomonas aeruginosa*, *Pasteurella multocida*	[24], [25]
Levan	Fructose	Neutral	β-2,6-D-fructan	*Bacillus subtilis*, *Zymomonas mobilis*, *Halomonas sp.*	[26], [27]
Xanthan	Glucose, Mannose, Glucuronic acid	Anionic	β-1,4-D-glucan with β-D-mannose-1,4-β-D-glucuronic acid-1,2-α-D-mannose sidechain. Approximately 50 % of terminal mannose residues are pyruvated and the internal mannose residue is acetylated at C-6	*Xanthomonas campestris*	[28]
Fungi					
Pullulan	Glucose	Neutral	α-1,6-linked α-1,4-D-triglucoside maltotriose units	*Aureobasidium pullulans*	[29], [30]
Scleroglucan	Glucose	Neutral	β-1,3-D-glucan with β-1,6-D-glucose linked to every third unit	*Sclerotium glutanicum*	[31]

the fermentation medium and directly recovered as a highly pure polymer free from lignin and other noncellulosic materials. It is now a high-value, specialty chemical with highly specific applications like skin regeneration [16]. Levan is a water soluble, strongly adhesive, and film-forming biopolymer with many potential uses as emulsifier, stabilizer and thickener, encapsulating agent, osmoregulator, and cryoprotector in food, cosmetics, pharmaceutical or chemical industries [26]. Fungal polysaccharides are still somewhat limited, with pullulan from *A. pullulans* [30] and scleroglucan produced by *Sclerotium glucanicum* [32] being the most known and already obtained at technical scales. Whereas the major market for pullulan is still in food sector, there are numerous reports for its potential applications in pharmaceutical, medical, and environmental remediation areas [30]. Similarly, due to its exceptionally high stability, the first application of scleroglucan was in the oil recovery; however, other applications in pharmaceutical, cosmetic, and agriculture sectors have also been proposed [31, 32].

2.3   Microbial EPS Production Processes

Fermentation is a very versatile process technology for producing value added products such as microbial biopolymers and since fermentation parameters have a high impact upon the viability and economics of the bioprocess, their optimization holds great importance for process development. Especially, microbial polysaccharide production is greatly influenced by fermentation conditions such as pH, temperature, oxygen concentration and agitation as well as by the composition of the culture medium [7, 10, 16]. Moreover, besides the fermentation conditions, the chemical structure, monomer composition, and physicochemical and rheological properties of the final product also change with the type of strain. This in turn allows the industrial production of polysaccharides with desired specifications via controlling the fermentation conditions, choosing feasible feedstocks, and using high-level producer strains.

EPS synthesis is a tightly regulated carbon and energy-intensive process resulting in a wide range of nutritional and environmental requirements of the EPS producer strains. Consequently, dependency of the production on microbial growth, nutrient availability, and fermentation conditions are subject to significant controversy in literature and hence generalizations should be avoided [7].

Fermentations for EPS production are batch, fed-batch or continuous processes depending on the microbial system used. In most cases, optimum values of temperature and pH for biomass formation and EPS production differ considerably so that typical fermentations start with the growth phase followed by the production phase. Moreover, considerable changes in the rheological properties occur during the course of fermentation due to EPS production. This results in a highly viscous and non-Newtonian broth which in turn may not only cause serious problems of mixing, heat transfer, and oxygen supply but also give rise to instabilities in the quality of the end product. Whereas this is a common technical difficulty in commercial xanthan

and pullulan production processes [30], it is not encountered in levan production due to the exceptionally low intrinsic viscosity of the polymer [33] as well as in microbial processes utilizing thermophilic microorganisms where production is realized at high temperatures [7]. Whether the production is small laboratory scale or large at industrial scale, the fermentation media are almost always designed to have high carbon to nitrogen ratio where nitrogen serves as the growth limiting nutrient [34]. Under conditions employed for industrial production of microbial polysaccharides, the same principle of high carbon to nitrogen ratios is used, but the substrates utilized are the cheapest available form.

2.3.1 Low-Cost Biomass Resources

Fermentation medium can represent almost 30 % of the cost for a microbial fermentation. Complex media commonly employed for growth and production are not economically attractive due to their high amount of expensive nutrients such as yeast extract, peptone, and salts. In order to reach high production titers at reasonable costs, fermentation medium should be carefully designed to make the end product compatible with its synthetic petrochemical counterpart.

