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review

Polysaccharides from Probiotics: New Developments as Food Additives

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Summary

Microbial polysaccharides with nutraceutical potential and bioactive properties have been investigated in detail during the last few decades. There is an increasing demand in food industries for live microbes or polysaccharides produced by them which assert health benefits other than dietetic constituents. Although there are a large number of exopolysaccharide (EPS)-producing bacteria, the titers are low for commercialization. This manuscript deals with the polysaccharides produced by probiotic strains, with major emphasis on the EPSs, their properties, applications and some of the strategies adopted which would be helpful in better understanding of the process in the near future. Research on the improved EPS biosynthesis is essential for obtaining high yields. Therefore, to reach commercialization, metabolic engineering must be applied.

Key words: polysaccharide, exopolysaccharide, probiotic, glucan, prebiotic

Introduction

Bacterial polysaccharides exhibit a wide range of macromolecules that comprise intracellular polysaccharides; structural polysaccharides, which are mostly cell wall components, *viz.* lipopolysaccharides (LPSs); peptidoglycan and capsular polysaccharides (CPSs), which are covalently associated with cell surface (O-antigen) or secreted as exopolysaccharides (EPSs). These polysaccharides perform different tasks for microbial integrity and survival. Intracellular polysaccharides take part in various biochemical reactions within the cell. Peptidoglycans and LPSs are structural components of the cell wall and outer cell membrane of the Gram-positive and Gram-negative bacteria, respectively. LPSs also act as an important virulence factor in some pathogenic organisms permitting the bacterium to survive in harsh environments (*1*). EPSs and other polysaccharides are used as food additives to enhance texture. The addition of synthetic texture-promoting agents to food and dairy products is prohibited in a number of countries of the European Union and the United States of America. EPSs play an important role in improving the appearance, stability and rheological properties of novel food products. The relevance of EPS-producing probiotic microorganisms as efficient starter cultures for the production of fermented foods has increased, *viz.* yoghurt or cheese have received increased interest due to fascinating technological properties of EPSs (*2*).

Exopolysaccharides (EPSs) of microbial origin are long chain, high-molecular-mass water-soluble polymers which may be ionic or non-ionic and have potential applications in food industries as texturizers, viscosifiers,

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emulsifiers and synerisis-lowering agents due to their pseudoplastic rheological behaviour and water-binding capacity (*3*). The high viscosity and unique rheological properties that EPS molecules confer to fermented milk, even at very low concentrations, make EPSs a potential ingredient in food products. Although microbial EPSs produced by non-food-grade bacteria are being widely applied as thickening, gelling and stabilizing ingredients in food industry, the consumers' opinion and country legislations restrict the use of genetically modified organisms (GMOs) for human food applications. Therefore, the developments of polysaccharides from probiotics hold high importance. Probiotics are health-promoting live microbes that accumulate in the host's gut and support their well-being. Lactic acid bacteria (LAB), propionibacteria and bifidobacteria are the most common, whereas certain bacilli and yeasts are also used as probiotics. Few *Bacillus* strains have also been recognized as good probiotics in recent years. Compared to the well-established LAB and bifidobacteria (*4,5*), they have also been found to be excellent EPS producers and they exhibit exceptional properties which are discussed in this review. *Bacillus subtilis* has already been provided with Generally Recognised as Safe (GRAS) status (*6*), and *Bacillus coagulans* has also been added by the European Food Safety Authority (EFSA) to their Qualified Presumption of Safety (QPS) list (*7*) and has been approved for veterinary purposes as GRAS by the US Food and Drug Administration's Center for Veterinary Medicine (*8*). The presence of protective layer of extracellular polysaccharides (EPSs) prolongs the survival of probiotic microorganisms during gut transition in gastrointestinal tract of the host (*9*). Regarding food safety concerns, it would be fascinating to avoid the employment of additives as texture improvers, emulsifying, stabilizing and gelling or water-binding agents in fermented foods and substitute them by the use of EPS-producing probiotic strains in the starter cultures. Chemical composition and availability of fermentable carbohydrates in the colon have a strong influence on the gut microbiota composition. Specific carbohydrates are now being used as prebiotics based on the concept that they stimulate selected beneficial bacteria (probiotics) in the colon. Prebiotics are defined as selectively fermented ingredients that allow specific changes, both in the composition and activity in the gastrointestinal microflora, which confers benefits upon the host's well-being and health. The primarily studied prebiotics are inulin and fructooligosaccharides (FOSs) (*10*). β-glucans have also been studied recently for the mentioned activities (*11*).

