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Review

Microbial exopolysaccharides: Main examples of synthesis, excretion, genetics and extraction

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ABSTRACT

Exopolysaccharides (EPSs) produced by microorganisms represent an industrially untapped market. Some microorganisms can produce and excrete over 40 g L^{-1} of EPS in simple but costly production conditions.

Approximately thirty strains of eukaryotic and prokaryotic microorganisms are notable for their EPS production. EPSs are produced in response to biotic and abiotic stress factors and/or to adapt to an extreme environment. The main function of EPSs is to aid in protection against environmental pressures.

Heteropolysaccharides and some homopolysaccharides are synthesised in microbial cells and then secreted into the extracellular environment. More currently, homopolysaccharide synthesis occurs outside of the cells after specific enzymes are exuded.

Although natural secretory mechanisms exist in microorganisms, it is often necessary to resort to physical or chemical extraction methods to improve the yield of EPSs at an industrial level.

In light of growing interest, our basic understanding of microbial EPSs needs to be improved.

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1. Introduction

Polysaccharides are industrially used as thickeners, stabilisers and gelling agents in food products. More recently they were used as depollution agents and there was a growing interest in their biological functions like antitumor, antioxidant or prebiotic activities (Liu et al., 2010). They are derived from a wide variety of sources: bacterial, fungal, algal and plant. Despite the many sources of polysaccharides, the world market is dominated by polysaccharides from algae and higher plants (Jonas & Farrah, 1998; Leung, Liu, Koon, & Fung, 2006). These biopolymers are obtained by direct extraction from biomass and may be subjected to chemical hydrolysis or fermentation to obtain the smallest molecules able to be polymerised (Pichavant, 2009).

Higher plants are the primary source of polysaccharides, which include starch, cellulose, pectins and "gums". Polysaccharides come

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from plant cell walls in the form of cellulose or lignin. Polysaccharides are also stored as starch for reserves (Reddy & Yang, 2005). Cellulose, mainly from cotton plants (*Gossypium sp.*), is the most exploited biopolymer today. Galactomannane, or "gum", is another commonly used polysaccharide in the food industry. It is produced from Guar (*Cyamopsis tetragonolobus*) and Locust Beans (*Ceratonia siliqua*) (Bourbon et al., 2010).

As for starch, 60 million tons are extracted per year from different cereal crops, including maize and wheat, and roots and tubers, such as manioc and potatoes. Starch is used in various applications: as a stabiliser for soups and frozen foods, a pill coating, a paper covering and as a raw material to produce ethanol.

The principal polysaccharides from red algae (Rhodophyceae) and brown algae (Phaeophyceae) are the following classes: carrageenans, derived from *Kappaphycus alvarezii* and *Euchema denticulum*; alginates, derived from *Laminaria sp*., *Pelvetia sp*., *Sargassum sp*., *Ecklonia sp*. and *Undaria sp*.; agar, derived from *Gelidium sp.* and *Gracilaria sp*.; and fucans, derived from *Stichopusc sp*. and *Laminaria sp*. (De Ruiter & Rudolph, 1997; Li, Chen, Yi, Zhang, & Ye, 2010). Polysaccharides were first marketed in the 1930s in the United States. Today, annual world production of polysaccharides from marine biomass is approximately 25 000–30 000 tons per year (Pichavant, 2009).

Polysaccharides derived from microorganisms, including bacteria, yeasts and moulds, represent an unexploited market (Sutherland, 2001). Polysaccharide biosynthesis and accumulation generally take place after the growth phase of the microorganism. The polysaccharides produced by microorganisms can be classified into three main groups according to their location in the cell: (i) cytosolic polysaccharides, which provide a carbon and energy source for the cell; (ii) polysaccharides that make up the cell wall, including peptidoglycans, techoïd acids and lipopolysaccharides and (iii) polysaccharides that are exuded into the extracellular environment in the form of capsules or biofilm, known as exopolysaccharides (EPSs). EPSs are divided into two groups: homopolysaccharides and heteropolysaccharides. Homopolysaccharides are made up of a single type of monosaccharide, like dextran or levan. Heteropolysaccharides are made up of several types of monosaccharide like xanthans or gellans, have complex structures and are usually synthesised inside the cell in the form of repeating units (Bergmaier, 2002; Lahaye, 2006; Roger, 2002). Heteropolysaccharides make up the majority of bacterial EPSs. EPS biosynthesis can be divided into three main steps: (i) assimilation of a carbon substrate, (ii) intracellular synthesis of the polysaccharides and (iii) EPS exudation out of the cell (Vandamme, De Baets, & Steinbüchel, 2002). EPSs aid the cell in various functions. EPSs protect against biotic stress, like competition, and abiotic stresses that might include temperature, light intensity or pH. In the cases of acidophilic or thermophilic species and Archaea, EPSs aid in adapting to extreme conditions. Despite the wide diversity of microbial EPSs with physicochemical properties that are industrially promising, only two EPSs are authorised for use as additives in the food industry in the United States and Europe: xanthan (30 000 tons/year) and gellan.

Due to the growing interest in renewable resources, industrial research, particularly in the biofuel sector that produces bioethanol, is increasingly well positioned to incorporate to EPS production and utilisation. Some microorganisms are capable of producing and excreting over 40 g L^{-1} of EPSs under conditions of stress (Lin & Chen, 2007; Papinutti, 2010; Ravella et al., 2010). EPS production from microorganisms has the following advantages: production in a matter of days compared to the 3–6 months in the case of plants; energy efficient, in the case of microalgae (production uses solar energy); possibility of utilising industrial wastes such as glycerol, hydrocarbon residue and CO_2 as carbon substrates (Gonzalez Lopez et al., 2009; Harada, 1965; Thompson & He, 2006) and the absence of competition with arable land. Furthermore, EPSs are naturally exuded by most microorganisms into the extracellular environment (Bejar, Llamas, Calvo, & Quesada, 1998; Chen, Hsu, Lin, Lai, & Wu, 2006; Chi, Pyle, Wen, Frear, & Chen, 2007; Li, Schenk, Srivastava, Zhurina, & Ullrich, 2006; Ravella et al., 2010; Survase, Saudagar, & Singhal, 2006; Tsujisaka & Mitsuhashi, 1993), facilitating their recovery. The main factors limiting EPS production by microorganisms are linked to the costs of production. The main costs consist of purchasing substrate in certain cases and acquiring the infrastructures required for production, which can include bioreactors and maintaining asepsis.

The purpose of this bibliographical review is to make an inventory of the EPSs of industrial interest produced by microorganisms including bacteria, yeasts, moulds and microalgae. This review will present the principal pathways of EPS biosynthesis and describe the mechanisms of naturally occurring excretion and of industrially induced extraction.

2. EPSs of microbial origin and their physiological roles

As the first step of this review, an inventory was made of the main EPSs produced by microorganisms, including yeasts, moulds, bacteria and microalgae (Tables 1 and 2).