Fermentation feedstock has been the most expensive constituent in microbial biopolymer production. Till the1990s, studies were generally focused on using defined culture conditions in order to recover ultra pure biopolymers with minimum batch-to-batch variation and free of impurities that would interfere with their chemical and biological characterization. However, to maximize the cost effectiveness of the process, recent work has shifted to use multi-component feedstock systems and the synthetic media were replaced by cheaper alternatives such as olive mill wastewater (OMW), syrups, and molasses [7, 16, 35]. Currently, a wide range of industrial and agricultural by-products and waste materials are used as nutrients for industrial fermentations. In Table 2.2, various biomass resources and the applied pretreatment methods have been listed for some microbial EPS producers together with the EPS yields obtained after a certain fermentation period.

2.3.1.1 Syrups and Molasses

Syrups and molasses have long been used as substrates for fermentative production of commercial polysaccharides such as pullulan [46–49], xanthan [57], dextran [38], scleroglucan [32], levan [33, 41, 42], and gellan [39] due to their many advantages like high sucrose and other nutrient contents, low cost and ready availability, and ease of storage. Using molasses in crude form resulted in low pullulan [46] and scleroglucan [32] yields which in turn pointed out the need for pretreatments. Pretreatment of sugar beet molasses with sulfuric acid has been reported for pullulan [47] and levan [33] production but, when acid treatment was combined with activated carbon treatment, significant improvements in pullulan [48, 49] and levan [33] yields

Table 2.2 Biomass resources and applied pretreatments for some microbial EPSs

EPS	Microorganism	Biomass	Pretreatment	Yield (Time)	Reference
Curdlan	*Agrobacterium* sp. ATCC 31749	CCS	Clarification by filtration	7.72 g/L (120 h)	[36]
Dextran	*L. mesenteroides* NRRL B512	Carob extract	Milling	8.56 g/L (12 h)	[37]
		Aqueous extraction			
Dextran	*L. mesenteroides* NRRL B512	Carob extract and cheese whey	Deproteinization of whey	7.23 g/L (12 h)	[37]
Dextran	*L. mesenteroides* V-2317D	Sugar beet M	No treatment	50 g/L (9 days)	[38]
Gellan	*S. paucimobilis* ATCC-31461	Sugarcane M	Dilution	13.81 g/L (48 h)	[39]
Gellan	*S. paucimobilis* ATCC 31461	Cheese whey	Neutralization	7.9 g/L (100 h)	[40]
		Heat treatment			
Levan	*Halomonas* sp. AAD6	Starch M	Clarification by centrifugation	12.4 g/L (210 h)	[33]
	Sugar beet M	pH adjustment			
		Acid hydrolysis			
		TCP treatment			
		AC treatment			
Levan	*Paenibacillus polymyxa* NRRL B-18475	Sugar beet M	Dilution	38.0 g/L (5 days)	[41]
		Gel filtration chromatography			
		Anion exchange chromatography			
Levan	*P. polymyxa* NRRL B-18475	Sugarcane syrup	Clarification by filtration	19.6 g/L (5 days)	[41]
Levan	*Zymomonas mobilis* ATCC 31821	Sugarcane M	Clarification by centrifugation and filtration	2.53 g/L (24 h)	[42]
Levan	*Z. mobilis* ATCC 31821	Sugarcane syrup	Clarification by centrifugation	15.5 g/L (24 h)	[42]
		Filtration			
Pullulan	*Aureobasidium* sp. NRRL Y	CCS	Clarification by centrifugation	4.5 g/L (9 days)	[43]
Pullulan	*A. pullulans* SU-M18	Carob extracts	Aqueous extraction	6.5 g/L (3 days)	[44]
Pullulan	*A. pullulans*	OMW	Clarification by filtration	8 g/L	[45]
Pullulan	*A. pullulans* NRRLY-6220	OMW	No treatment	10.7 g/L (7 days)	[46]
Pullulan	*A. pullulans* NRRLY-6220	Grape pomace	Aqueous extraction	22.3 g/L (7 days)	[46]
Pullulan	*A. pullulans* NRRLY-6220	Sugar beet M	Dilution	6.0 g/L (7 days)	[46]
Pullulan	*A. pullulans*	Sugar beet M	Acid hydrolysis	32.0 g/L	[47]

Table 2.2 (continued)

