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The importance of carbon and nitrogen sources on exopolysaccharide synthesis by lactic acid bacteria and their industrial importance

La importancia de las fuentes de carbono y nitrógeno en la síntesis de exopolisacáridos y su importancia industrial

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Abstract

Exopolysaccharides (EPSs) are classified into two groups, homopolysaccharides (HoPs) and heteropolysaccharides (HePs). They are produced by lactic acid bacteria (LAB) and used in a range of industrial applications, including the medical and food industries. The HoPs are extracellular EPSs, and their production depends on extracellular enzymes, whereas HePs are intracellular EPSs. Their nature (extra or intracellular) directly impacts production rates, with HoPs achieving higher yields. The development of processes for producing EPSs has attracted great interest since novel application trends have emerged due to the great diversity of recent information generated on EPS properties. The HoPs have been synthesized by fermentation using bacterial cells and a cell-free enzymatic process, whereas HePs have been produced only by fermentation. Analysis of the EPS production processes indicates that macronutrients, such as the carbon and nitrogen sources used in the culture media, are crucial for the synthesis of EPSs and the enzymes involved, and understanding their importance can assist in the designing of processes for the production of EPSs with desirable characteristics and yields, according to the needs of the processes and products to which they are applicable. This review emphasizes the analyses of carbon and nitrogen sources used for EPS production and their functional applications and productive aspects.

Keywords: exopolysaccharides, homopolysaccharides, heteropolysaccharides, lactic acid bacteria, extracellular enzymes.

Resumen

Los exopolisacáridos (EPS) se clasifican en dos grupos, homopolisacáridos (HoPs) y heteropolisacáridos (HePs), son producidos por bacterias acido lácticas (BAL) y se utilizan en diversas aplicaciones industriales donde destacan la industria farmacéutica y de alimentos. Los HoPs son EPS extracelulares y su producción depende de enzimas extracelulares, los HePs son EPS intracelulares. La naturaleza (extra o intracelular) influye directamente en la tasa de producción, teniendo mayor rendimiento los HoPs. El desarrollo de procesos para producir EPS ha generado gran interés ya que han surgido nuevas aplicaciones debido a la información generada recientemente sobre sus propiedades. Los HoPs han sido producidos por fermentación, utilizando bacterianas y mediante procesos enzimáticos libres de células, mientras que los HePs solo se han producido por fermentación. El análisis de los procesos de producción de EPS muestra la importancia de los macronutrientes tales como las fuentes de carbono y nitrógeno para la síntesis de EPS y enzimas involucradas, su entendimiento puede ayudar al diseño de procesos para la producción de EPS con características deseadas y rendimientos acordes a las necesidades de los procesos y productos a los que son aplicables. Esta revisión enfatiza sobre el análisis de las fuentes de carbono y nitrógeno utilizadas para la producción de EPS y sus aplicaciones y aspectos funcionales productivos.

Palabras clave: exopolisacáridos, homopolisacáridos, heteropolisacáridos, bacterias ácido lácticas, enzimas extracelulares.

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1 Introduction

Exopolysaccharides (EPSs) can be synthesized by a wide variety of sources including algae, plants, fungi, and bacterial strains such as lactic acid bacteria (LAB) (Donot *et al*., 2012; Mahapatra and Banerjee, 2013; Li *et al*., 2015; Cheng *et al*., 2019), which are "Generally Recognized as Safe" (GRAS) microorganisms. Recently, EPSs have received considerable attention due to their diverse use in industrial applications, based on their properties such as biocompatibility, biodegradability, and environmental and human compatibility (Ates, 2015; Osińska-Jaroszuk *et al*., 2015).

Exopolysaccharides are molecules that have been defined in the literature in different ways; usually, according to the microorganisms used for their production and the method of separation or purification used, they present different chemical structures and water and alkali solubility (Kagimura *et al*., 2015; Osińska-Jaroszuk *et al*., 2015). The chemical structure, molecular weight, charge, presence of side chains, and rigidity are characteristics that define the application of these molecules (Zannini *et al*., 2016).

The synthesis of EPSs by bacteria can be described by four mechanisms: a) the Wzx/Wzy-dependent pathway, where individual repeating units are assembled by several glycosyltransferases, is exclusively found in most LAB strains, including the genera *Lactococcus*, *Lactobacillus*, and *Streptococcus* (Hergerle *et al*., 2018; Zhou, Cui and Qu, 2019); b) the ATP-binding cassette (ABC) transporter-dependent pathway (Gram-negative bacteria), similar to the Wzx/Wzy-dependent pathway, uses a lipid acceptor to initiate polysaccharide synthesis and a synthase-dependent pathway for which the requirement for a lipid acceptor molecule depends on the polysaccharide in question and uses similar protein families to facilitate EPS export across the periplasm and through the outer membrane (Whitney and Howell, 2013); c) the synthase-dependent pathway, in which homopolymers are produced using a membrane-embedded, multi-protein complex typically consisting of an inner membrane-embedded glycosyltransferase and co-polymerase, often called the synthase complex, which polymerizes the EPSs and facilitates translocation across the inner membrane (Low and Howell, 2018); and d) extracellular synthesis using a single glycosyltransferase, in which the EPSs are assembled from precursors obtained from the cleavage of sucrose molecules; a monosaccharide unit is transferred to a primer molecule, producing fructans (levan) or glucans (dextran), which can be branched at distinct levels (Schmid, 2018; Barcelos *et al*., 2020).

The EPSs synthetized by LAB represent an alternative to EPSs synthetized from plant and algae sources since they show similar characteristics to the gums currently used, representing many advantages (Mahapatra and Banerjee, 2013). The EPSs produced by LAB are natural biopolymers and, depending on the conditions of the culture medium, are encapsulated or secreted into their environment (Dertli *et al*., 2016). They are classified in two distinct groups, homopolysaccharides (HoPs) and heteropolysaccharides (HePs) (Vaningelgem *et al*., 2004; Dertli *et al*., 2016; Saadat *et al*., 2019). The EPS yield is controlled by multiple factors such as the LAB strain used (genes involved in EPS synthesis), the growth conditions (pH, temperature, dissolved oxygen), and the medium composition, where C concentration and C/N ratio play an important role (Haj-Mustafa *et al*., 2015; Nouha *et al*., 2018). An adequate C/N ratio is required for EPS yield as the carbon source is transformed into lactic acid to produce energy, as well as for the synthesis of cell wall components and EPS, and nitrogen is necessary for the synthesis of essential cellular components such as enzymes, proteins, and nucleic acids. Therefore, a higher C/N ratio and sufficient amounts of both carbon and nitrogen increase EPS production (Harutoshi, 2013).