Several probiotic strains are recognized to produce EPSs. These high-molecular-mass compounds consist mostly of various polysaccharides, which either remain attached to the outer surface of the cell or are secreted by microorganisms into their extracellular environment (*12*). EPSs have been classified by different criteria, *viz.* the composition of one or different types of sugar monomers into homopolysaccharides (*viz.* cellulose, dextran, pullulan, levan, and curdlan) and heteropolysaccharides (*viz.* gellan and xanthan). The repeating units are branched or unbranched, and are connected by glycosidic linkages. EPSs are usually comprised of monosaccharides but sometimes also contain non-carbohydrate substituents (*viz.* succinate, pyruvate, acetate and phosphate). Heteropolysaccharides (HePSs), *e.g.* kefiran, are used as additives in milk products, whereas homopolysaccharides (HoPSs), *e.g.* levan, are introduced in sourdough products where they contribute with other components to the improvement of structural quality, baking capacity and to the reduction of bread staling factors (*13*).

Although EPSs from probiotics have several positive aspects, the negative ones are linked to their spoilage properties. The EPS production by LAB during wine and cider preparation results in the products with detrimental rheological properties. The LAB EPSs are accountable for biofilm formation, which leads to biofouling thereafter (*14,15*). A biofilm is an aggregate of embedded microorganisms on a self-secreted matrix surface. These adherent matrices are extracellular polymeric substances, *viz.* DNA, protein and polysaccharide, sometimes also known as slime. Biofouling in dairy industry occurs due to biofilm formation in the equipment used for the processing of dairy products and it causes numerous technical and hygiene problems. Numerous probiotic strains from various genera for EPS biosynthesis and their industrial applications are discussed in this review.

Exopolysaccharide Biosynthesis

Biosynthesis of EPSs differs from one genus to another and is an energy-dependent process. Production pathway and utility of substrate molecule also differ based on the production of EPS type. EPS biosynthesis involves glycosyltransferases (EC 2.4) that link intracellularly a sugar from nucleotide sugars to a lipid carrier molecule. Availability of sugar nucleotides affects greatly the biosynthesis of certain EPSs (*viz.* alginate, gellan, *etc*.). The inter-conversion of glucose-6-phosphate (glycolysis intermediate) into glucose-1-phosphate (sugar nucleotide precursor), which is catalyzed by phosphoglucomutase (PGM), is a key step in sugar nucleotide biosynthesis. In contrast, biosynthesis of other EPSs such as levan, mutan, alternan, reuteran and dextran is catalyzed extracellularly by levansucrase (EC 2.4.1.10), mutansucrase (EC 2.4.1.5), alternansucrase (EC 2.4.1.140), reuteransucrase (EC 2.4.1.5) and dextransucrase (EC 2.4.1.5), respectively, from sucrose. The mechanism involved here is the transfer of D-glucose moiety of sucrose to an acceptor monosaccharide or oligosaccharide.

The amount of EPS production depends on the medium and cultural conditions used for the growth of microbes. EPS production is generally favoured by high carbon and low nitrogen substrate ratio (*16*). Complex media support the production of EPSs (*3*), which indicates the necessity of rich nutrients like peptone, beef extract and yeast extract for the biomass development prior to EPS production. A large number of media listed in Table 1 (*17–29*) have been employed to produce EPSs from various probiotic strains. The higher EPS production in a mixed milk culture was discussed by Cerning (*3*). Carbohydrate components of the culture media affect the yield of EPSs but do not influence their chemical structures. They also affect viscosity of the EPSs, possibly owing to the heterogeneity in their molecular mass distribution.

L. delbrueckii ssp. *bulgaricus* grew less in a simplified medium than in a complex medium, but EPS production

Table 1. Production media used for probiotic EPS biosynthesis

Name of medium	Reference
basal salts solution (BSS) medium	*17*
basal minimal medium	*18*
minimal salts medium (MSM)	*17*
LBS medium	*19*
semi-defined medium (SDM)	*20*
nitrogen-free medium (NFM)	*17*
chemically-defined medium (CDM)	*17,21*
milk medium (MLM)	*17*
BMM medium	*22*
de Man-Rogosa-Sharpe (MRS) medium	*23,24*
EPS medium	*25,26*
whey-based medium	*27*
glucose-yeast extract-acetate (GYEA) medium	*28*
skimmed milk-based medium	*29*

was about double and it continued even after the cell growth was stopped. After the addition of a mixture of vitamins, the EPS production increased, although the responsible factor for this enhancement is not clear (*30*). Maximum EPS production in bacilli was achieved after 24 to 36 h depending on the strains and media used. The study of Kodali *et al*. (*28*) revealed that the pH of glucose yeast extract acetate (GYEA) broth was unstable before EPS production, during exponential and late exponential phase. In stationary phase, the pH of the medium became constant at 7.4–7.5, after the EPS production. The production was maximum at the highest cell biomass at 36 h. The majority of the cell population involved in higher EPS production was found to be the endospore-forming ones (*28*). EPS production from various food grade organisms is listed in Table 2 (*31–43*).