EPS	Microorganism	Biomass	Pretreatment	Yield (Time)	Reference
Pullulan	A. pullulans P 56	Sugar beet M AC treatment	Acid hydrolysis	24 g/L (144 h)	[48]
Pullulan	A. pullulans P56	Sugar beet M K$_3$[Fe(CN)$_6$] treatment AC treatment	Acid hydrolysis	35 g/L (96 h)	[49]
Scleroglucan	Sclerotium rolfsii MTCC 2156	Sugarcane juice	Dilution	23.87 g/L (72 h)	[32]
Scleroglucan	S. rolfsii MTCC 2156	Sugarcane M	Dilution	19.21 g/L (72 h)	[32]
Scleroglucan	S. rolfsii MTCC 2156	Coconut water	Dilution	12.58 g/L (72 h)	[32]
Scleroglucan	S. rolfsii MT-6	Waste loquat kernel Acid Hydrolysis Detoxification	Milling	12.08 g/L (72 h)	[50]
Scleroglucan	S. glucanicum NRRL 3006	CCS	Dilution	14.8 g/L (144 h)	[51]
Xanthan	X. campestris	Carob extracts Pressing Heat treatment	Aqueous extraction	0.126 g/L/h	[52]
Xanthan	X. campestris PD 656	Apple pomace Alkaline treatment	Drying and crushing	52.1 g/L (6 days)	[53]
Xanthan	X. campestris	Grape pomace Alkaline treatment	Drying and crushing	10 g/L (6 days)	[53]
Xanthan	X. campestris PD 656	Tangerine peels	Alkaline treatment	32.9 g/L (6 days)	[53]
Xanthan	X. campestris NRRL B-1459	Sugar beet pulp	No pretreatment	1.19 g/L (4 days)	[54]
Xanthan	X. campestris NRRL B-1459	OMW	Clarification	4 g/L (5 days)	[55]
Xanthan	X. campestris T646	OMW	Clarification	7.7 g/L (5 days)	[56]
Xanthan	X. campestris ATCC 1395	Sugar beet M	No pretreatment	53 g/L (24 h)	[57]
Xanthan	X. campestris EBK-4	Ram horn hydrolysate Heat treatment Clarification by filtration	Acid hydrolysis	25.6 g/L (48h)	[58]
Xanthan	X. campestris 1182	Cheese whey	No pretreatment	26.35 g/L (72 h)	[59]
β-Glucan	Botryosphaeria rhodina	OMW	Clarification by centrifugation	17.2 g/L (120h)	[60]
EPS	Paenibacillus jamilae CECT 5266	OMW	Clarification by filtration	2.5 g/L (100h)	[61]
EPS	P. jamilae CP-38	OMW	Clarification by filtration	5 g/L (72 h)	[62]
EPS	Halomonas sp. AAD6	Sugar beet pulp	Drying and milling	2.22 g/L (3 days)	[63]

AC: activated carbon, CCS: condensed corn solubles, M: molasses, OMW: Olive mill wastewater, TCP: tricalcium phosphate