The microbial species are presented with their optimal EPS production quantities and a description of the associated substrates and growth conditions.

The molecules are varied in nature and are produced in variable concentrations, ranging from 0.0022 to 86.3 g L^{-1} (Tables 1 and 2).

Both eukaryotic and prokaryotic microbial groups are represented, but bacteria produce the greatest diversity of molecules and produce quantities of over 10 g L^{-1}.

Of the 35 inventoried strains, 15 belong to fungal or algal species. Among these species, only 4 are capable of producing over 10 g L^{-1}. Half of the 20 bacterial strains cited produce EPSs in concentrations of over 10 g L^{-1}.

The physiological role of EPSs depends on the biotope of the microorganisms producing them. EPS production is a direct response to selective environmental pressures, including temperature, pressure and light intensity (Dudman, 1977; Otero & Vincenzini, 2003). These EPSs affect the way in which microorganisms interact with the external environment, whether the environment is liquid or solid.

Microorganisms are often associated in a biofilm of high cellular density. The glycocalyx, which is mainly composed of EPSs, is essential for the formation of a biofilm. The EPSs can influence the stability of a biofilm through interactions between the polysaccharide chains (Higgins & Novak, 1997). EPSs allow the microbial flora to adhere to a biological support, which may constitute a substrate for the microorganism growth. Apart from playing a role in adhesion, biofilm formation occupies an important place in the adaptation of bacteria to the physicochemical conditions of the environment.

EPSs do not appear to function as energy reserves, and microorganisms are unable to catabolise the EPSs produced (Cerning et al., 1994). The major role of EPSs is to protect the cell in its environment.

Surrounding itself with a layer of extracellular polysaccharides containing high water content, the microorganism ensures greater resistance against desiccation and predation by protozoans. Moreover, the anionic nature of the exterior polysaccharide layer can help to capture essential minerals and nutrients. Exopolymers also help to degrade certain metals due to their anionic character and their capacity to chelate metals and ions (Beech & Tapper, 1999; Iverson, 1987; Ozturk, Aslim, & Suludere, 2009; Zinkevich et al., 1996). The polysaccharide envelope also regulates the diffusion

Table 1
Principal exopolysaccharides produced by eukaryotic microorganisms.

Microorganisms	Exopolysaccharides	Microbial strains	Substrates	EPS concentrations ($g L^{-1}$)	Growing conditions	References
Yeasts and filamentous fungi	Pullulan	*Aureobasidium pullulans*	Sucrose	1.3–52.5	pH = 4–4.5; 30 °C; 100 h[a]	Duan et al. (2008), Jiang (2010), Ravella et al. (2010), Seo et al. (2004), Tsujisaka and Mitsuhashi (1993), Wu, Jin, Kim, et al. (2009), and Youssef et al. (1999)
	Scleroglucan	*Slerotium sp.*	Sucrose/glucose	7–21	pH = 4,5; 28 °C; 48–120 h	Wang and McNeil (1995a, 1995b), Survase et al. (2006), and Survase, Saudagar, and Singhal (2007)
	Schizophyllan	*Schizophyllum commune*	Glucose/sucrose	1.62–8.03	28 °C; 168 h	Kumari, Survase, and Singhal (2008)
	Galactan	*Sporobolomyces sp.*	Sucrose	5.63	pH = 5.3; 22 °C; 168 h	Pavlova, Koleva, Kratchanova, and Panchev (2004)
	Glucan	*Rhodoturula sp.*	Glucose/sucrose	1–5	pH = 5.5; 22–26 °C; 3–4 d[b]	Martin, Lu, and Patel (1993), Pavlova and Grigorova (1999), and Pavlova, Panchev, and Hristozova (2005)
		Tremella fusiformes	Glucose		pH = 8; 28 °C; 48–72 h	Cho, Oh, Chang, and Yun (2006)
		Crytptococcus sp.	Sucrose	1–5.75	pH = 5,3; 24 °C; 6 d	Pavlova, Panchev, Krachanova, and Gocheva (2009)
		Tremella mesenterica	Glucose	7.6	25 °C; 1 bar; 7 d	Cheng et al. (2005)
		Ganoderma lucidum	Glucose	15	pH = 3.5; 30 °C; 21 d	Papinutti (2010)
		Tremella aurantia	Xylose/glucose	1.8–7.6	25 °C; 175 h	Chen et al. (2006)
Microalgae		*Porphyridium cruentum*	CO_2	0.1–0.3	pH = 7.5; 20 °C	Rebolloso Fuentes et al. (1999) and Fabregas, Garcìa, Morales, Lamela, and Otero (1999)
		Botryococcus braunii	CO_2	2.5	pH = 7; 25 °C; 14 d	Lupi, Fernandes, Tome, S.-Correia, and Novais (1994)
(Diatoms)		*Amphora holsatica*	CO_2	0.027	19 d	Leandro, Gil, and Delgadillo (2003)
		Navicula directa	CO_2	0.026	9 d	Leandro et al. (2003)
		Melosira nummuloïdes	CO_2	0.0022		Leandro et al. (2003)

[a] Hours.
[b] Days.

of certain molecules between the extracellular and intracellular environments. This regulated diffusion allows certain bacteria to resist surfactants and antibiotics (O'Toole, Kaplan, & Kolter, 2000; Schwarzmann & Boring, 1971).

Apart from its adhesive and protective functions, the formation of a biofilm promotes the association of different microbial species, as in the case of intestinal mucosa (Yegorenkova, Tregubova, Matora, Burygin, & Ignatov, 2011). Once associated in the biofilm, the metabolic products of one species can act as substrates for metabolic processes of another species. Additionally, adherence of one species to a biofilm can create attachment sites for other species (Dunne, 2002).

After analysing the literature, 6 EPS-producing microorganisms were identified with EPS productions above or equal to $50 g L^{-1}$. The EPSs obtained in 24–120 h may be of interest to various industrial sectors seeking renewable resources.

Five bacteria and one mould were identified as strong producers: *Agrobacterium sp.*, producing $76 g L^{-1}$; *Alcaligenes faecalis*, producing $72 g L^{-1}$; *Xanthomonas campestris*, producing $53 g L^{-1}$; *Bacillus sp.*, producing $86.3 g L^{-1}$; *Zymonas mobilis*, producing $50 g L^{-1}$ and *Aureobasidium pullulans*, producing $52.5 g L^{-1}$.

These 6 microorganisms produce 4 different EPSs: levan, produced by *Z. mobilis* and *Bacillus sp.*; pullulan, produced by *A. pullulans*; curdlan, produced by *Agrobacterium sp.* and *A. faecalis* and xanthan, produced by *X. campestris*.

The biosynthetic pathways of all 4 major EPSs, as well as the factors that influence these pathways, are representative of the production mechanisms of microbial EPSs.