Table 2. EPSs produced by food-grade microorganisms

Exopoly-saccharide	Microorganism	Reference
kefiran	*Lactobacillus hilgardii*	*31*
hyaluronic acid	*Streptococcus equi*	*32*
dextran	*Leuconostoc mesenteroides*	*33,34*
	Lactobacillus hilgardii	*35*
β-D-glucan	*Pediococcus parvulus* 2.6	*36*
cellulose	*Escherichia coli* Nissle 1917	*37*
mutan	*Streptococcus mutans, S. sobrinus*	*38*
alternan	*Leuconostoc mesenteroides*	*39*
inulin	*L. reuteri* 121, *L. citreum* CW28	*40*
levan	*L. reuteri* 121, *L. sanfranciscensis*	*13,41*
fructan	*L. frumenti*	*42*
reuteran	*L. reuteri* ATCC 55730	*43*

Isolation and Purification of Exopolysaccharides

Isolation of polysaccharides is apparent prior to their further characterization. The method should not affect the chemical and physical properties of the polysaccharides. Various probiotic strains are involved in the synthesis of EPS mixtures of various structures. For example, *L. reuteri* 121 was found to produce several HoPSs under the same culture conditions (*44*). The recovery of EPSs with identical structure but diverse molecular mass has also been described (*45*). If a single strain secretes mixtures of HePSs with different structures, it should be studied carefully, although there are no adequate reports on diverse HePS production from a single probiotic strain. Often the identification of such polysaccharide fractions comes from the analysis of monomer composition. Immense care should be paid when interpreting such data. This is particularly true when small samples are analyzed. The preparation and isolation methods may add contaminating polysaccharides that are either originally present in the medium components, like mannans from yeast extract, or that could be added as cell wall debris from source organisms as a result of mechanical disruption or lysis during fermentations.

Ultrafiltration is a reliable method for the removal of interfering compounds such as low molecular mass polysaccharides present in the media. Further ultrafiltration helps to purify EPSs partially after fermentation (*46*). Casein removal from a milk-based medium using pronase or trichloroacetate (TCA) is commonly used. Gel permeation chromatography (GPC) helps to determine EPS concentration smaller than 20 mg/L in cell-free culture supernatants produced in less than 2 h, which proves it to be an appropriate method for rapid screening of EPS producers.

Exopolysaccharide Characteristics

For polysaccharide characterization, it is necessary to determine their monosaccharide composition and linkage patterns. Probiotic EPSs show an ample deviation in molecular structures and action complexity, which results in physical alteration of food components and elicits bioactive properties (*47*). EPSs are comprised of large monomer chains composed of 'stiff' linkages that exhibit high specific volumes. The general properties related to flexibility of different linkages are well studied. β(1,4) links are stiffer as compared to β(1,3) and β(1,2). Alpha linkages are typically more flexible than β linkages, therefore EPS chains comprising large repeating units with more α(1,2) and α(1,3) links are more flexible (*48*).

Textural properties of EPSs are due to molecular characteristics and also the ability to interact with proteins at low pH (*49*). Various techniques have been used for the determination of their molecular mass. Few of them were based on retention time of polysaccharide eluted by size exclusion chromatography coupled with refractometry. This method also facilitates an immediate estimation of molecular mass and greatly assists the selection of high EPS-producing strains (*50*). The size exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS) is a recent and more efficient method for the determination of polysaccharide molecular mass (*51*) which can provide accurate molecular mass determination in the oligomeric range.

Numerous analytical strategies are available for determining the monomer composition of EPS samples. Methanolysis and pertrimethylsilylation provide samples that can be analyzed by gas chromatography. A simpler

method required for acid hydrolysis followed by monomer detection using high-pressure anion exchange chromatography with pulsed amperometric detection is usually used in the studies (*52*). The linkage pattern of the monomers is determined after methylation of all free hydroxyl groups, polysaccharide hydrolysis, reduction of methyl glycosides by $NaBD_4$ and acetylation, which provides *O*-acetyl group at linkage points. The partially methylated alditol acetate is separated by gas chromatography (GC) and fragmented by mass spectrometry to give specific primary fragment ions. This analysis is completed by ^1H and ^{13}C NMR spectrometry (*53*). These studies include spectrometric data, chemical shifts, and coupling constants and are extremely useful for assigning spectra of complex carbohydrates. The use of spin diffusion to provide information related to linkage and the technique which requires shaped pulses is complementary to the heteronuclear multiple bond correlation (HMBC) experiments. The use of deuterium-induced differential isotope shifts is an important tool in the determination of linkage type (*54*).