were obtained, most probably due to the removal of heavy metals and colored substances. Activated carbon is particularly known for its efficiency in removing heavy metal pollutants [64]. However, after a systematic study on the effect of different pretreatments on the heavy metal distribution of starch and beet molasses samples, Küçükaşık et al. [33] reported a drastic increase in the dissolved iron (Fe^{2+}) content after the activated carbon treatment. This has been attributed to the reduction of iron from its impregnated Fe^{3+} form to its soluble form since this increase in soluble iron was more profound when acid treated samples were subjected to activated carbon treatment [33]. Same authors suggested tricalcium phosphate ($Ca_3(PO_4)_2$, TCP) treatment as an effective method for selective removal of iron and zinc from molasses or other mixtures of comparable composition. Heavy metals like iron, zinc, and nickel are known to enter the apatite crystal structure of TCP by replacing the Ca atom [65]. Göksungur et al. [49] applied potassium ferrocyanide ($K_3[Fe(CN)_6]$) treatment to precipitate heavy metals. For the microbial levan production with *Paenibacillus polymyxa* NRRL B-18475, clarified sugar cane syrup and crude sugar beet molasses resulted in very low yields. Therefore, peptone was added to the cane syrup and beet molasses was subjected to various expensive pretreatments like passing it through gel filtration and anion exchange columns in order to increase the levan yields to levels comparable with sucrose [41]. To produce levan from *Zymomonas mobilis*, both sugarcane molasses and sugarcane syrup were clarified by centrifugation followed by filtration and then used at 250 g/L carbohydrate concentration [42]. For levan production by halophilic *Halomonas* sp. cultures, sugar beet molasses, and starch molasses were subjected to five different physical and chemical pretreatment methods and their combinations, that is, clarification, pH adjustment, sulfuric acid, TCP and activated carbon treatment [33]. In both molasses types, pretreatments like clarification, pH adjustment were not adequate as also reflected by the low EPS production yields due to the retained undesirable constituents (e.g., heavy metals, impurities) which influence the growth of microorganism and associated polysaccharide production [47, 66, 67]. Highest levan yields were obtained with sugar beet molasses pretreated with TCP followed by acidification with sulfuric acid and then subjected to activated carbon pretreatment [33]. On the other hand, Kalogiannis et al. [57] applied various treatment methods to sugar beet molasses including aeration, acid, activated carbon, $K_3[Fe(CN)_6]$ treatments, and ion exchange chromatography, however, none of the pretreatments improved the xanthan yield of *X. campestris* ATCC 1395 cultures and the highest production was obtained with the untreated crude molasses. Crude beet molasses is also used to produce dextran by *L. mesenteroides* bacterial cultures and the yields were comparable to those of media containing pure sucrose [38]. Banik et al. [39] used Response surface methodology to optimize the production of gellan gum by *S. paucimobilis* ATCC-31461 using crude sugarcane molasses and reported a maximum yield of 13.81 g/L gellan. Survase et al. [32] used various dilutions of coconut water, sugarcane molasses, and sugarcane juice for scleroglucan production by filamentous fungi *S. rolfsii* MTCC 2156 and obtained the highest yields (23.87 g/L in 72 h) from sugarcane juice that was obtained from a local market and hence did not require any pretreatments before use. Coconut water and sugarcane juice were also used for EPS production by *Lactobacillus confusus* cultures [68].

2.3.1.2 Sugar Beet Pulp

Sugar beet pulp (SBP), a by-product of the sugar beet industry, is the fibrous material left over after the sugar is extracted from sugar beets and is mainly composed of cellulose, hemicellulose, and pectin. Beet pulp is used in countries with an intensive cattle raising industry, as livestock feed. In other countries, it is dumped in landfills. However, SBP can be an important renewable resource and its bioconversion appears to be of great biotechnological importance [69].

There are several reports on the pretreatments applied to SBP. Autoclaving of SBP at 122 °C and 136 °C for 1 h was reported to change its composition and physico-chemical properties causing increased swelling and improved solubility of pectins and arabinans [70]. Ammonia pressurization depressurization (APD) pretreatment where SBP is exploded by the sudden evaporation of ammonia, was found to substantially increase hydrolysis efficiency of the cellulose component [71]. Recently, Kühnel et al. [72] examined the influence of six mild sulfuric acid or hydrothermal pretreatments at different temperatures on the enzymatic degradability of SBP and they found that optimal pretreatment at 140 °C of 15 min in water was able to solubilize 60 % w/w of the total carbohydrates present, mainly pectins.

Taking into consideration that SBP is carbohydrate-rich with a high carbon-to-nitrogen ratio (C/N, 35–40), that sugar beet farming is a widespread and already mature industry, and that beet pulp is abundant and cheap, this coproduct has potential for use as a renewable biomass feedstock for microbial fermentations for biopolymer production [71]. On the other hand, there are very limited numbers of reports on the use of SBP as a resource for microbial polysaccharide production. Yoo and Harcum investigated the feasibility of using autoclaved SBP as a supplemental substrate for xanthan gum production from *X. campestris* and they reported a production yield of 0.89 g xanthan per gram of SBP in 4 days of fermentation time [54]. Söğütçü et al. [63] investigated the effects of autoclaving, reducing the particle size by milling and accessibility of SBP for EPS production by halophilic *Halomonas* sp. AAD6 cultures. In this study, milling of dried SBP in a mortar grinder, supplying SBP in dialysis tubes rather than directly in culture media and autoclaving SBP separately, and then adding to the fermentation media were all found to increase the EPS yields.