Currently, sucrose can be used as inductor for HoP production because it is abundant in nature, easy to obtain, and inexpensive (Soncar *et al*., 2020). Recently, health issues resulting from the excessive absorption of sucrose have received attention; in this context, the production of functional EPSs using sucrose as feedstock has become of practical significance and has attracted considerable interest (Ni *et al*., 2019). Sugarcane (*Saccharum officinarum*), a C4 plant, is a major crop for sucrose production in tropical and subtropical areas and a major and economically important cash crop in the world in terms of production, accounting for approximately 75% of the sugar production in the world (Anur *et al*., 2020; Soncar *et al*., 2020). For these reasons, it may be an attractive option for EPSs production as it is an agricultural culture adequate for diversification and adaptation of production systems (Gonzáles-Leos *et al*., 2020).

To improve EPS production methods, it is

necessary to design cheap processes. For this, it is essential to understand the inherent factors of the production systems, such as specific LAB producers and the carbon and nitrogen sources used in production systems and their importance in LAB metabolism. The aim of the present study was therefore to determine the carbon and nitrogen sources used for EPS production and the diversity of LAB used for this purpose, in addition to reviewing their applications of industrial importance, from classical to new trends.

2 Homopolysaccharides

The HoPs are composed of repeated units of one type of sugar monomer, such as D-glucose or D-fructose, and classified as α-glycans or β-fructans with variable molecular weights (Huang *et al*., 2015; Ryan *et al*., 2015; Saadat *et al*., 2019). Characterization of HoPs has shown that they can replace or reduce the use of more expensive hydrocolloids and could find applications as, e.g., texturizing agents (Zarour *et al*., 2017); they also play an important role in the stabilization of frozen foods and can be used as edible films (Davidović *et al*., 2018), prebiotics, texturing and gelling agents (Kanimozhi *et al*., 2017), to preserve the surface of fish, meat, vegetables, or cheese from oxidation and other chemical changes, in cosmetics and therapeutics as natural antioxidants, among other applications (Zarour *et al*., 2017; Yang *et al*., 2018; Zhou *et al*., 2018). The principal advantages of bacterial exopolysaccharides over conventional ones are diverse. However, it can be highlighted that they show great potential due to their natural origin, biocompatibility, biodegradability, and functional groups, which can be targeted to undergo further functionalization or bioconjugation processes (with proteins or markers) (Taberno and Cardea, 2020).

The HoP biosynthesis by LAB involves extracellular enzymes that are induced by the presence of sucrose (Leemhuis *et al*., 2013), such as as glucansucrases (Fig. 1) or fructansucrases (Fig. 1b), which are extracellular enzymes (Galle and Arendt, 2014; Saadat *et al*., 2019). The HoPs are classified as α-D-glucans, β-D-glucans, and polygalactans; α-D-glucans, including dextran (α-1,6), alternan (α-1,3 and α-1,6), mutan (α-1,3), and reuteran (α-1,4); the production of these EPSs depends on the extracellular enzyme dextransucrase (EC 2.4.1.5) belonging to glycoside hydrolase family 70 (GH70). This family includes the alternansucrases (EC 2.4.1.140), mutansucrases (EC 2.4.1.373), and reuteransucrases (EC 2.1.4.5), enzymes necessary for the production of alternan, mutan, and reuteran, respectively (Siddiqui *et al*., 2013; Vuillemin *et al*., 2016). Dextransucrases catalyze the synthesis of dextran from sucrose, transferring D-glucopyranosyl groups from sucrose to other carbohydrates that are present, or even to dextran chains that can serve as polymerization primers (Mori *et al*., 2011). They also catalyze the transfer of mono, di, or higher-order glucose units to other carbohydrate acceptors via a variety of glycosidic linkages (Kang *et al*., 2013).

On the other hand, β-D-glucans include fructans such as levan (β-2,6 and β-2,1 linkages) and inulin types (β-1,2 and β-2,6 linkages); the production of these EPSs depends on extracellular fructosyltransferases belonging to the glycoside hydrolase family 68 (GH68). These enzymes are categorized into two groups, levansucrases (EC. 2.4.1.10) and inulosucrases (EC 2.4.1.9), where the product of the catalytic activity is levan and inulin, respectively (Ortiz-Soto *et al*., 2019; Tezgel *et al*., 2020). Fructansucrases catalyze the synthesis of polysaccharides by transferring fructosyl units to oligosaccharides or to a sucrose molecule, producing fructooligosaccharides with the formula GFn, (with n ranging from 1 to 10) (Santos-Moriano *et al*., 2015). Fructansucrases catalyze three different reactions according to the acceptor molecule: 1) sucrose hydrolysis, when water is used as the acceptor molecule; 2) transfer reactions, when sucrose or kestose ($C_{18}H_{32}O_{16}$) act as acceptor molecules (fructooligosaccharide synthesis); and 3) polymerization reactions, when the growing fructan chain acts as an acceptor molecule (fructan synthesis), depending on the origin and selectivity of the enzyme (Galle and Arent, 2014; Núñez-López *et al*., 2019).

The most reported culture medium for HoP production is Man Rogosa and Sharpe (MRS), which contains sucrose as carbon source and yeast, peptone, and beef extract as a nitrogen source (Feng *et al*., 2018; Yang *et al*., 2019; Zhou *et al*., 2018). In 2020, Ale *et al*. mentioned that the carbon and nitrogen sources used have more influence on EPS yield and composition than other nutrients contained in the culture medium; however, it depends on the LAB used and the EPS synthesized (Midik *et al*., 2020). It is important to mention that in EPSs production, it should be considered that yeast extract may interfere with the quantification (when characterizing a system)