3. Biosynthesis, exudation and genetics

3.1. Homopolysaccharides

Extracellular homopolysaccharide synthesis is carried out by a specific secreted enzyme, and synthesis can occur either outside the cell or within the cell wall (Roger, 2002). One example is the synthesis of dextran, which is composed of units of glucose with α-(1–6) linkages and branches composed of α-(1–2) or α-(1–3) linkages. Dextrans are synthesised by *Leuconostoc sp.*, a Gram-positive bacterium, where the only intervening enzyme is dextranesucrase, also known as D-glycosyl-transferase (Petit, 2005).

Table 2
Principal exopolysaccharides produced by prokaryotic microorganisms.

Microorganisms	Exopolysaccharides	Microbial strains	Substrates	EPS concentrations ($g\,L^{-1}$)	Growing conditions	References
Bacteria	Cellulose	*Acetobacter xylinum*	Fructose/glucose	7–23.6	pH = 4–5; 30 °C; 40 h	Hwang, Yang, Hwang, Pyun, and Kim (1999), Kouda, Naritomi, Yano, and Yoshinnaga (1997), Naritomi, Kouda, Yano, and Yoshinaka (1998), Choi, Choi, Lee, and Lee (1996), and Choi, Choi, and Lee (1996)
		Acinetobacter sp.	Ethanol/diesel	4.7	pH = 7; 30 °C; 1 bar	Huang, Chen, and Chen (2008), Huang, Tang, and Shang-Tian (2007), and Kang et al. (2009)
	Alginate	*Pseudomonas aerugina*	Xylose	0.4	30–37 °C; 1 bar; 72 h[a]	Celik, Aslim, and Beyatli (2008)
		Azobacter sp.	Glucose/fructose	1.1–7.5	pH = 7; 35 °C; 1 bar; 72 h	Quagliano and Miyazaki (1999), Celik et al. (2008), Emtiazi, Etemadifar, and Tavassoli (2003)
	Dextran and derivatives	*Leucomostoc sp.*	Sucrose	8.17	pH = 5.5; 35 °C; 1 bar	Santos, Teixeira, and Rodrigues (2000)
	Curdlan	*Agrobactérium*	Glucose/sucrose	5.02–76	pH = 7.5; 30 °C; 5 d[b]	Shih et al., 2009, Stredansky, Conti, Bertocchi, Matulova, and Zanetti (1998), Wu, Zhan, Liu, and Zheng (2008)
		Alcaligenes faecalis	Glucose	30–72	pH = 7; 30 °C; 120 h	Wu et al. (2008)
	Gellan	*Shingomonas*	Starch	13.2–35.7	pH = 7–7.5; 30–32 °C	Nampoothiri, Singhania, Sabarinath, and Pandey (2003)
	Hyaluronic acid	*Sreptococcus sp.*	Glucose	5.0–10.0	pH = 7; 37 °C	Jagannath and Ramachandran (2010)
	Xanthan	*Xanthomonas campestris*	Molasse	53	pH = 7; 28 °C; 1 bar; 24 h	Kalogiannis et al. (2003)
	Levan	*Erwinia sp.*	Sucrose	15		Shih et al. (2010)
		Bacillus spp.	Glucose/sucrose	0.32–86.3	pH = 5.6–5.8; 37 °C; 1 bar; 3 d	Larpin, Sauvageot, Pichereau, Laplace, and Auffray (2002), Liu and Shen (2008), Shih et al. (2010), and van Geel-Schutten, Flesch, ten Brink, Smith, and Dijkhuizen (1998)
		Zymonas mobilis	Sucrose	22–50	30 °C; 120 h	Bekers et al. (2005) and de Oliveira, da Silva, Buzato, and Celligoi (2007)
	Other EPS	*Enterobacter sp.*	Glycerol/glucose	6–18	pH = 7; 30 °C, 4 d	Alves et al. (2010) and Prasertsan, Wichienchot, Doelle, and Kennedy (2008)
		Edwardsiella tarda		0.2	28 °C; 3–7 h	Guo et al. (2010)
		Vibrio diabolicus sp. nov.	Glucose	2.5	pH = 7–8; 30–45 °C; 48 h	Raguénès, Christen, Guezennec, Pignet, and Barbier (1997)
		Geobacillus sp.	Sucrose/maltose	0.114	pH = 6.8–9.8; 54–87 °C	Kambourova et al. (2009)
		Halomonas sp.	Sucrose/glucose	1.6–4.5	pH = 7; 32–37 °C	Bejar et al. (1998) and Poli et al. (2009)
		Nigrospora oryzae var. glucanicum	Sodium acetate	4.5 and 5.3		Sudhakaran and Shewale (1988)
(Cyanobacteria)		*Synechocystis sp.*	CO_2	0.35–0.55	pH = 6.8; 25 °C; 20 d	Ozturk et al. (2009)

[a] Hours.
[b] Days.

3.1.1. Levan

Levan, composed of D-fructofuranosyl residues joined by β-(2–6) linkages, is synthesised outside the cell (Fig. 1).

Levan can be obtained by fermenting saccharose with bacteria, such as *Zymomonas mobilis* or *Bacillus subtilis* (Monsan et al., 2001; Shih, Chen, & Wu, 2010) or by enzymatic synthesis using saccharose as substrate.

Levan biosynthesis depends on an extracellular enzyme with saccharose specificity named levansucrase, also known as sucrose 6-fructosyltransferase, β-(2–6)-fructosyltransferase, and

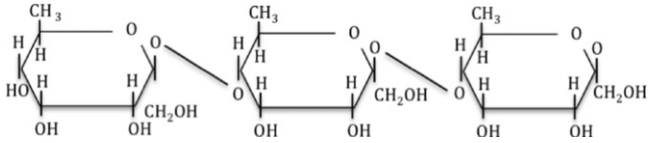

Fig. 1. Chemical structure of levan.

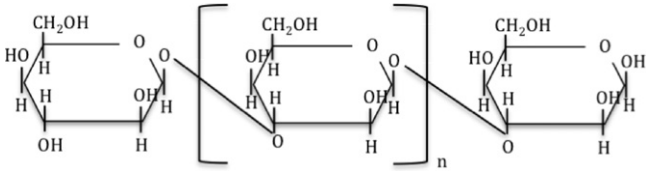

Fig. 3. Chemical structure of curdlan.