2.3.1.3 Olive Mill Wastewater

The manufacture of olive oil produces large amounts of a dark colored juice called OMW that consists of a mixture of water from the olive, machinery cooling waters, fruit washings, and remainder of the fruit. Typically, OMW comprises about 15 % organic material that is composed of carbohydrates, proteins, and lipids as well as a number of other organic compounds including monoaromatic and polyaromatic molecules [62] and the toxic effects are mainly derived from its extremely high organic load and the presence of recalcitrant organic compounds such as polyphenols with strong antimicrobial properties. Hence valorization of OMW produced by the olive oil industry has long been an environmental concern in Mediterranean

countries [61]. Beside various conventional technological treatment methods applied, biovalorization of OMW to value-added chemicals is considered as the most cost-effective and environmentally compatible option [73]. Due to its composition with high carbon-to-nitrogen ratio, its use as a suitable substrate for microbial polymer production has been proposed [45] and applied to produce pullulan [45] and xanthan gum [74]. By studying the resource variability factors [55] and then by use of a high-producer strain and medium optimization, Lopez et al. [56] reported significant improvements in xanthan yields, reaching 7.7 g/L in 5 days. In all these studies, in order to reduce the inhibitory effect of phenols, the OMW obtained from the industry was clarified by filtration, diluted with distilled water or saline, neutralized, sterilized by autoclaving, and then used in microbial fermentation. OMW pretreated by this approach has also been used for the microbial production of a metal-binding EPS by *Paenibacillus jamilae* bioreactor cultures. Due to the high phenol biodegradation ability of *Paenibacillus* genus, these cultures were not only proposed for the production of an EPS that could be used as a biofilter but also for the bioremediation of OMW [62]. The main constraint associated with the use of OMW is the need for dilution in order to lower the amount of phenols which in turn limits the concentration of the used waste as culture medium [62]. On the other hand, for the β-glucan production from the fungus *Botryosphaeria rhodina* DABAC-P82, OMW was only clarified by centrifugation and then after steam sterilization, directly applied as substrate without dilution. Due to the lack of oxidase activity, high biopolymer yields and decreases in phenol content of the culture were attributed to the adsorption action of the fungal biofilm [60]. Undiluted OMW was found to be a poor substrate for pullulan production by *A. pullulans* [46]. Besides EPSs, OMW has also been used as a fermentation substrate for the microbial production of other biopolymers including polyhydroxyalkanoates (PHAs) [75].

2.3.1.4 Cheese Whey

Whey is the major by-product obtained during the preparation of dairy products such as cheese. The nutrient composition of whey is based on the nutrient composition of milk from which it is derived, which in turn is affected by many factors including how the milk was processed. Lactose is the major component comprising about 70 % of the total solids of whey. Whey also contains a pool of nutrients and growth factors that have the potential to stimulate the growth of microorganisms but the suitability of whey for EPS production highly depends on the ability of the microorganism to utilize lactose. Cheese whey has been used as carbon and nitrogen source for xanthan [59] and gellan [40] production. Mozzarella cheese whey has been used for xanthan production by two different *X. campestris* strains and although both strains reached comparable yields, the polymers were found to differ in their chemical characteristics [59]. The low yields were attributed to the low capacity of the *X. campestris* strains to utilize lactose. On the other hand, Fialho et al. [40] evaluated the gellan gum production by the *S. paucimobilis* ATCC 31461 strain in media containing lactose, glucose, and sweet cheese whey as substrates. The strain was known to grow on

lactose and to produce highly viscous gellan directly from lactose [76]. Sweet cheese whey obtained from the industry was neutralized and disinfected by three cycles of heat treatment at 80 °C for 30 min. A maximum gellan yield of 7.9 g/L could be recovered from the flask cultures after 100 h of fermentation period [40]. Cheese whey has also been investigated as a potential substrate for dextran production by *L. mesenteroides* NRRL B512 cultures [37]. For this, proteins were removed from whey by precipitation through autoclaving and then centrifugation. Though lactose in the supernatant was found to repress the dextransucrase activity, 7.23 g/L dextran could be produced when carob extract was also present in the medium [37].

2.3.1.5 Pomace

Only few researchers have published work on grape pomace, which today is a very significant waste product in agriculture industries. Grape pomace is the residue left after juice extraction by pressing grapes in the wine industry. Globally, about 10 million tons of grape pomace (seeds, skin, and stem) is produced each year. In Spain alone, over 250 million kg of this by-product are used every year either as animal feed (with low nutritional value) or for ethanol production by fermentation and distillation (low level benefit). This material is underexploited and most of it is generally disposed in open areas, leading to serious environmental problems [77]. Israilides et al. [46] extracted the sugars in the grape pulp by using hot water at 65–70 °C and then clarified the solution and used for pullulan production by *A. pullulans* NRRLY-6220 cultures. Since the grape pomace extract mainly contained sugars and very low amounts of protein, the polymer produced was very similar in its amount as well as molecular weight to the pullulan produced in defined medium. Moreover, the pullulan yields were high reaching 22.3 g/L after 7 days of fermentation period. Stredansky and Conti [53] tested grape pomace, tangerine peels, and apple pomace as substrate for xanthan production by solid state fermentation (SSF) with *X. campestris* NRRL B-1459 cultures. These substrates were soaked in alkaline solution to neutralize the organic acids and then added to the fermentation media. Performances of these feedstocks were also evaluated in the presence and absence of spent malt grains as inert support and apple pomace proved to be a superior substrate yielding high amounts of xanthan under both conditions. Low xanthan yields with grape pomace were attributed to the low sugar content used and the low absorption capacity of the solid material.