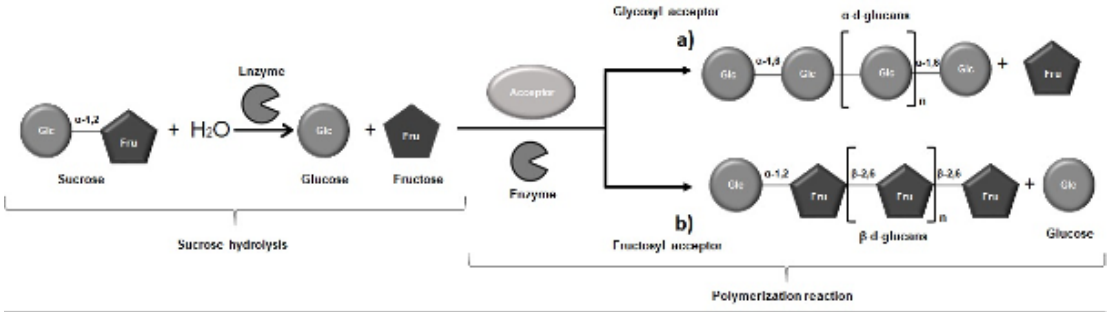

Fig. 1. General enzymatic mechanisms for HOPs synthesis a) Sucrose in presence of glucansucrases and b) Sucrose in presence of fructansucrases.

of EPSs because it contains mannans and other carbohydrates that co-precipitate with EPS (Ale *et al*., 2020). However, mannans can serve as a primer molecule for EPSsynthesis as the EPS produced by *Weissella confuse* MD1 and *Bacillus circulans* contains mannans (Lakra *et al*., 2020; Vidhyalakshmi *et al*., 2016). However, in addition to the carbon and nitrogen sources, an important factor is the concentration and relationship (C/N ratio). In some cases, EPS production is ideal at high concentrations of carbon and low concentrations of nitrogen (Borgio *et al*., 2009; Degeest *et al*., 2001). When the C/N ratio decreases (high nitrogen concentration), the EPS concentration decreases in the case of *Pediococcus damnosus* 2.6 (Seesuriyachan *et al*., 2011). On the other hand, in some cases, growth factors are required (Midik *et al*., 2020), and yeast extract can meet these requirements (Moghannem *et al*., 2018).

Some authors have demonstrated the negative and positive effects of some nutrients on culture medium for EPS production (MRS). In the case of yeast extract and peptone (source of nitrogen), positive effects have been reported; yeast extract provides growth factors such as vitamins and amino acids, which support bacterial growth (Moghannem *et al*., 2018). Seesuriyachan *et al*. (2011) studied the effect of yeast extract concentration (1 to 5 g/L) on EPS production and stated that it is an excellent source of nitrogen (4 g/L) for this purpose; however, both the concentration and the relationship with the carbon source (C/N) must be considered, since they are factors that have a direct influence on yields.

In the case of carbon sources, they also influence both biomass production and EPS. Seesuriyachan *et al*. (2011) indicate that EPSs and biomass production are affected at high sugar concentrations (125 g/L) of the MRS culture medium, caused by osmotic stress. Also, the carbon source used in the culture medium is important for initiation and chain elongation in the polymerization reaction. For example, the content of EPSs produced by *Lactobacillus casei* CRL 87 was higher when galactose was used as carbon source instead of glucose; most likely, galactose was the best primer molecule or it can be metabolized by two alternative biosynthetic pathways (Mozzi *et al*., 2001). Other sugars involved in EPS synthesis are maltose, fructose, lactose, and sucrose, with sucrose being the substrate most commonly used to synthesize HoPs (Guérin *et al*., 2020). The carbon and nitrogen sources depend on the bacterial strain used for EPS production.

2.1 Dextran production

Dextran is an EPS that belongs to the group of α-D-glucans, in which the main backbone chain consists of α (1→6) glycosidic linkages (Fig. 2b) and may also be branched through various secondary linkages such as α (1→2), α (1→3), and α (1→4) (Zannini *et al*., 2016; Oropeza-De la Rosa *et al*., 2019). Dextran is a biodegradable EPS synthetized principally by LAB species, chiefly *Leuconostoc* and *Weissella* (Onilude *et al*., 2013; Zarour *et al*., 2017); however, the literature reports for the latter are not as numerous as those for *Leuconostoc*. Dextran production involves dextransucrase activity, which consists of two principal steps: sucrose hydrolysis and a polymerization reaction; the second step requires an initiator or acceptor molecule as can be seen in Figure 1.

Dextran production by *Leuconostoc* spp. and *Weissella* spp. is strongly influenced by carbon and nitrogen sources and is induced by the presence of sucrose (carbon source) (Xu *et al*., 2017). It also needs an efficient nitrogen source and inorganic salts in the culture medium (Rosca *et al*., 2018; Srinivas and Padma 2014); in the particular case

of *Weissella confuse*, the physical and chemical culture conditions affect the molecular weight of the produced dextran (Rosca *et al*., 2018). In some cases, dextran production is affected by a high sucrose content in the culture medium, resulting in a low amount of dextran, probably due to dextransucrase repression (Xu *et al*., 2017). Furthermore, in the case of *Leuconostoc mesenteroides*, the capacity for dextran synthesis can be lost in culture media with a high salt content (Zannini *et al*., 2016). Among the physical and chemical conditions of the culture for dextran production (temperature, sucrose, and acceptor concentrations), nitrogen and phosphorous contents play a crucial role not only in the production but also in the molecular weight of the dextran synthesized (Du *et al*., 2017). However, dextran production and requirements depend on the LAB species used. For example, Onilude *et al*. (2013) indicated that the nitrogen source used is important; organic sources produced higher EPS yields than inorganic sources. According to Siddiqui *et al*. (2013), peptone is important as it contains growth factors and specific minerals, such as phosphate, in trace amounts.

In LAB metabolism, the glucose obtained from hydrolyzed sucrose (Fig. 1) is first used for microbial growth during the exponential phase and then for dextran production by polymerization during the stationary phase, liberating fructose (Fig. 1a). The liberated fructose can further be reduced to mannitol in the case of *Leuconostoc* spp. by the action of mannitol dehydrogenase, whereas in *Weissella* spp., it is regularly fermented but not reduced to mannitol (Xu *et al*., 2017). According to Castilla-Marroquín *et al*. (2020), the possibility of the flux of the carbon source by LAB depends on the culture conditions and can result in the production of lactic acid, mannitol, fructose, and dextran.