β-(2–6)-fructan: D-glucose 1-fructosyltransferase or EC 2.4.1.10. (Meng & Futterer, 2003). Numerous Gram-positive bacteria, including *Bacillus sp*., and Gram-negative bacteria, including *Z. mobilis*, produce levansucrase. This enzyme is the key point in levan production. Levansucrase is an extracellular enzyme whose function is to catalyse levan synthesis from saccharose by transfructosylation. The enzyme accumulates in the periplasm before being excreted and appears to adopt its final conformation in the periplasm. The optimal temperature for levansucrase synthesis depends on the bacterial strain: for *Z. mobilis*, it is 0 °C whereas for *B. subtilis*, it is over 10 °C. Levansucrase is produced in the extracellular environment at acidic pHs (Castillo & Lopez-Munguia, 2004). Levansucrase activity can be inhibited by the presence of glucose and temperatures above 45 °C. Levansucrases seem to use different excretion mechanisms. In some Gram-positive bacteria, levansucrase is reported to be secreted by a two-step mechanism which depends on a signal peptide cleavage and protein folding. The presence of external effecters, such as the metallic ions Fe or Ca and/or pH, completes the signal for the excretion of levansucrase. This enzyme excretion mechanism is common to many strains of *Bacillus sp*., including *Bacillus stearothermophilus*, *Bacillus amyloliquefaciens* and *B. subtilis* (Hernandez et al., 1999; Vandamme et al., 2002). Conversely, in some Gram-negative bacteria, including *Z. mobilis*, *Erwinia amylovora*, *Rahnella aquatilis*, *Pseudomonas syringae pv. glycinea* and *P. syringae pv. phaseolicola*, levansucrase secretion is activated by a signal peptide independent pathway. First kinetics studies suggested a ping-pong mechanism involving a stabilised fructosyl-enzyme complex (Ben Ammar et al., 2002). Details of the catalytic action of the *B. subtilis* fructosyltransferase is now well known (Seibel et al., 2006).

3.1.2. Pullulan

Pullulan is a linear homopolysaccharide composed of glucose. Pullulan is described in the majority of cases as a polymer composed of a succession of maltotriose trimers with following linkages: α-(1–4)-Glu-α-(1–4)-Glu-α-(1–6) (Fig. 2). Several other types of linkages can be found in pullulan, such as a succession of maltotretraose units (α-(1–4)-Glu-α-(1–4)-Glu-α-(1–6)-Glu-α-(1–6)) (Singh, Saini, & Kennedy, 2008). The mould *A. pullulans* (*A. pullulans*) is described in the literature as producing high pullulan concentrations of 52.5 g L^{-1} (Jiang, 2010; Wu, Jin, Kim, Tong, & Chen, 2009).

Unlike levan, pullulan is biosynthesised in the cytosol then secreted into the extracellular environment. Although the biosynthetic pathways of pullulan are not clearly understood, some authors (Shingel, 2004; Singh & Saini, 2008; Singh et al., 2008) have proposed a biosynthesis pathway model where synthesis of the precursors is followed by polymerisation to form pullulan.

The model is based on the action of the glycolipid intermediate, LPh-Glu. The precursors of the pullulan molecule are formed in 3 main stages. The first stage is the formation of LPh-Glu, through the intermediary uridine-diphosphate-glucose (UDPG), which is catalysed by ATP. The second stage transfers an additional D-glucose, produced by UDPG, to form a molecule of isomaltose (LPh-Glu-(1–6)-Glu). In the final stage, the isomaltose interacts with the glycosyl lipid precursor from stage 1 to produce the molecule of isopanosyl (LPh-Glu-(1–6)-Glu-(1–4)-Glu). The isopanosyl molecules are then polymerised into a pullulan chain (Catley & McDowell, 1982; Shingel, 2004). Enzymes involved in the synthesis of pullulan has not been identified yet.

Numerous studies have been carried out to define the optimum conditions for pullulan synthesis (Jiang, 2010; Ravella et al., 2010; Singh & Saini, 2008; Singh et al., 2008; Singh, Saini, & Kennedy, 2009; Vijayendra, Bansal, Prasad, & Nand, 2001; Wu, Jin, Kim, et al., 2009; Wu, Jin, Tong, & Chen, 2009; Youssef, Roukas, & Biliaderis, 1999; Zhang, Chi, Zhao, Chi, & Gong, 2010). These studies have revealed that maximal pullulan synthesis occurred at pH 4.5, but the maximal growth of *A. pullulans* occurred at pH 6.5. The optimal temperature for pullulan production depends on the *A. pullulans* strain and ranges between 24 and 30 °C. Trace elements like vitamins, including biotin and thiamine, and mineral salts, including Cl, Mn and Fe, can also affect the production of EPS (West & Reesd-Hamer, 1992; West & Strohfus, 1997). In addition to growth conditions, pullulan synthesis is also affected by the stage of growth and age of the culture. Pullulan synthesis appears to be concomitant with the formation of chlamydospores (Ravella et al., 2010; Simon, Caye-Vaugien, & Bouchonneau, 1993; Singh et al., 2008).

The choice of carbon source, which may be glucose, saccharose or dextran, and its concentration in the culture medium play a key role in pullulan production (Duan, Chi, Wang, & Wang, 2008; Seo et al., 2004; Singh et al., 2008). Ravella et al. (2010) showed that saccharose is more effective than xylose, glucose, fructose or cel-

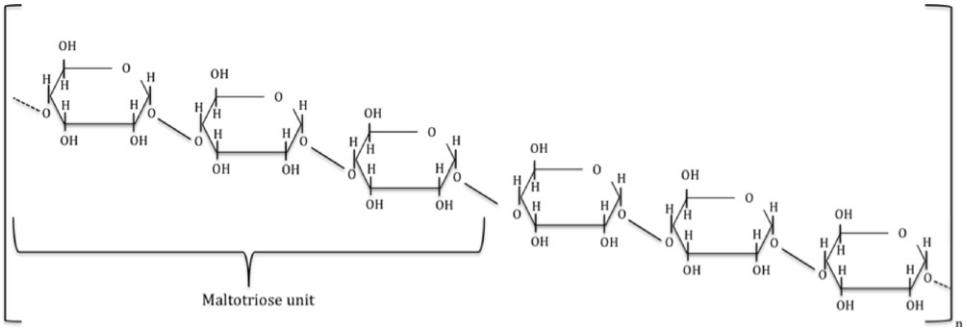

Fig. 2. Chemical structure of pullulan with maltotriose as repeating unit.