Exopolysaccharides from Lactic Acid Bacteria

Exopolysaccharide-producing LAB, including *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Streptococcus*, synthesize numerous kinds of HoPSs (glucans and fructans) and HePSs, diverse in molecular mass, linkages, solubility, and degree of branching. EPS production levels in LAB are relatively low (40–800 mg/L) compared to xanthan gum (10–25 g/L) produced by *X. campestris* (*55*). The molecular mass of EPSs from LAB range from 10 to 1000 kDa (*54,56*). Most of the lactic acid bacteria produce polysaccharides extracellularly from sucrose by glycansucrases or intracellularly by glycosyltransferases from sugar nucleotide precursors. *Lactobacillus*, *Leuconostoc* and *Streptococcus* synthesize glucansucrases and fructansucrases. Glucansucrases are able to synthesize various α-glucans depending on the position of glycosidic bounds: α(1,6) linkage (dextran production in *Leuconostoc* spp.), α(1,3) (mutan production in *Streptococcus* spp.), alternating α(1,3) and α(1,6) (alternan production in *Leuconostoc mesenteroides*) and α(1,2) glycosidic bonds (in *L. mesenteroides*). On the basis of glucansucrase specificity, elongated oligosaccharides can be formed with various glycosidic linkages at the non-reducing end (*57*).

Lactobacillus fermentum produced 100 mg/L of EPS in MRS broth. The 1 % (*m*/*V*) solution of the purified EPS from this strain showed high apparent viscosity of 0.88 Pa·s at a shear rate of 10 s^{-1} (*58*). *Lactobacillus rhamnosus* yielded from 931 to 1275 mg/L of purified EPS with glucose or lactose media at 32 or 37 °C (*59*). The exopolysaccharide from *L. rhamnosus* was a heptasaccharide repeating unit composed of D-glucose, D-galactose and *N*-acetyl-D-glucosamine in the molar ratio of 3:3:1. It showed the presence of 2- and 3-substituted galactofuranose, 2-substituted galactofuranose, 3-substituted glucopyranose, 6-substituted glucopyranose and 6-substituted galactopyranose residues in a molar ratio of 0.5:0.4:0.5: 1.0:0.9 (*60*). EPS from *L. delbrueckii* contained 72 % of glucose equivalents and 1.1 % protein contents. At the highest EPS concentration (20 g/L), the consistency of this solution was 17.71 mPa·s (*61*). Monosaccharide components of EPS in lactobacilli comprised glucose, galactose and rhamnose. The rhamnose and glucose fractions were generally higher than the galactose fraction in EPS from human gut flora (*62*).

Lactococcus lactis ssp. *cremoris* produced two EPSs with different sugar compositions and molecular mass: a neutral EPS of 10^6 Da and a smaller one of 10^4 Da with negatively charged phosphate groups (*63*). This strain produced 600 mg/L of EPSs, which consists of rhamnose, glucose, galactose and phosphates in a lactose-containing medium (*64*), while EPSs that consist of only glucose and galactose were produced with other carbohydrate substrates. *Streptococcus thermophilus* produced 20–100 mg/L of EPSs in milk medium. EPSs synthesized by this strain differed in their molecular mass, ranging from 10 to more than 5000 kDa. They were composed of galactose and rhamnose in a molar ratio of 2.5:1. Streptococcal EPSs are classified into six groups according to their monomer compositions. Apart from galactose and glucose, other monomers, such as (*N*-acetyl) galactosamine, (*N*-acetyl) glucosamine and rhamnose are also found as repeating unit constituents (*65*). *Streptococcus thermophilus* strains are known to produce HoPS EPSs, which are categorized into high-molecular-mass (HMM) and low-molecular-mass (LMM) polymers. Few streptococcal strains are known to produce either HMM EPS or LMM EPS alone and some strains secrete both EPSs in combination (*66*). EPSs from *S. thermophilus* contained 94 % of glucose equivalents whereas protein content was 0.6 % and at highest EPS concentration (20 g/L) the consistency of solutions of EPS was 13.14 mPa·s (*61*).

EPSs produced by *Lactococcus lactis*, *Lactobacillus sakei*, *S. thermophilus* and *L. helveticus* are not degraded by faecal microorganisms (*67*). *Streptococcus macedonicus* polysaccharide contains trisaccharide sequence of β-D-Glc*p*NAc-(1,3)-β-D-Gal*p*-(1,4)-β-D-Glc*p* (*68*). This corresponds to an internal EPS backbone of lacto-*N*-tetraose and lacto-*N*-neotetraose. In fact, the same two trioses have been identified in the structure of several human milk oligosaccharides that are important for healthy infant nutrition. Another LAB, *Leuconostoc*, produces more biomass in the MRS medium than in the EPS medium, but the quantity of EPS produced in the EPS medium (22.5 g/L) is much higher than in the MRS (14 g/L) (*25*). LAB isolates W3 from whey and MC-1 from yoghurt were found to produce EPSs with a maximum production of 2.0 and 8.79 g/L, respectively (*69,70*). Two strains of *Pediococcus pentosaceus* were found to produce dextran type of EPSs in sucrose-containing liquid media. The concentration of EPSs produced by these AP-1 and AP-3 strains was 6.0 and 2.5 g/L, respectively. Both EPSs differed from linear dextran by branching through 3,6-di-*O*-substituted α-D-glucopyranosyl residues. EPS from strain AP-3 exhibited higher degree of branching and higher molecular mass than that from strain AP-1 (*71*).