As seen in Table 1, sucrose is a unique carbon source for dextran production in LAB cultures. The nitrogen sources most frequently reported are yeast extract, peptone, and beef extract, and the most frequently reported culture medium for dextran production is MRS supplemented with sucrose, containing the nitrogen sources mentioned. Table 1 shows that the dextran yields obtained by some authors are variable, ranging from 0.14 g of dextran per g of sucrose (*W. cibaria* CMGDEX3) to 0.86 g of dextran per g of sucrose (*L. pseudomesenteroides* DRP-5), with a productivity from 0.26 to 1.19 g of dextran/h/L for *L. pseudomesenteroides* YF32 and *L. pseudomesenteroides* DRP-5, respectively. It can also be observed that the yields do not depend on the bacteria used but can be impacted by the nitrogen source because the highest yields were obtained with yeast extract, meat extract, and peptone, except for the 13.5 g/L obtained by Yang *et al*. (2018). The majority obtained yields greater than 23.5 g/L when using the three sources of nitrogen mentioned; likewise, the lowest yield was obtained when only yeast extract was used (Baruah *et al*., 2017). Similarly, different carbohydrate acceptors (Fig. 1) such as glucose, lactose, and maltose can be used as initiators of the enzymatic reaction, and these molecules have no impact on the molecular weight and structure of the by-product dextran, only on yield (Huang *et al*., 2020).

On the other hand, in addition to yield, characteristics such as the molecular weight of the dextrans produced are variable regardless of the bacteria and nitrogen source used, ranging from 13 to 200,000 kDa; they also present a variable composition (α (1→2), α (1→3) and α (1→6)), as can be seen in Table 1. Kanimozhi *et al*. (2017) indicated that the molecular weight of dextran produced at different concentrations of sucrose using *Weissella cibaria* as inoculum is directly proportional to the concentration of sucrose contained in the culture medium. Likewise, Rosca *et al*. (2018) produced dextran under different culture conditions, obtaining dextrans with molecular weights of 13, 140, and 990 kDa using *Weissella confuse*, indicating that the variability was associated with the culture conditions. In both studies, the same nitrogen sources were used, indicating that the control of the production process can influence the characteristics of the dextran obtained.

2.2 *Industrial applications of dextrans*

Dextran has important commercial value due to its structural characteristics, in particular its molecular weight. Currently, the production of dextran is of great industrial importance due to a variety of applications in the food, pharmaceutical, and chemical industries as a prebiotic, antioxidant, therapeutic, food additive, texturing, and gelling agent as well as an antisynergetic, antifungal, and stabilizing compound (Table 1).

In addition to the applications listed in Table 1, dextran is widely used in diverse applications such as dextran produced by *Leuconostoc mesenteroides*, which is used to fabricate electrochemical double-layer capacitors as it possesses oxygen-containing functional groups attached to the polymer backbones; the oxygen atom has a single pair of electrons forming a dative bond with the cation from the salt

Table 1. Characteristics and applications of dextran production by different microorganisms.

Microorganism	**Carbon source/ Nitrogen source**	**Dextran characteristics**	**Yield (g dextrant/ g substrate)**	**Productivity (g/ L h)**	**Application**	**Reference**
L. mesenteroides NRRL B-1426	Sucrose/ Yeast extract, peptone, and beef extract	85.5 % α (1→6), 14.5 % α (1→3), >2000 kDa	-	-	Prebiotic, functional foods	Kothari and Goyal (2013); Kothari and Goyal (2016)
L. mesenteroides BD1710	Sucrose / Yeast extract, peptone	α (1→6), 6 % α (1→3), 635 kDa	0.36	0.67	-	Han *et al*. (2014)
Leuconostoc NM105	Sucrose/ Yeast extract, peptone, and beef extract	α (1 → 6), α (1 → 2), 100,000 kDa	0.47	0.49	Food and cosmetics	Yang *et al*. (2015a)
L. pseudomesenteroides XG5	Sucrose/ Yeast extract, tryptone and beef extract	α-(1 → 6), 2 600 kDa	0.28	0.74	Food and cosmetic	Zhou *et al*. (2018)
L. citreum B-2	Sucrose/ Yeast extract, tryptone and beef extract	75% α-(1→ 6), 19% α-(1→3), α-(1→2), 3770 kDa	0.37	0.58	Food and cosmetics	Feng *et al*. (2018)
L. pseudomesenteroides DRP-5	Sucrose/ Yeast extract, peptone, and beef extract	α-(1→6), 6230 kDa	0.86	1.19	Food and therapeutics as new natural antioxidant	Du *et al*. (2018)
L. pseudomesenteroides YF32	Sucrose/ Yeast extract, peptone, and beef extract	α-(1→6), 5500 kDa	0.25	0.26	Food industries, such as texturing, bioflocculant and drug delivery agents.	Yang *et al*. (2018)
L. citreum	Sucrose/ Yeast extract, soy peptone and beef extract	α-(1→6), 6070 kDa	0.49	0.51	Food additive	Yang *et al*. (2019)
L. mesenteroides	Sucrose/ Yeast extract, polypeptone and beef extract	(1→6), α (1→3), 1176 kDa	-	-	-	Park *et al*. (2013)
W. confusa		(1→6), α (1→3), 1158 kDa	-	-		
W. cibaria CMGDEX3	Sucrose/ Yeast extract and bactopeptone	α (1→6), 3.4 % α (1→3), >2000 kDa	0.14	1.0	Textural properties of bread, to improve the quality of conventional and gluten free bread	Ahmed *et al*. (2012)
W. cibaria RBA12	Sucrose/ Yeast extract	97 %α (1→6), 3 % α (1→3), >2000 kDa	0.41	0.34	Prebiotic	Baruah *et al*. (2017)
W. cibaria NITCSK4	Sucrose-Glucose/ Yeast extract and peptone	High percentage of α (1→6), α (1→3), 200 000	-	0.91	Texturing and gelling agent in foods	Kanimozhi *et al*. (2017)
W. confusa	Sucrose/ Yeast extract, polypeptone and beef extract. In milk	990 kDa 140 kDa 13 kDa	0.315	0.52	Antifungal	Rosca *et al*. (2018)
W. confusa QS 813	Sucrose/ Yeast extract, peptone, and beef extract	(1→6), 160 000 kDa	-	-	stabilizer in the wheat starch?based frozen food industry	Tang *et al*. (2018)
W. confusa	Sucrose/ Yeast extract, peptone, and beef extract	(1→6), 100 000 kDa	-	-	Texturing agent Antisyneresis	Benhouna *et al*. (2019)

for the conduction process (Hamsan *et al*., 2020). Dextran with a molecular weight of 200 kDa and the presence of an α (1→3) linkage, produced by *Leuconostoc mesenteroides*, shows potential to be used as a prebiotic due to its resistance to α-glucosidase and α-amylase activity (Khotari *et al*., 2013; Khotari *et al*., 2016). A 100,000-kDa dextran with α (1→2) branches produced by *L. citreum* NM105 shows prebiotic properties due to its branch distribution and has potential in the preparation of plasticized films (Yang *et al*., 2015), similar to 3,770-kDa α (1→6) dextran with α (1→3) branches and few α (1→2) branches (Feng *et al*., 2018). A dextran produced by *Leuconostoc pseudomesenteroides* with a molecular weight of 5,500 kDa and an α (1→6) backbone has potential for application in the food industry as a texturizing or bioflocculant agent, based on its high thermal and emulsifying activity, and can be used as a stabilizing and emulsifying agent; it also has potential in the pharmaceutic industry as a drug delivery agent (Yang *et al*., 2018). A dextran produced by *Leuconostoc mesenteroides* T3 combined with sorbitol forms an edible coat with mechanical and water vapor permeability properties (Davidović *et al*., 2018).