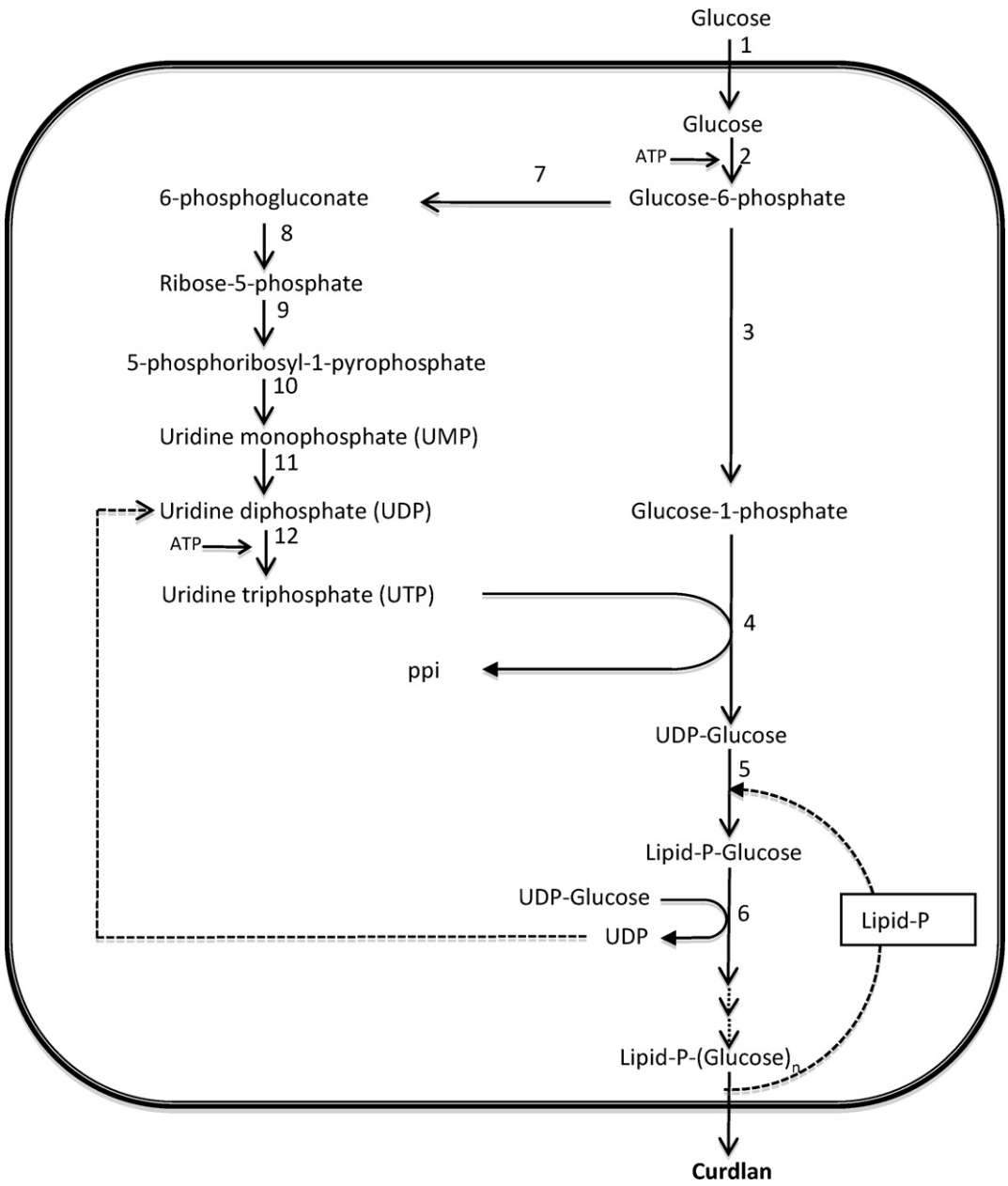

Fig. 4. Metabolic pathway for the synthesis of curdlan (adapted from Vandamme et al. (2002) and Ruffing et al. (2006)). 1: Glucose-binding-protein for glucose uptake, 2: hexokinase, 3: phosphoglucomutase, 4: UDP-glucose phosphorylase, 5: UDP-galactose-4′-epimerase, 6: β-(1–4)-galactosyltranferase, 7: glucose-6-phosphate deshydrogenase, 8: 6-phophosgluconate deshydrogenase, 9: ribose phosphate diphosphokinase, 10: orotate phosphoribosyltranferase and orotidine-5′-phosphate decarboxylase, 11: uridylate kinase, 12: UDP kinase.

lobiose in stimulating EPS production. The same authors showed that pullulan production is promoted by the addition of $NaNO_3$ to the culture medium. Conversely, pullulan synthesis may be inhibited by cycloheximide (Singh et al., 2008).

Pullulan production varies widely among *A. pullulans* strains ranging from 1.3 g L^{-1} to 52.5 g L^{-1} (Duan et al., 2008; Jiang, 2010; Ravella et al., 2010; Seo et al., 2004; Tsujisaka & Mitsuhashi, 1993; Wu, Jin, Kim, et al., 2009; Youssef et al., 1999).

Catley and Hutchison (1981) suggested that pullulan secretion may be associated with the cell wall, the plasma membrane and/or the periplasmic space. However, Finkelman and Vardanis have demonstrated evidence to the contrary. To date, no enzyme involved in this process has been identified (Finkelman & Vardanis, 1982).

3.1.3. Curdlan

Curdlan is an insoluble, linear homopolysaccharide with no branching. It is composed of 400–500 D-glucose residues joined by β-(1–3)-glucosidic linkages (Shih, Yu, Hsieh, & Wu, 2009) (Fig. 3). Curdlan is mainly produced by certain strains of *Agrobacetrium sp*., e.g. *Agrobacterium radiobacter*, and by *A. faecalis var myxogenes*.

During the first stage in curdlan synthesis, glucose enters the cell via an active transporter, which may be the phosphoenolpyruvate-glucose phosphotransferase system (PEP-PTS) and/or permease (Laws, Gu, & Marshall, 2001). When the substrate enters the cell, it is phosphorylated into glucose-6-phosphate. Phosphoglucomutase then converts glucose-6-phosphate into glucose-1-phosphate (Fig. 4). The next step is the synthesis of UDP-glucose, a key precursor of curdlan. UDP-glucose is formed from glucose-1-phosphate and uridine triphosphate (UTP), catalysed by UDP-glucose

pyrophosphorylase. Curdlan synthase catalyses polymerisation, transferring a molecule of glucose from the UDP-glucose to the nascent polymer chain, to produce a molecule of UDP. In the cytosol, UDP kinase uses the ATP from glycolysis or the tricarbolic acid (TCA) cycle to convert UDP into UTP (Jin, Um, Yin, Kim, & Lee, 2008; Ruffing, Mao, & Ruizhen Chen, 2006; Vandamme et al., 2002).

The main factors affecting curdlan production are the following: concentration of nutrients, chiefly carbon, nitrogen and phosphate; the pH of the culture medium and the aeration of the culture (McIntosh, Stone, & Stanisich, 2005; Shih et al., 2009; Vandamme et al., 2002). Jin et al. (2008) defined the optimal pH to be 7 for culturing *Agrobacterium sp*. and optimal pH to be 4.5 for the production of curdlan, with an optimal temperature between 30 and 32 °C. It has been shown that nitrogen deficiency favours the production of curdlan during the stationary phase of growth, provided that the sulphate and phosphate concentrations are optimal (Kim, Jung, Choi, Kim, & Rhee, 2001). The microbial growth rate diminishes with an increase in the concentration of ammonium (Vandamme et al., 2002). The addition of uracil, the precursor of UDP-Glucose, also appears to favour curdlan production. Today, glucose is the chief industrial carbon source used to produce curdlan (McIntosh et al., 2005). However, tests carried out on other carbon sources have shown that saccharose and molasses are also suitable for curdlan production (McIntosh et al., 2005; Shih et al., 2009; Vandamme et al., 2002).