Bifidobacterial Exopolysaccharides

EPSs isolated from different human intestinal bifidobacteria can act as fermentable substrates for microbial intestinal populations (*72*). Bifidobacterial strains did not produce an excessive amount of acetic acid that could

adversely affect the sensory properties of fermented milk. EPSs obtained from 24-hour-old cultures of three different bifidobacterial isolates ranged from 0.32 to 0.53 g/L (*73*). EPS production from *B. longum* BB-79 was in the equal range (0.47 g/L) (*74*).

A study reveals that bile affects the synthesis of EPS by *Bifidobacterium animalis* ssp. *lactis*. EPS of *B. infantis* contains 55 % of glucose equivalents and 0.7 % of protein content at high solution concentration (20 g/L). The consistency of this solution was 9.518 mPa·s (*61*). Bile induces the *gtf01207* expression, which encodes a putative priming of glycosyltransferase involved in EPS biosynthesis (*75*). Bile is a toxic compound so microbes synthesize EPS against it for their survival.

Propionibacterial Exopolysaccharides

EPS yields were observed at 500, 250 and 400 mg/L from *Propionibacterium freudenreichii*, *P. thoenii* and *P. freudenreichii*, respectively, and they were composed of D-glucose (Glc), D-mannose (Man), and D-glucuronic acid (GlcA) in a molar ratio of 2:2:1. However, methylation analysis demonstrated the presence of terminal Glc*p*, 3-substituted Glc*p* and 3,6-disubstituted Man*p* in a molar ratio of 1:1:2 (*76*).

Propionibacterium acidipropionici produced two polysaccharides which differ in their compositions, charges and molecular mass. EPS from *P. acidipropionici* was composed of galactose, mannose, and glucosamine with traces of glucose, galactosamine and phosphate. It was fractionated into a major acidic fraction and a minor neutral one. The major fraction was a high-molecular-mass polyelectrolyte, rich in galactose, which accounts for more than 78 % of the total polysaccharide. Viscosity determinations in the absence and presence of salt showed a polyelectrolyte behaviour and an intrinsic viscosity of 2.21 g/L in 0.01 M NaCl (*77*).

Exopolysaccharides from *Bacillus* Strains

Several EPS now production studies have used bacilli as hosts, which are now regarded as potentially safe probiotics. EPS production from *Bacillus polymyxa* was found to be growth associated, signifying that the supply of adequate nutrients increased the EPS production especially with the increased sucrose or potassium nitrate concentration as a carbon source. Maximum EPS synthesis by *B. polymyxa* was 54 g/L (63 % yield) and it was obtained after 48 h of fed-batch fermentation with sucrose and potassium nitrate (*78*). Nitrogen-free medium (NFM) favoured higher EPS production in bacilli. The maximum EPS production by *B. subtilis* was approx. 206 μg/mL in nitrogen-free medium after 7 days of incubation at 37 °C (*17*). Ropy *B. licheniformis* strain produced maximum EPS concentration at mid exponential growth phase. EPS biosynthesis does not depend on sugar concentration. Incubation of *B. licheniformis* cells in ethanol-supplemented MRS media (1–7 %, by volume) resulted in enhanced EPS production in the exponential and stationary phases. The produced EPS was a heteropolymer predominantly constituted of mannose (80 %) (*79*).

EPSs synthesized by *Bacillus* spp. have comparatively elevated viscosity and superior pseudoplastic properties. EPS produced by *Bacillus polymyxa* was composed of glucose, mannose, and glucuronic acid (*80*). The concentration of the EPS obtained from *B. polymyxa* was about 15 g/L after 114 h of cultivation, resulting in its productivity of 0.13 g/(L·h) (*81*). EPS production from *B. coagulans* was also associated with biomass growth. This bacterium exhibited high affinity for growth-limiting substrate. Maximum yield of EPS obtained at 36 h of fermentation was estimated to be (0.33±0.005) g/L in glucose yeast extract acetate (GYEA) medium. A heteropolymer composed of galactose, mannose, fucose, glucose and glucosamine from bacilli showed significant emulsifying activities in different vegetable oil/hydrocarbon substrates. EPS from *Bacillus coagulans* comprises fucose as a constituent sugar, which is noticeable (*28*).