2.3.1.6 Carbon Dioxide

CO_2 is a nontoxic, nonflammable, abundant, and renewable feedstock and its biotransformation into industrially important chemicals can not only have a positive impact on the global carbon balance but also provide novel routes for the green biotechnology. As one of the oldest life forms on earth, microalgae have very high CO_2 biofixation capacity, grow fast, and accumulate large quantities of lipids and

carbohydrates and hence became the most promising feedstock for production of next generation biofuels like biodiesel and bioethanol [78]. Considering the fact that CO_2 is a very cheap carbon source, microalgal systems should also be considered as potential resources for EPS production. However, in the literature, there are very few reports on microalgal polysaccharide production. In general, for production of value added products, the biggest advantage in using open microalgae culture is the direct use of solar energy which in turn is highly energy efficient and cheap [79]. Actually, these systems applied to phototropic and mixotrophic cultures are considered to be the most technically and economically feasible methods at commercial scale [78]. On the other hand, this advantage does not hold for EPS production where use of monocultures, closed, and controlled cultivation systems are required to reach high levels of productivity [80]. Although photobioreactors and fermenters are advantageous in providing optimum conditions for biomass growth and EPS production, these systems are expensive and energy intensive when compared with open systems [81].

There are various types of bioreactors that can be used for EPS production such as airlift flat plate photobioreactors (well reviewed by Zhang et al. [82]). Generally, culture conditions for lipid-rich biomass production and EPS production are remarkably different. A systematic study conducted with the green colonial fresh water microalgae *Botryococcus braunii* strains on the effect of culture conditions on their growth, hydrocarbon and EPS production also revealed two distinct culture conditions so that cultivation in 16:8 h light dark cycle yielded higher hydrocarbons whereas continuous illumination with agitation yielded higher amounts of EPSs with 1.6 g/L—maximum yield obtained from *B. braunii* LB 572 strain [83]. In a study on the effect of salinity with the same strain, EPS yields of 2–3 g/L were also reported [84]. The difference in cultivation conditions could also be used for the high-level EPS production by use of a two-stage culture as reported for spirulan production by *Spirulina platensis* [85]. In this method, whereas the first stage focuses on rapidly increasing microalgal biomass, culture conditions in the second stage are modified to maximize the polysaccharide yield. *Rhodella violacea* [86] and *Porphyridium cruentum* [87] are well known as producers for viscous bioactive EPS [88] and the highest yield of 543.1 mg/L EPS production was reported for *P. cruentum* after optimization of initial pH, light intensity, inoculation ratio, and liquid volume of shaking batch cultures [89]. By culturing *P. cruentum* semi-continuously in flat plate photobioreactors, a production rate of 68.64 mg/L per day could be reached by Sun et al. [90]. Very low EPS concentrations (less than 30 mg/L) were reported for planktonic diatoms like *Amphora holsatica*, *Navicula directa*, and *Melosira nummuloïdes* [5, 91]. However, these yields can be improved by further studies on optimizing the bioreactor conditions in favor of EPS production.

Another important issue for microalgal cultivation is the need for using high concentrations of chemical fertilizers as a source for nitrogen and phosphorus. Whereas high nitrogen concentrations in the cultivation medium favors polysaccharide synthesis pathways and biomass formation, lipid accumulation is favored under nitrogen limited conditions where polysaccharide pathways are blocked and the photosynthetically fixed carbon is directed towards fatty acid synthesis [92]. Microalgae could

become a favorable source for EPS production if the high expenses associated with fertilizers could be reduced by replacing them with their low cost alternatives. Besides the use of wastewater as an inexpensive source, the literature is very limited in such studies.