The highly linear dextran from *Weissella* finds use in several food applications such as thickening, viscosifying, and emulsifying agents; it is also a potential soluble fiber which can act as a prebiotic as it can only be metabolized by selected microbiota of the human gastrointestinal tract (Baruah *et al*., 2017; Amaretti *et al*., 2020). Regarding dextran produced by *Weissella* LAB species, numerous studies have been performed with diverse results. For example, Ahmed *et al*. (2012) reported a dextran produced by *Weissella cibaria* CMGDEX3 with a molecular weight of 2,000 kDa, an α (1→6) backbone, and 3.4% (1→3) branches that showed potential for application as a precursor in sourdough baking and for improving the quality of gluten-free bread. A high-molecular-weight dextran of 160,000 kDa with an α (1→6) backbone produced by *Weissella confuse* QS-813 has potential for application in the food industry, with a significant influence on the freeze-thaw cycles of wheat starch by reducing water mobility and syneresis (Tang *et al*., 2018). A similar case of dextran produced by *Weissella confuse*, with a molecular weight of 100,000 kDa and an α (1→6) backbone, showed antisynergetic and antioxidant activity in yogurt (Benhouna *et al*., 2019). It needs to be considered that, regardless of the use of several *Weisella* strains for biotechnological, probiotic, and even medical purposes, certain species of this genus do not have GRAS status as they behave as opportunistic pathogens (Abriouel *et al*., 2015; Kang *et al*., 2019).

In general, the literature shows that the physicochemical characteristics of the dextran produced by LAB depend, to a great level, on the control of the production bioprocess, where the concentration of sucrose used in the culture can be highlighted, in addition to the sources and, probably, amounts of nitrogen used. Likewise, characteristics such as the molecular weight and ramifications of this EPS are decisive in determining its application. Also, some authors have shown that a process can be designed to produce dextran of a desired molecular weight; one method to achieve this is the use of mutated dextransucrases extracted from *Leuconostoc mesenteroides* 0326 (Wang *et al*., 2017).

2.3 Alternan, mutan and reuteran production

The metabolism of LAB producers of alternan, mutan, and reuteran is similar to that of dextran-producing strains; production is induced by the presence of sucrose (carbon source) and the same nitrogen sources in the culture medium (Boddapati *et al*, 2020; Gangoiti *et al*., 2020). However, it is particularly important to consider that the requirements for biomass production are different from those for EPS production (Santos-Moriano *et al*., 2015).

Alternan, originally considered to be a dextran with better solubility (Zikmanis *et al*., 2020), is an EPS belonging to the group of α-D-glucans, with a unique backbone structure of alternating α (1→6) and α (1→3) D-glycosidic linkages (Fig. 2a) (Zanini *et al*., 2016; Gangoiti *et al*., 2020). Alternan is mainly synthetized by LAB such as *Leuconostoc* strains because of extracellular alternansucrase activity, using sucrose as substrate (Sanalibaba and Çakmak, 2016; Zanini *et al*., 2016; Wangpaiboon *et al*., 2020). The synthesis process for alternan is similar to that for dextran; however, the physical characteristics of alternan are different from those of dextran, including high solubility, low viscosity, and significant resistance to microbial and enzymatic hydrolysis (Zanini *et al*., 2016). Its production is complicated because all *Leuconostoc* ssp. strains produce dextran simultaneously; however, the best strategy to produce pure alternan is using an enzymatic system, isolating alternansucrases for this purpose (Zikmanis *et al*., 2020). In the case of alternan production by *Lactobacillus reuteri* E8, brain

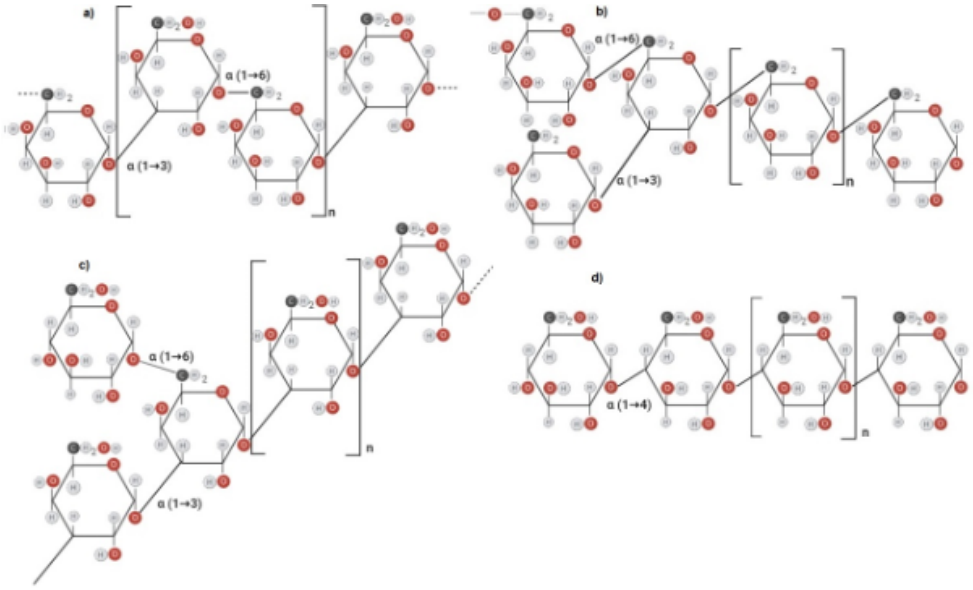

Fig. 2. Chemical structure of HoPs classified as α-d-glucans (Saadat et al., 2019). a) Dextran, b) Alternan, c) Mutan and d) Reuteran-n, written below chemical structure brackets indicate the polymerization degree for each polymer, which can vary remarkably depending on the growing conditions and species used.

heart infusion medium was used as culture medium, supplemented with sucrose as inductor (Yilmaz *et al*., 2020). For enzymatic systems, it is necessary to produce alternansucrases, and for this purpose, the culture medium used is the same as that used to produce dextran, namely MRS supplemented with sucrose, maltose, and glucose (Musa *et al*., 2014), similar to reuteran production using reuteransucrases (Yang *et al*., 2020).