Physicochemical Properties of Exopolysaccharides

Ropiness

Buchanan and Hammer (*82*) described ropiness in milk as a change from its normal consistency to a condition in which long threads of viscous masses are observed. They attributed this condition to the production of polysaccharide derivatives. Ropiness is of high importance in forming polysaccharide properties. It is manifested by an increase in viscosity and a slimy or fatty mouthfeel of a polysaccharide. Usually ropiness (mucoidness) is generated by a strain during EPS production and contributes to adhesiveness, whereas the parameters like firmness and elasticity of a polysaccharide are more related to the properties of protein matrix. EPS-protein interactions are very important for exhibiting textural properties. EPSs synthesized by *B. infantis* and *S. thermophilus* are polyelectrolytes, while those produced by *L. delbrueckii* are neutral polysaccharides (*61*). EPSs with ropy thread of 11.3–21.0 mm produced from *L. helveticus* in 32–60 h demonstrate the ropy nature of the strain. These EPSs have high molecular mass of 26 500 kDa. The highest EPS yield of 0.73 g/L from this strain was observed in fermentation after 60 h (*83*).

Ropy *L. helveticus* strain was found to produce EPSs with various molecular masses (3–26 500 kDa) in 0–84 h at 37 °C and pH=5, whereas non-ropy *L. helveticus* and *Streptococcus thermophilus* produced EPSs of 3 to 395 kDa under the same conditions. An increase in the molecular mass of EPSs produced by a ropy strain from 26 to 26 500 kDa was observed after 12 to 60 h of fermentation, which also increased ropiness from 11.3 to 21.0 mm (*83*). After 72–84 h of fermentation, the decrease in ropiness up to 5.6–6.0 mm and in molecular mass up to 2700 kDa was observed, which might have been caused by enzymatic degradation (*84*).

Rheology: viscosity and elasticity

The rheological properties of EPSs carry a great importance in their overall characteristics but they are significantly affected by molecular features which need to be understood better before their possible consumption as food ingredients is approved. EPS structural studies

are united with the rheological ones and reveal significant differences among exopolysaccharides, hence their rheological properties depend principally on intrinsic physicochemical characteristics, *viz.* molecular mass, polydispersity, monosaccharide composition and degree of substitution. EPSs exhibit remarkable thickening and shear-thinning properties and display high intrinsic viscosities. Hence several slime-producing strains of lactic acid bacteria and bifidobacteria and their biopolymers have interesting functional and technological properties. Therefore, they have a huge application in bakery products.

The physicochemical properties of EPSs including viscosity depend on their molecular mass, monosaccharide composition, primary structure and interaction with milk constituents, mainly ions and proteins. It can be expected that the rheological properties of a purified EPS will differ from that observed in the context of a fermented product. Since both, the formation of gel and the biosynthesis of EPS occur during fermentation, the resulting networks are highly cross-linked. Two rheological characteristics can be distinguished: viscosity is the property of a material to resist deformation. In the context of fermented dairy products, this attribute can be described as slimy and fluid. Elasticity is the property of a material to recover its initial conformation after a deformation occurred. These attributes correspond to a firm body and gum-like fermented milk product. Both properties are important for the organoleptic quality of a product and for its appealing appearance and pleasant mouthfeel (*85*).

Viscosity is a very significant characteristic of EPS solutions. Emulsification and viscosity are desirable features of a biopolymer for its application under extreme conditions, *e.g.* pH, temperature and salinity. Viscosity of EPS solutions depends on a number of characteristics related to the EPS structure, *viz.* composition of the polysaccharide, chain stiffness, branches and side groups in the polysaccharide chain (*2*). Two kinds of viscosities have been discussed the most: intrinsic viscosity and apparent viscosity. Intrinsic viscosity is an important property of a polysaccharide which helps to isolate it from culture solutions. Polysaccharide exhibits high intrinsic viscosity with a high molecular mass and a rigid structure (*65*). Two different *S. thermophilus* strains have been found to produce equal amounts of EPSs with similar composition and structure but with different molecular mass. They can vary in their effects on the viscosity of stirred yoghurts due to the differences in intrinsic viscosity as mentioned above (*86*). Hence, various EPS-producing strains may enhance the viscosity of yoghurt differently. A straightforward relationship between the apparent viscosity and EPS production has not been detected yet. Apparent viscosity measurements are performed at 25 °C and expressed in mPa·s.

The radius of gyration is used to demonstrate the dimensions of a polysaccharide chain. The EPS of $2.4 \cdot 10^6$ Da from *L. lactis* NIZO B891 exhibits smaller gyration radius as compared to the EPS of $1.4 \cdot 10^6$ Da from *L. lactis* NIZO B40 (*87*). The backbone of *L. lactis* NIZO B40 EPS is composed of β(1,4)-linked residues that demonstrate a rigid structure and a high specific volume, whereas the backbone of *L. lactis* NIZO B891 EPS has α- and β(1,6)-linked residues that impart a degree of flexibility, hence this EPS exhibits smaller radius of gyration.