2.3.1.7 Lignocellulosic Biomass

Lignocellulosic biomass is also a cheap and abundant alternative for microbial biopolymer production, especially for microbial systems with hydrolytic capability via endoglucanases or cellobiose. Otherwise, it is utilized to a limited extend during the fermentation and hence requires pretreatments beforehand. The filamentous fungi *S. rolfsii* and other medicinal mushrooms (*Basidiomycetes*) can naturally metabolize different five carbon sugars like xylose and arabinose and hence are especially well suited for EPS production from lignocellulosic substrates. Some examples include the simultaneous production of schizophyllan and arabinoxylan by *Schizophyllum commune* strain ATCC 38548 cultures grown on alkaline H_2O_2-pretreated corn fiber as a sole carbon source [93]. Same strain was also used for schizophyllan production from activated charcoal detoxificated rice hull hydrolysate [94]. Influences of individual or combined inhibitors as well as the importance of detoxification step in EPS production were systematically investigated in this study. Under SSF conditions, a high temperature tolerant white rot fungus *Lentinus squarrosulus* MBFBL 201 was reported to degrade cornstalks very fast and up to 5 g/L EPS could be recovered from the fermentation media [95].

On the other hand, there are only very few reports on the bacterial EPS production using cellulose-rich biomass. Acid hydrolaysates of wood were used for succinoglycan production by *Pseudomonas* sp. ATCC 31260 cultures. The produced EPS was found to be rheologically comparable with commercially available xanthan [96]. When cultured on acid-hydrolyzed sawdust, *Brevundimonas vesicularis* LMG P-23615 and *Sphingopyxis macrogoltabida* LMG 17324 bacterial strains were found to accumulate high amounts of PHA with yields ranging from 64 to 72 % of the dry cell weight [97]. In another study, lignocellulosic fibers with 58–63 % cellulose content were used as a low cost natural complex carbon source for EPS production by *Bacillus megaterium* RB-05 cells with known cellulase activity. The fibers immersed in production medium were pretreated by autoclaving for 15 min, however, once inoculated with cells, EPS production was found to be driven solely by the bacterial cellulase activity. Moreover, recovery of the EPS from the culture required several steps due to the biofilm formed along the fibers [98]. In another recent study, rice bran was subjected to serial enzymatic treatment using amylase, amyloglucosidase, alcalase, and lipase enzymes and then the hydrolysate was used for the co-production of intacellular and extracellular polymers by nitrogen-fixing *Sinorhizobium meliloti* MTCC 100 shaking bacterial cultures. Supplementation of the medium with 20 % rice bran hydrolysate resulted in maximum yields of 11.8 and 3.6 g/L EPS and PHA, respectively [99].

2.3.1.8 Others

Carob (*Ceratonia siliqua* L.), which has long been regarded as just a nitrogen-fixing tree grown in the Mediterranean region, has recently found its place in the food industry as a biomass substrate due to its very high sugar content [100]. Moreover, it has been established as a viable biomass resource for bioethanol production [101]. Carob extracts have also been used for microbial production of xanthan [52] and pullulan [44] polysaccharides. Roseiro et al. [52] developed a multistep pretreatment process for carob-based feedstocks that involves aqueous extraction of carob pulp followed by pressing. By recycling of press liquor, the final sugar content of the carob extract was improved however, as a result of esterasic activities, the syrup was found to contain increasing concentrations of isobutyric acid with time in a pH-dependent manner. Though accumulation of isobutyric acid could be controlled by an additional heat treatment step, its presence was found to inhibit the growth of *X. campestris* cells [52]. When carob extracts with 25 g/L initial sugar content were used for pullulan production by a pigmented strain of *A. pullulans* (SU-M18), a pullulan productivity of 2.16 g/L/day could be reached at pH 6.5 and 25 °C [44]. For dextran production, carob pod residues obtained from the galactomannan industry were milled and the sugars were extracted at 70 °C by use of an acetate buffer. A dextran yield of 8.56 g/L could be reached by *L. mesenteroides* NRRL B512 cultures within 12 h of fermentation period [37].