As for pullulan the secretory mechanisms of this intracellular homopolysaccharide have not yet been elucidated (Lee, Bohm, Krug, & Boos, 2007).

3.2. Heteropolysaccharides: the example of xanthan

The biosynthetic pathways of heteropolysaccharides are more complex than those of homopolysaccharides. The synthesis of heteropolysaccharides can be divided into three steps: (i) assimilation of simple sugars and conversion into nucleotide derivatives; (ii) assembly of *pentasaccharide* subunits attached to a lipid transporter, probably the undecaprenyl phosphate or isoprenoid phosphate, and (iii) polymerisation of repeating units of *pentasaccharide* and secretion into the extracellular environment (Whitfield, Valvano, & Rose, 1993). Xanthan, one of the most studied heteropolysaccharides, is produced by *X. campestris* (*X. campestris*). Xanthan is formed from two D-glucoses, two D-mannoses and a D-glucuronic acid (Fig. 5) (Rosalam & England, 2006). This EPS generally has a very high molecular weight ranging from 500 to 2000 kDa, varying according to the bacterial genus and species. Xanthan is formed from repeating units of 2–8 monomers.

The biosynthetic pathway of xanthan (Fig. 6) has been described by several authors (Becker, Katzen, Puhler, & Ielpi, 1998; Leigh & Coplin, 1992). The synthesis of xanthan starts with the assembly of repeating *pentasaccharide* units. These units are then polymerised to produce the macromolecule.

The repeating xanthan units are formed by the sequential addition of monosaccharides, involving acetyl-CoA and phosphoenolpyruvate. The first step of the *pentasaccharide* assembly is the transfer of glycosyl-1-phosphate from an UDP-glucose molecule to a polyisoprenol phosphate of a lipid transporter. To form the lipid-linked *pentasaccharide* unit, this transfer is followed by sequential transfer of the other sugar residues: D-mannose, D-glucuronic acid from GDP-mannose and UDP-glucuronic acid (Rosalam & England, 2006; Vandamme et al., 2002).

The acetyl groups attach to the internal mannose residue, and pyruvate is added to the terminal mannose. The acetyl groups contribute to the texturing properties of xanthan. Once xanthan is synthesised, it is exuded into the extracellular environment.

Each of these steps requires substrates, like glucose and mannose, as well as enzymes, such as a polymerase and a transferase, specific to xanthan synthesis. If the required substrate or enzyme is missing, the step will be blocked.

Studies conducted on optimising xanthan production show that three factors strongly influence the EPS concentration: the carbon source and its concentration, phosphate concentration and pH (Casas, Santos, & Garcia-Ochoa, 2000; Chantaro & Pongsawatmanit, 2010; Ielpi, Couso, & Dankert, 1981a, 1981b; Rosalam & England, 2006). The optimal temperature for producing xanthan is between 30 and 33 °C (Garcia-Ochoa, Santos, Casas, & Gomez, 2000; Vandamme et al., 2002). Xanthan production is maximal at a neutral pH, between 6 and 8. The carbon sources currently used in industry are glucose and saccharose, with the addition of nitrate in the form of glutamate (Garcia-Ochoa et al., 2000). Kalogiannis, Iakovidou, Liakopoulou-Kyriakides, Kyriakidis, and Skaracis (2003) showed that *X. campestris* exuded xanthan at a concentration of 53 g L^{-1}, when grown with molasses in the presence of KH_2PO_4. When grown in glucose, a decreased concentration of 40 g L^{-1} xanthan was observed. As *X. campestris* is a strictly aerobic bacterium, oxygen levels are crucial and may be increased by agitating the culture (Vandamme et al., 2002).

Lipid transporters play an important role in heteropolysaccharide synthesis which is combined with the EPS excretion. These transporters are long-chain phosphate esters and isoprenoide alcohols, identical to those described in the biosynthesis of lipopolysaccharides, O-antigen and peptidoglycans (Sutherland, 1990). In EPS synthesis, lipid transporters provide an anchor to the extracellular membrane and facilitate the precise, orderly formation of the carbohydrate chain and the transport of the chain through the cell membrane. Repeating subunits are assembled on the internal side of the membrane then transferred through the membrane. Once outside the cell, several hundred or even thousand repeat units are assembled by a polymerase. Some polysaccharides are polymerised on the inner side of the cytoplasmic membrane then directly exported through the intermediary of a lipid transporter. Certain proteins linked to lipids may act as flippase-exporters and polymerases of the repeat unit (De Vuyst, De Vin, Vaningelgem, & Degeest, 2001). After excretion, the intervention of an enzyme specific to the EPS may liberate the polymer and, in this case, contribute to the possible formation of a biofilm. The polymer may also remain fixed to the surface of the cell to form a capsule specific to the microorganism, for purposes of adhesion, pathogenicity or protection. The mechanisms of polymerisation, determinants of chain length and mechanisms of exportation have not yet been clearly elucidated.

3.3. Genetics of EPS synthesis

References concerning EPS biosynthesis genetics are disparate and generally refer to a specific product or a specific microorganism production. Indeed microbial strains don't usually produce pure substances but often produce a mixed of polymers whose synthesis involve several gene clusters (Hay, Ur Rehman, Ghafoor, & Rehm, 2010; Orr, Zheng, Campbell, Mcdouqall, & Seviour, 2009; van Kranenburg, Boels, Kleerebezem, & de Vos, 1999). While genetic data on certain EPS like xanthan is abundant, information on genetics of other EPS synthesis (i.e. pullulan) is still scarce. Vorhölter et al. (2008) have demonstrated that the xanthan biosynthesis requires nucleotide sugars (UDP-glucose, UDP-glucoronate and GDP mannose) from which xanthan repeated units are built under the control of the *gum* genes. In the strain *X. campestris*, the biosynthesis is encoded by a single gene cluster of 12 kb (*gumBCDEEFGHIJKLM* genes). The biosynthesis of glycosyltransterase is encoded by the gum genes *D*, *M*, *H*, *K* and *I* (van Kranenburg et al., 1999; Vorhölter et al., 2008). In the case of alginates, 24 genes were identified in *P. aeruginosa* as being involved in this production. The cluster consists of 12 structural genes (*algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*,