Mutan, a sticky, colorless, water-insoluble EPS that belongs to the group of α-D-glucans containing α (1→3) linkages in the main backbone (Fig. 2c), is produced by LAB species such as *Streptococcus* and *Lactobacillus* as a result of extracellular mutansucrase activity (Boddapati, *et al*., 2020), induced by the presence of sucrose. The structure, i.e., the degree of polymerization, branching, and proportion of α (1→3) and α (1→6) linkages, varies with the organism and the type of enzyme involved in the production (Boddapati, *et al*., 2020). Among the mutan-producing strains reported in the literature are *Streptococcus mutans* 6715, *Streptococcus salivarius* HHT, and the mutant strains *Leuconostoc mesenteroides* NRRL B1118, *Lactobacillus reuteri* ML1, and *Leuconostoc mesenteroides* NRRL B-1355 (Li *et al*., 2020). Reports in the literature indicate that mutan solubility in water can be achieved by structure modification such as carboxymethylation, one of the effective chemical modifications reported to enhance solubility, decrease viscosity, and improve the bioactive properties of glucan polymers (Boddapati *et al*., 2020).

Reuteran is a water-soluble EPS that belongs to the group of α-D-glucans containing α (1→4) linear segments (Fig. 2d) interconnected with α (1→6) glycosidic linkages in the main chain, with no repeating units present (Gangoiti *et al*., 2020, Dijkhuizen, 2018). Reuteran is generally associated with fermented milk and is produced only by *Lactobacillus reuteri* as a result of extracellular enzymatic reuteransucrase activity (Zanini *et al*., 2016). The characteristics of EPSs depend on the LAB and the reuteransucrase expressed. For example, the enzyme expressed by *Lactobacillus reuteri* 121 can produce reuteran with 58% α (1→4) and 42% α (1→6) linkages, and the enzyme produced by *Lactobacillus reuteri* ATCC 55730 has 79% α (1→4) and 21% α (1→6) linkages. This indicates that the enzymes expressed by different LAB have different specificities (Chen *et al*., 2019). The production of reuteran is similar to that of dextran: synthesis is induced by the presence of sucrose, and the most reported culture medium is MRS containing yeast extract, peptone, and beef extract as a nitrogen source. The culture conditions cited include anaerobic conditions; however, the nitrogen sources used are diverse, i.e., wheat and corn flour (Yang *et al*., 2015a; Yang *et al*., 2015b). Reports on reuteran synthesis by *Lactobacillus reuteri* 121 GtfA GS using

different sucrose concentrations show that the ratio of polysaccharide to oligosaccharide can be regulated by the sucrose concentration of the culture (Gangoiti *et al*., 2020).

2.4 Industrial applications of alternan, mutan and reuteran

Alternan has been used for the synthesis of silver nanoparticles with antibacterial and antifungal activity against pathogenic strains (Yilmaz *et al*., 2020) and as a food ingredient due to its health-promoting properties: it improves bread quality and texture, acts as a biothickener, is high in dietary fiber, and induces satiety in humans and animals (Gangoiti, Pijning and Dijkhuizen, 2018; Gangoiti *et al*., 2020). It is also used in the diet of weaned piglets as 20-50% of the total diet as it reduces the number of *Escherichia coli* and the amount of heat-stable enterotoxin in the small and large intestines without negatively impacting piglet growth (Dai *et al*., 2020). Due to high resistance to microbial and mammalian enzymes and because it can only be hydrolyzed by isomaltodextranases and alternanases (resistance to digestion), alternan can be used for the production of ingredients for functional foods such as prebiotics and can also be applied as a low-glycemic index sweetener (Gangoiti *et al*., 2020). It can be used in a wide variety of foodstuffs, e.g., sauces, cream, yogurt, jelly, ice cream, soups, and baked products; also, alternan derivatives such as alternan-carboxylic acid esters are particularly suitable for use in foods, pharmaceutical products, or cosmetics as emulsifiers and surfactants (Taylan, Yilmaz and Dertli, 2019).

The principal application of mutan is as an inducer of the enzyme mutanase, which can hydrolyze α (1→3) glycosidic bonds and degrade dental biofilms, but it can also be used as a heavy metal adsorbent (Boddapati *et al*., 2020, Gummadi, 2020).

The EPS reuteran has been used as a food ingredient due to its health-promoting properties and improvement of bread quality and texture. In-vitro digestibility assays, using either pancreatic α-amylase and amyloglucosidase or rat intestinal maltase-glucoamylase and sucrase-isomaltase, simulating the digestive power of the gastrointestinal tract, revealed that reuteran is high in dietary fiber and can induce satiety in humans and animals (Gangoiti *et al*. 2020; Dijkhuizen, 2018; Gangoiti *et al*., 2020). It has also been used in the diet of weaned piglets as 20-50% of the total diet as it reduces the number of *Escherichia coli* and the amount of heat-stable enterotoxin in the small and large intestines and has no effect on piglet growth (Dai *et al*., 2020).

2.5 Inulin and levan production

Inulin, classified as an oligo- or polysaccharide depending on its chain length, belongs to the fructan carbohydrate group since it is composed of β-D-fructosyl subgroups linked by (2→1) glycosidic bonds, with the molecule usually ending with a (1→2)-bonded α-D-glucosyl group (Fig. 3a). The length of fructose chains varies from 2 to 60 monomers; those containing a maximum of 10 fructose units are also referred to as oligofructoses (Mensink *et al*., 2015).

The production of inulin and levan has not reached industrial levels; however, they can both be produced by either fermentation or enzymatic systems (via inulosucrases), using sucrose as an acceptor (Porras-Domínguez *et al*., 2014; Mensink *et al*., 2015; Ni *et al*., 2018). Inulin synthesis via inulosucrases has been reported for LAB species such as *Lactobacillus gasseri* DSM 20604 (Ni *et al*., 2018), *Lactobacillus reuteri* 121 (Charoenwongpaiboon *et al*., 2018), *Lactobacillus jensenii* JV-V16 (Ni *et al*., 2020), *Lactobacillus* johnsonii NCC533 (Saadat *et al*., 2019), *Leuconostoc citreum* CW28 (Saadat *et al*., 2019), and *Streptococcus mutans* (Saadat *et al*., 2019). In the particular case of inulin synthesis by *Lactobacillus jensenii* JV-V16, the yield of inulin obtained (278 g/L) is high, showing its capability to be applied in industrial-scale processes; however, it is important to note that the enzyme was overexpressed using molecular biology (Ni *et al*., 2020).