Condensed corn solubles (CCS) is a by-product of bioethanol industry. While ethanol is separated from the fermentation broth via distillation, the remaining solids are first recovered by centrifugation and then concentrated using evaporators. The final product CCS contains changing levels of carbohydrates, proteins, vitamins, and nutrients [102]. CCS obtained from a dry-mill ethanol plant has been diluted and used for the cost-effective production of scleroglucan by *S. glucanicum* [51, 103]; however, the yields were lower than those of *S. rolfsii* cultures grown on sugarcane juice or molasses [32], coconut water [32], and waste loquat kernel [50]. In another study, CCS was diluted, neutralized, clarified by centrifugation and filtration, and then used for the poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) production by *Rhodospirillum rubrum* cultures [102]. CCS from a wet-mill ethanol production plant has been clarified by centrifugation and then used as substrate for pullulan production by *Aureobasidium* sp. strain NRRL Y-12974 cultures and the yields (4.5 g/L in 9 days) were found to be comparable with those of soluble starch (5.4 g/L in 9 days), however, much lower than glucose containing medium (10.1 g/L in 9 days) [43]. CCS has also been used for curdlan production by *Agrobacterium* sp. ATCC 31749 shake flask cultures. A maximum curdlan yield of 7.72 g/L was recovered after 120 h of fermentation in media containing 400 g/L CSS [36].

Ram horn hydrolysates were also reported to be a suitable enhancer for xanthan production by *X. campestris* EBK-4 because of their high amino acid and mineral content. To obtain the hydrolysates, ram horns, which are usually discharged as waste in slaughterhouses, were subjected to acid hydrolysis with sulfuric acid followed by heat treatment at 130 °C. After neutralization, the hydrolysates were clarified by filtration and then added to the fermentation medium [58].

Waste loquat kernel is another potential biomass resource for EPS production due to its high protein and carbohydrate content. Waste kernels are dried and milled and then subjected to acid hydrolysis with 2 M HCl using an autoclave. Then the hydrolysates were detoxified with $Ca(OH)_2$, neutralized, and then used for scleroglucan production by *S. rolfsii* MT-6 [50] and EPS production by *Morchella esculenta* [104].

Other biomass residues used for microbial EPS production include corn-steep, spent grain and spent sulfite liquors, hydrolyzed potato starch, peach pulp, and peat hydrolysate [28, 30].

2.4 Summary

Synthesis of value added biochemicals from biomass using microorganisms serves as a promising alternative to harsh chemical synthesis processes that employ expensive, hazardous, and non-renewable raw materials. Though functional characteristics of a biopolymer establish its market potential, a useful biopolymer cannot find its proper place in the polymer market unless it can be produced economically. In microbial polysaccharide production, the shift in feedstock utilization requires intensive research activities for the application of innovative concepts on a large scale. These concepts could involve novel resources and pretreatments as well as fermentation and downstream processing techniques.

Suitability of the feedstock is largely determined by the metabolic needs of the microorganism for EPS production. If the production relies on glucose or sucrose, then syrups and molasses of varying origin are established substitutes. However, other biomass resources rich in pectin, sucrose or glucose like ram horn, pomace, and pulps from food industry could also be used along with an appropriate pretreatment to extract the precursors for polysaccharide synthesis. Similarly, suitability of cheese whey for gellan rather than xanthan and dextran production is a direct outcome of the lactose metabolism of the producer strain. Cellulose-rich biomass is an already established resource for fungal bioactive EPSs; however, more studies on the isolation of novel EPS producing cellulolytic strains, development of pretreatment methods, and optimization of fermentation conditions are needed for feasible production of bacterial EPSs from lignocellulosic biomass resources.

Another very important issue in microbial EPS production is that the chemical and physical properties of the polymers are largely determined by the cultivation conditions and this variation in polymer properties is more pronounced when biomass residues are used as feedstock. This point becomes more important when the product is launched to the market. Hence precise characterization of the EPS as well as mechanism of its biosynthesis is of utmost importance in the search for suitable biomass resources.

For biopolymers with high-value applications, rather than the production yields, consistency in both product quality and yield are important which in turn can be

ensured to a high extend by preventing the carryover of impurities and metabolic by-products. In such cases, chemically defined medium conditions are usually preferred for cultivation over complex medium with varying composition. Microalgae enable the use of CO_2 as a cheap, simple, and abundant carbon source and hence cell-free microalgal cultivation media could be a good source for the recovery of bioactive polysaccharides. With accumulating knowledge and awareness on the biological importance of microalgal polysaccharides, a growing number of studies are now focused on optimizing the fermentation conditions for their production. Problems associated with the diversity of culture conditions for lipid-rich biomass production, and EPS production could be overcome by further studies on the development of multi-stage process strategies. Such studies on integration of value added chemical production to microalgal biofuel production based on biorefinery approach hold great importance for the long-term sustainability of the whole process.

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