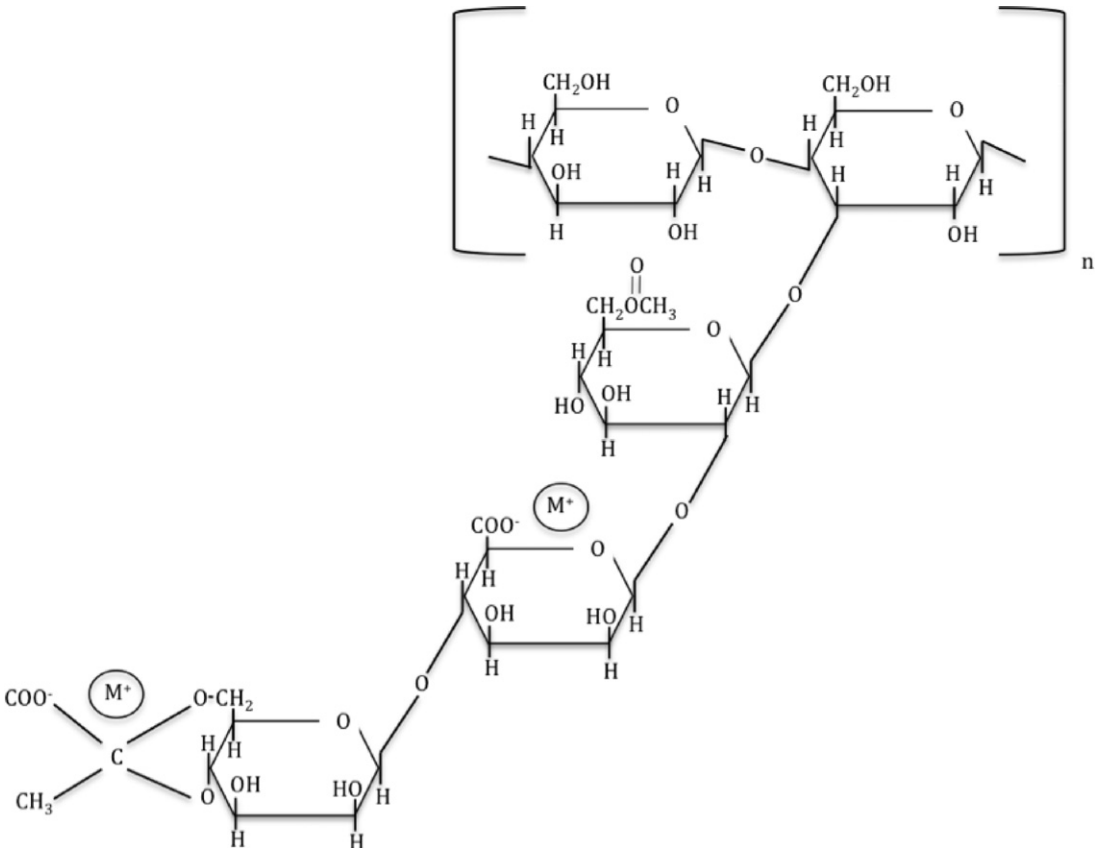

Fig. 5. Chemical structure of xanthan. M^+: Na, K, 1/2Ca.

algL, *algI*, *algJ*, *algF and alga*) which are clustered in a single operon approximately estimated to 3.96 Mb on the genome map PAO1. Only the *algC* gene is located at separately on the chromosome. This gene encodes for a phosphomannomutase which is involved in rhamnolipid and lipopolysaccharide biosynthesis. This operon contains all the genes coding for proteins involved in alginate biosynthesis (*algD* and *algA* for precursor synthesis; *algI*, *algJ* and *algF* for acetylation; *algG* for epimerization, *algL* for degradation, etc.) (Hay et al., 2010).

In *Azetobacter vinelandii*, the alginate biosynthesis gene cluster is organized in 3 operons (Kumar, Mody, & Jha, 2007).

As for alginates, only few genes encode the proteins implicated in levan biosynthesis. In *B. subtilis*, 16 genes of the *eps* operon (*yveK-yvfF*) are involved in the polysaccharide biosynthesis, modification and export. Recently, two genes has been identified: *eps G* (*yveQ*) and *epsH* (*yveR*) may be involved in the EPS biosynthesis. *EpsG* encodes a protein that is probably involved in the EPS polymerisation and *epsH* encodes a glycosyltransferase (Marvasi, Visscher, & Casillas Martinez, 2010).

Rhizobium succinoglycan biosynthesis is one of the most studied genetic mechanisms. The genes and enzymes involved in succinoglycan synthesis are clearly identified. About 30 genes are implicated in the biosynthesis of this EPS: *ndvAB* genes, *exoABCDFGHIJKMNOPQRSTUVWXYZ* genes and *exsABH* genes. The regulatory gene of succinoglycan biosynthesis is regulated by two-component system (chromosomal and plasmidic) (Kumar et al., 2007; Sutherland, 2001).

In most lactic acid bacteria (LAB), the exopolysaccharide synthesis genes are located on plasmids rather than the chromosome (Kumar et al., 2007). For example, the information for EPS biosynthesis *by Lactobacillus lactis* NIZO B40 is located in a single12-kb gene cluster on a single 40-kb plasmid (Welman & Maddox, 2003). Gene clusters for EPS present an high level of similarity among different LAB strains (Van der Meulen et al., 2007).

4. EPS extraction methods

As presented by Sheng, Yu, and Li (2010), a number of methods have been developed and applied to extract EPS from microbial cultures and sludges. Chemical, physical methods, and combinations of physical and chemical methods are used. These different methods can be compared according to two criteria: quantity and quality of extracted EPS. Indeed, extraction products can be contaminated by chemical extracting reagents or proteins due to extraction treatments (Comte, Guibaud, & Baudu, 2006). During EPS extraction, cell lysis might occur at different levels which are difficult to evaluate, either by measuring the protein or nucleic acid content of EPS or the release of intracellular compounds (D'Abzac, Bordas, Van Hullebusch, Lens, & Guibaud, 2010; Garcìa Becerra, Acosta, & Allen, 2010; Sheng, Yu, & Yu, 2005). Changes in the composition and properties of EPS might also occur with the macromolecule disruption (Wang, Cheung, Leung, & Wu, 2010).

Comparisons between different chemical methods (i.e. cationic exchange resin (CER), formaldehyde/NaOH, EDTA, glutaraldehyde or alkaline) have been realized by different authors. Domìnguez, Rodrìguez, and Prats (2010a) showed that the EPS extracted with resin (158 ± 3 mg/g total EPS) were almost the same that were obtained with formaldehyde/NaOH (150 ± 3 mg/g total EPS). Alkaline extraction was compared with CER extraction by Garcìa Becerra et al. (2010). They obtained 3 times more organic materials (proteins, carbohydrates, etc.) with alkaline extraction than CER. The CER method is preferred largely because the resin is easy to remove,