Levan is an EPS produced by several microorganisms including LAB; it contains fructosyl residues linked by β (2→6) carbons (Fig. 3b) and packed into nano-sized spherical forms, providing it with a remarkably low intrinsic viscosity (Öner *et al*., 2016). Reports indicate that sucrose is an inductor of levan production (Ua-Arak *et al*., 2016); according to other studies, levan can be produced by *Streptococcus salivarius*, *Streptococcus mutans*, *Leuconostoc mesenteroides* NRRL-B512F, *Lactobacillus sanfranciscensis* LTH2590, *Lactobacillus sanfranciscensis* TMW 1.392, *Lactobacillus reuteri* LB121, and *L. reuteri* LTH5448 (Lynch *et al*., 2018; Saadat *et al*., 2019; Seitter *et al*., 2020). The culture medium used for levan and inulin production is the same as that used for dextran production; the nitrogen sources more often used are tryptone, yeast extract, and meat extract, whereas sucrose is used as a carbon source or inductor

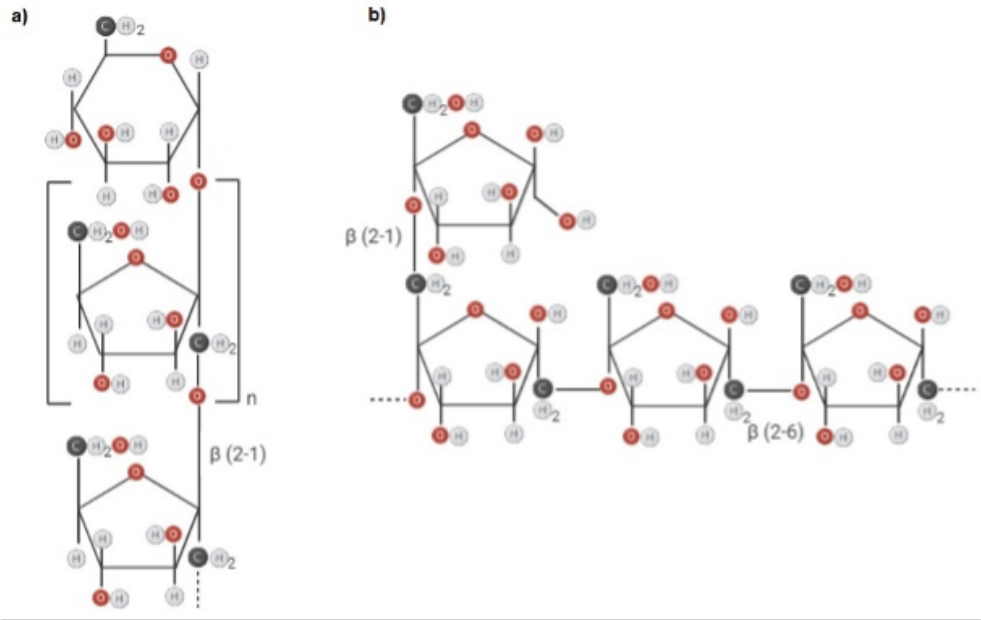

Fig. 3. Chemical structure of HoPs classified as β-d-glucans (Saadat *et al*., 2019). a) Inulin and b) Levan-n, written below chemical structure brackets indicate the polymerization degree for each polymer, which can vary remarkably depending on the growing conditions and species used.

(Seitter *et al*., 2020). Nitrogen plays a crucial role during levan production by *Bacillus subtilis* and *Paenibacillus* sp 2H2 because the enzymes required for polymer synthesis are produced simultaneously with cell growth (Freitas *et al*., 2017). Levan is generally produced in situ on wheat doughs and sourdoughs (Lynch *et al*., 2018; Seitter *et al*., 2020).

2.6 *Industrial applications of inulin and levan*

Currently, inulin is used in the food industry as a food and feed additive as well as in agricultural and pharmaceutical applications. In particular, short-chain inulin is used as a sweetener (low calorie), whereas long-chain inulin is used to form gels, increase viscosity, and improve organoleptic properties. As a non-digestible fiber, it is used as a sugar and fat replacement in dairy products and prebiotics; in pharmacology, inulin is used against colon cancer as an inhibitor of pathogenic adherence, to stabilize drugs, improve the dissolution and release of drug, and in conjugates as a pH-sensitive intestinal controlled-release vehicle for NSAID drugs (non-steroidal anti-inflammatory drugs) (Porras-Domínguez *et al*., 2014; Mensink *et al*., 2015; Mandracchia *et al*., 2018; Saadat *et al*., 2019; Kulcarni *et al*., 2021). Moreover, according to animal experiments, inulin can inhibit diabetes-induced hyperglycemia, diabetes, obesity, inflammatory bowel disease, and colon cancer and regulate glucose and lipid metabolism, the endocrine system, and oxidative stress (Porras-Domínguez *et al*., 2014; Mensink *et al*., 2015). Inulins are mostly used as industrially formulated prebiotics because of their ability to promote the growth of beneficial bacteria in the human colon (Charoenwongpaiboon *et al*., 2018); these properties have been widely confirmed in both *in vitro* and *in vivo* experiments. Inulin has also shown antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* in conjugated polyvinyl alcohol composite nanofibers (inulin/PVA CNFs) (Wabhi *et al*., 2020).

Levan, with characteristics such as biocompatibility, biodegradability, renewability, flexibility, and eco-friendliness, is used in a wide variety of medical applications as a hypo-cholesterol, antitumor, immunomodulatory, anti-inflammatory, antioxidant, anti-carcinogenic, anti-AIDS, hyperglycemic inhibitory, and plasma substitute agent. Bahroudi *et al*. (2020) have shown that levan at 5% (w/w) promotes the abundance of bifidobacterial, facilitates body weight control, and diminishes total cholesterol and glucose in Wistar albino rats, suggesting its potential application in veterinary. In the food industry, it

is used as a source of di-fructofuranoses, fructose, and fructooligosaccharides and as an emulsifying and encapsulating agent, color and flavor vehicle, biothickener, fat substitute, and an alternative to produce gluten-free products since levan improves rheology and texture, reducing the use of bread improvers (Srikanth *et al*., 2015; Sanalibaba and Çakmak, 2016; Ua-Arak *et al*.,2016; Daba *et al*., 2021).