EPS from *S. thermophilus* is composed of random coils as opposed to sequenced rigid polymers (*54*). EPS from *L. lactis* ssp. *cremoris* is also confirmed to be composed of random coils (*88*). These random coil polymers do not have permanent shape and acquire uncertain fluctuating tertiary structures. The viscosity of the solutions of EPS polymers comprising such coils is based on EPS concentration and specific volume (*89*).

Fermentative Conditions

To optimize the fermentation parameters, numerous challenges have been resolved prior to enhancing the EPS production but still there are various unsolved assignments. One of the key problems is the strain-specific behaviour (*90*) altering the fermentation conditions. Fermentation conditions are crucial and largely influence the EPS production. Non-optimal growth conditions of mesophilic LAB strains affect the production of EPS most (*91*). In *Propionibacterium*, maximum EPS is synthesized at the incubation temperature of 23 °C (*92*). Optimum temperatures for EPS production were determined for diverse LAB strains as follows: 25 °C for *L. lactis* (*93*), 40 °C for *S. thermophilus* ST 111 (*29*) and between 20 and 25 °C for *L. rhamnosus* (*91*). Fermentation was carried out with *Leuconostoc* sp. CFR 2181 at 30 °C for 72 h and 200 rpm and the obtained EPS production was 25.4 g/L in EPS media (*25*). The EPS production by *L. delbrueckii* ssp. *bulgaricus* RR was found optimum in both ranges above 45 and below 30 °C along with the optimum growth (*94*). In contrast, EPS productions by *L. acidophilus* and *L. casei* were maximum at the optimum growth temperature (*21*). High-molecular-mass EPSs are produced at low temperature fermentative process. Several reports revealed that the molecular structure and sugar composition of the EPSs depend on the fermentation conditions (*95*).

Ricciardi and Clement (*96*) emphasized the importance of medium composition and pH optimization for EPS production. Fermentations were performed with or without controlled pH prior to studying its influence on EPS production. Optimum pH values vary from one species to another and also for specific species. For *Propionibacterium*, optimum pH of 6.0 has been observed with 3.0 g/L of yeast extract in skimmed milk-based media, when EPS concentration varied from 0.1 to 0.3 g/L of glucose equivalent (*92*). EPS production from *S. thermophilus* ST 111 was low at various pH values in milk-based media, where additional nitrogen source did not have a positive effect. However, with a controlled pH of 6.2, the addition of whey protein hydrolysate enhanced EPS production fivefold (*29*). Optimal pH of 7–8 and a sufficient supply of oxygen are needed for the EPS production in bacilli. When cells are cultivated in the media containing ammonium, the growth rate of the cells is much faster than that in the nitrate-containing media.

Production of EPS was usually observed during the logarithmic phase. For some LAB, it continued into the stationary phase. Kinetic studies of EPS production were carried out every 4 h during 16–24 h of fermentation using *Lactobacillus rhamnosus* LR32 alone or in combina-

tion with other probiotics. In such studies, the media comprised fructooligosaccharides and polydextrose with or without sucrose. Batch fermentations were conducted for 16 h using soybean extract inoculated with 1 to $5{\cdot}10^3$ and 1 to $5{\cdot}10^5$ CFU/mL of pure and mixed cultures. Less growth was observed in *L. rhamnosus*-inoculated medium containing 12 % sucrose (>10^6 CFU/mL), followed first by *L. rhamnosus* in soybean extract without sucrose (>10^7 CFU/mL) and then by *L. rhamnosus* mixed with other probiotics and 12 % sucrose (>10^9 CFU/mL). At the same time, the control fermented product from the probiotic mixture with 12 % of sucrose reached >10^7 CFU/mL. EPSs had molecular mass between 9 300 and 73 000 Da, similar to tri- and tetrasaccharides (*97*). The study of the effect of different gaseous conditions on the physicochemical properties of yoghurt revealed that EPS production by LAB was maximum under the extreme oxidoreduction potential (oxidizing condition) and under N_2^-–H_2^- reducing condition (*98*).

Insight into the biological role of EPSs could offer a better understanding prior to designing physiological parameters leading to enhanced EPS production. Moreover, such information would enable us to find optimum parameters for mutants prior to increased EPS production. However, the physiological function of EPSs has not been established clearly. It is not likely that EPSs serve as a reserve source of energy and carbon since EPS-producing bacteria are mostly not capable of degrading their own EPS, although unrelated microbes sometimes catabolize polysaccharides of other bacteria (*99*). EPS production by *L. lactis* is very unstable under laboratory conditions, therefore, it would be significant to consider different regulatory aspects in metabolic engineering such as physiological and genetic for closer insight of sugar degradation and EPS biosynthesis pathways (*100*).