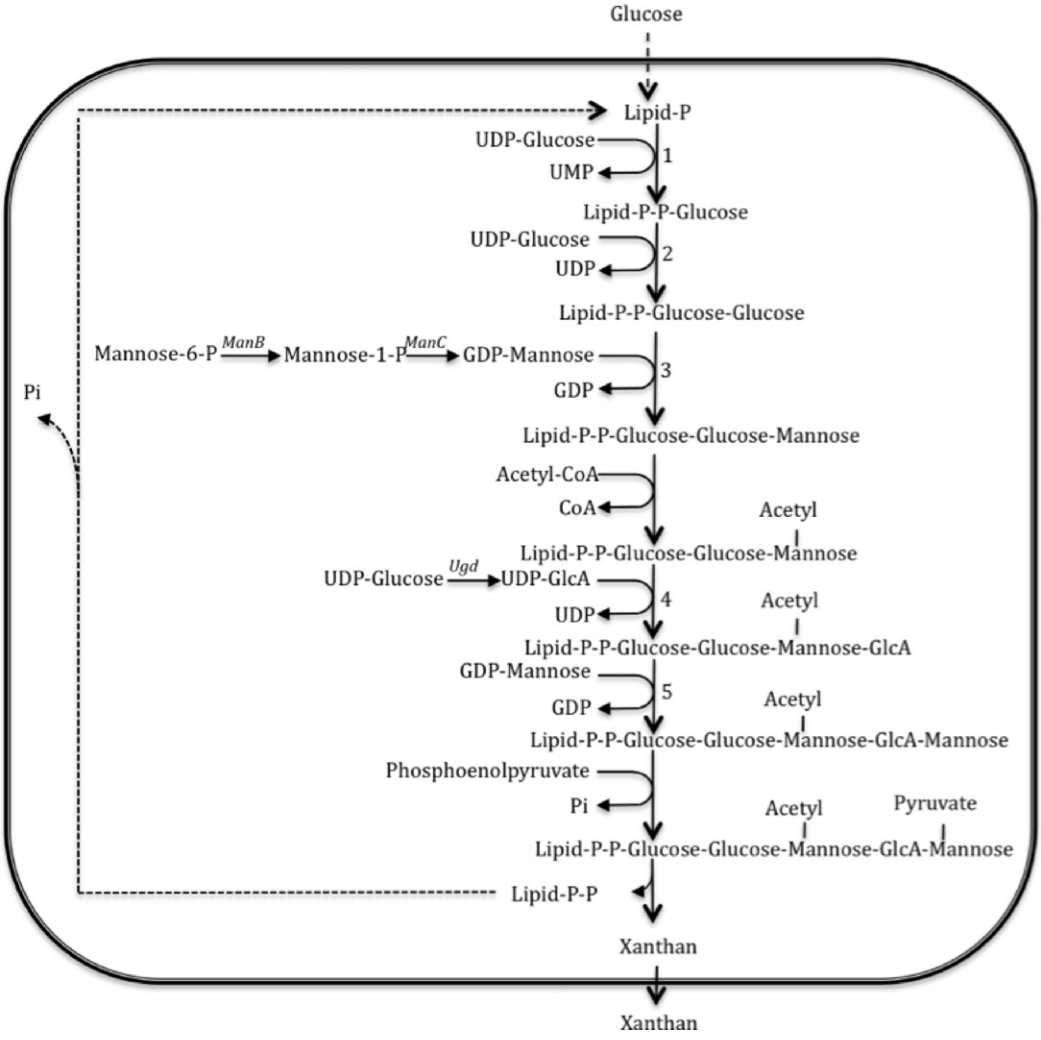

Fig. 6. Biosynthesis of xanthan (adapted from De Baets, Vandamme, and Steinbüchel (2002)). *ManB*: phosphomannomutase; *ManC*: GDP mannose pyrophosphorylase; *Ugd*: UDP glucose deshydrogenase, 1,2,3,4,5: glycosyltransferases.

and it avoids EPS pollution by chemical reagents. Methods based on cation exchange have been shown to be highly selective for EPS linked to magnesium and calcium molecules. Alkaline extraction allowed to obtain up to 75% organic materials. However, the alkaline treatments can cause a severe disruption in the polymer composition and severely damage the cells. EDTA leads to slight cell lysis, despite highly effective extraction capacity and EPS extraction contamination. In the case of the formaldehyde/NaOH method, the formaldehyde dose modifies characteristics of the EPS and cause substantial interferences when determining the constituent carbohydrates (Sheng et al., 2005, 2010).

The physical extraction methods (ultrasonic, centrifugation, microwave treatment or heating) allow to separate the EPS from cells. Usually, the physical treatment efficiencies are lower than those of the chemical extraction methods. Comte et al. (2006) have compared eight EPS extraction methods, three chemical methods (EDTA, formaldehyde + NaOH, glutaraldehyde) and four physical methods (sonication, CER, sonication + CER, heating) and a control method (centrifugation alone). They demonstrated that the EPS extraction efficiency was superior with the three chemical methods (96–318 mg of EPS DW per gram of sludge) compared to the four physical methods (21–64 mg of EPS DW per gram of sludge) and control (16–21 mg of EPS DW per gram of sludge). Domìnguez et al. (2010a) have also shown that the CER method was 1.1–6 times more effective than thermal treatment. Many authors showed that the physical treatments influenced only the molecular weight distribution but not the high-performance size exclusion chromatography (HPSEC) fingerprints of EPS contrarily at the chemical methods (Comte, Guibaud, & Baudu, 2007; Domìnguez, Rodrìguez, & Prats, 2010b). However, Villain, Simon, Bourven, and Guibaud (2010) have pointed out some differences in HPSEC chromatograms when EPS were extracted from different types of sludges.

Many studies have been carried out to optimize EPS extraction coupling ultrasonic treatments with other chemical and physical parameters (temperature, time, pH, ethanol, ozone, etc.) (Deng et al., 2011; Erden, Demir, & Filibeli, 2010; Meng et al., 2010; Yan et al., 2011; Yuan et al., 2010; Zhong & Wang, 2010). Actually, the extraction processes do not use a single method but the combination of several techniques. For example, Domìnguez et al. (2010a) compared the CER extraction method which was 1.1–1.5 times more effective than using formaldehyde + NaOH + sonication.

There is no simple method for qualitatively and quantitatively extracting all microbial EPS. An extraction technique must be chosen and optimized for each case, taking into account the characteristics of the EPS to be extracted. Several extraction methods need to be compared and the methods need to be chosen according to the final aim, be it of quality or of quantity. Combined and repeated extractions are required for the recovery of all the EPS fractions contained in microorganisms.

5. Prospects

As described in this bibliographic review, numerous studies have been carried out in the recent years on the biosynthesis of microbial EPSs and their role in ecosystems. For some strains, the sequence of genes coding for EPS production, the structure of the corresponding EPS and the metabolic pathway of EPS synthesis have been identified. However, data are lacking on the secretory mechanisms of these microbial EPSs and the relationship between gene sequences and EPS production, as well as the enzymes involved in synthesis and excretion. EPS production can already be controlled in numerous cases by growth conditions, including the concentration and type of carbohydrate, temperature and pH. However, a better understanding of the mechanisms involved in synthesis and excretion is still needed. The overexpression of certain genes may lead to increased EPS production and may allow to control of the structure and properties of EPSs for future use. The optimisation of EPS extraction methods has also emerged as a subject for study. No method currently exists to extract all microbial polysaccharides.

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