3 Heteropolysaccharides

Heteropolysaccharides have a complex structure. The most common monosaccharides are galactose and glucose, followed by rhamnose; they can be branched or unbranched (e.g. kefiran) and are composed of three to eight repeated monosaccharide subunits (D-glucose, D-galactose, and L-rhamnose), monosaccharide derivatives such as N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), glucuronic acid (GlcA), or substituted monosaccharides (containing phosphate, acetyl and glycerol) (Galle and Arendt, 2014; Miao *et al*., 2015; Zarour *et al*., 2017; Hilbig *et al*., 2019; Saadat *et al*., 2019). The mechanisms of HeP synthesis (Wzx/Wzy, ABC, synthase-dependent pathway, and glycosyltransferase) are usually more complex than those of HoP synthesis, involving encoded regulatory proteins, multiple glycosyltransferases, polysaccharide length regulation proteins, and polymerization and export proteins (Zhou *et al*., 2019), which are produced intracellularly, whereas HoPs are synthesized extracellularly by glycosyltransferases (Hilbig *et al*., 2019). Their nature (extra- or intracellular) directly impacts production rates: it has been reported that some LAB can produce over 43.79 g/L of HoPs (Kanimozhi *et al*., 2017); in contrast, the maximum HeP production recorded is 10 g/L (Galle and Arendt, 2014).

In contrast to HoPs, HePs are produced from sugar nucleotides by the activity of intracellular glycosyltransferases (Galle *et al*., 2011). The LAB known as HoP producers, *Lactococcus* lactis subsp. lactis, *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus casei*, *Lactobacillus sake*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, and *Streptococcus thermophilus*, are also recognized as HeP producers (Baruha and Goyal, 2016). The culture media used for HeP production are diverse and depend on the LAB used. The HeP structures are complex and dependent on the LAB producer, i.e., the cell wall polysaccharide produced by *Lactococcus lactis* IL1403 is predominantly composed of L-rhamnose, D-glucose, D-glucosamine, and D-galactosamine in a ratio of 4.6:1.0:0.8:0.6, respectively (Vinogradov *et al*., 2018). The EPSs synthetized by *Lactobacillus acidophilus* LA5 consist of glucose, galactose, glucuronic acid, and xylose, and in optimized conditions, a yield of 0.35 g/L can be obtained (Amiri *et al*., 2019). In a similar case, *Lactobacillus plantarum* KX041 produced 0.59 g/L of EPSs composed of arabinose, mannose, glucose, and galactose in a molar ratio of 0.95:12.94:7.26:3.31, respectively (Wang *et al*., 2017). On the other hand, *Lactobacillus rhamnosus* shows the capacity to produce an EPS with similar characteristics using glucose, galactose, sucrose, maltose, and lactose as carbon source, but its molecular weight (195 to 11,130 kDa) is dependent on the carbon source used. When cultivated on a medium with galactose, lactose or sucrose, heterogenic EPSs contain high- and low-molecular-weight fractions, whereas those synthesized on a medium with maltose and glucose contain only a high-molecular-weight fraction (Polak-Berecka *et al*., 2015). As in the case of HoPs, the carbon and nitrogen sources used in the culture medium and their ratio (C/N) are important to produce HePs, but also the growth factors and the pH, temperature, and oxygen tension conditions in the culture are important. In the case of LAB, an optimal balance between the carbon and nitrogen source is necessary to achieve high HeP yields (Deegest *et al*., 2001). On the other hand, Midik *et al*. (2020) reported that the NaCl concentration negatively impacts the EPS yield. At higher NaCl concentrations, EPS yield decreased for some *Lactobacillus plantarum* strains, producing a maximum of 488.57 mg/L of EPS with NaCl at 0% and a minimum of 38.73 mg/L of EPS with NaCl at 6% (Midik *et al*., 2020). Macedo *et al*. (2002) reported the highest EPS yields when yeast extract supplemented with whey permeate, amino acids, and salts was used as nitrogen source, producing 2767 mg/L of EPS, indicating the importance of nitrogen sources in the culture medium for *Lactobacillus rhamnosus* RW-9595M.

The most widely known HeP is kefiran, which has a defined structure and is composed of equal amounts of glucose and galactose. It is water-soluble and is used in a wide range of pharmaceutical applications, e.g., as coating agent, target delivery vehicle for clinical therapies, biologically active agent, and drug conjugate (Tan *et al*., 2020) Kefiran is produced by

LAB such as *Lactobacillus kefiranofaciens*, using sucrose as a carbon source and yeast extract as a nitrogen source (Dailin *et al*., 2016). Kefiran can be produced using different carbon sources such as sucrose and glucose, but better yields have been observed when lactose was used (Dailin *et al*., 2015). On the other hand, kefiran has also been produced in situ using *Lactobacillus kefiranofaciens* on cheese, using glucose, sucrose, galactose, and lactose as the carbon source; the best yields were obtained in the presence of glucose. Kefiran has been associated with several bioactive properties, including antitumor, antibacterial, antioxidant, and anti-inflammatory activity (Blandón *et al*., 2018).

The information gathered in this review can help in the design of both enzymatic and fermentative processes using the new trends in genetic engineering or proteomics, helping in the design of processes that are economic and competitive with chemical processes.

Conclusions

Knowledge of the metabolic processes to synthesize EPSs has generated great expectations since it indicates that it is possible to increase the yields and produce EPSs with the desired characteristics for each process as required. The understanding of metabolism has advanced considerably in recent years; nevertheless, there are still many challenges to achieving better production processes. One of the challenges is to increase the yields of HoPs and HePs, for which the production systems currently used must be further characterized, from considering the LAB to be used to the process conditions and nutrients, considering the inducers, micro- and macronutrients as the case of carbon and nitrogen sources. In the particular case of HoPs, everything that allows greater stability and enzymatic activity needs to be considered. The correct C/N ratio can allow the rational design of economic processes where higher concentrations of EPSs can be produced and with the desired characteristics, it is also important to control their concentrations and balance according to the LAB used and the desired product. Considering that EPS synthesis occurs during different growth phases, depending on the LAB used, is crucial. One of the main advantages of EPSs produced by LAB over conventional ones is the high diversity; however, it can be highlighted that they show great potential due to their natural origin, biocompatibility, biodegradability, and functional groups, which can be targeted to undergo further functionalization or bioconjugation processes according to the specific needs.

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