Genetics

Exopolysaccharide production is well studied in LAB, bifidobacteria and propionibacteria. Identification of EPS gene clusters and the functional role of the gene products have been reported for a number of food-grade microorganisms, especially *Streptococcus thermophilus* (*101*) and *Lactococcus lactis* (*102*). These bacteria contain specific *eps* gene clusters in their genome which are involved in EPS production, transportation, polymerization, and chain length determination. The function of the glycosyltransferase genes has been well recognized and it improved the EPS engineering immensely. EPS biosynthesis needs housekeeping genes (*galE, galU, rfbABCD*) in addition to the *eps* genes. The classification and characterization of numerous housekeeping genes (*galE, galU, rfbABCD*) allows the design of metabolic engineering strategies that would lead to enhanced EPS production. Studies with mathematical modelling refined the approach in order to predict the physiochemical rate of EPS synthesis into the system. Genetic modifications have been shown to be successful in enhancing the production of EPS (*103*).

The genetic instability of EPS production is a serious problem for industrial applications. Several studies have reported low production, or a change in the EPS composition (*104,105*). The instability was observed both of strains harbouring plasmids that encode genes for EPS production (*3,102*) and of strains which do not contain such plasmids (*106*). Although transposable elements have been identified in the vicinity or even within EPS gene clusters, it is for the time being not substantiated if they are the main cause of the observed genetic instabilities. When the EPS production is plasmid encoded, conjugal transfer of this EPS plasmid can be applied to obtain new EPS-producing strains, as has been demonstrated for the *L. lactis* EPS plasmid pNZ4000 (*102*). Plasmids are comprised of replicons that are functional in restricted host-range, which limits the transformation of feasible recipients (*107*). To overcome limited host range, one approach is to clone the *eps* gene cluster on a broad host-range plasmid, as has been done for the streptococcal Sfi6 *eps* gene cluster (*106*).

Genes involved in the synthesis of numerous EPSs produced by probiotic strains have been cloned and many of them have been characterized. Genes directing repeating unit synthesis and those involved in polymerization are putative targets for metabolic engineering studies to modify the physicochemical properties of the EPS by introducing new groups in the repeating unit or modifying the chain length. Moreover, rising and shuffling the pool of nucleotide sugars may have an encouraging effect on EPS production levels. Genes involved in the EPS biosynthesis in *Lactococcus* spp. reside in clusters and apparently these are much conserved. The genes coding for the enzymes and regulatory proteins required for EPS synthesis are of plasmid origin in the mesophilic LAB strains, *e.g. Lactococcus*, and chromosomally based in the thermophilic strains of *Streptococcus* and *Lactobacillus*.

For genetic engineering approach, the biosynthetic pathway can be divided into four reaction steps. These are the reactions involved in sugar transport into the cytoplasm, the synthesis of sugar-1-phosphates, activation and coupling of sugars, and processes involved in the EPS export. Each of these steps must be evaluated and explored in developing strategies for the engineering of EPS products. Genetic engineering could be used as a tool to direct the EPS synthesis and introduce desired properties by altering the composition or chain length. Such approach has been made in order to enhance the activities of certain enzymes involved in the biosynthetic pathway. For genetic manipulation prior to enhancing the EPS production, a comprehensive understanding of the flux of metabolites in each of the enzyme-catalyzed reactions is required. Current research is focused on the flux of carbon feed to the sugar nucleotides that can be altered either by deletion of genes coding for key enzymes, *e.g.* the PGMs, or by functional over-expression of the UDP-glucophosphorylases and/or uridyl-transferases.

Sugar nucleotide concentration is crucial for EPS biosynthesis. The over-expression of *cps3D* (analogue of UDP-glucose dehydrogenase) and *cps3S* (CPS synthase type 3) genes from *S. pneumonia* into *L. lactis* resulted in a low production of CPS type 3: a linear structure composed of -3-β-D-GlcUA-(1,4)-β-D-Glc-1 repeating units (*108*). The CPS type 3 is increased by the coexpression of *cps3U* gene, which encodes an UDP-glucose pyrophosphory-

lase (GalU) analogue (*109*). The UDP-glucose level in *L. lactis* is controlled by the level of GalU enzyme. Increased UDP-glucose and UDP-galactose levels were obtained due to homologous overexpression of the galU gene, which supports the concept that GalU enzyme certainly controls the production of these sugar nucleotides in wild-type cells (*102*). UDP-galactose epimerase (GalE) is another vital enzyme in sugar nucleotide and EPS biosyntheses. Assessment of EPS production in *L. lactis galE* mutant strain exhibited microscopic levels of UDP--galactose which stopped the EPS biosynthesis in glucose--supplemented media, whereas EPS production could be achieved by the addition of galactose. Furthermore, such mutants are not dividing appropriately when grown on glucose alone, which specifies a significant role for UDP--galactose as a precursor in cell wall biosynthesis (*102*). Sometimes the EPS production level could be reliant on the regulation of the EPS biosynthesis machinery, rather than on that of the level of sugar nucleotides and this prospect is supported by over-expression of glycosyltransferase *epsD* in *L. lactis*. Consequently, EPS production has been increased (*110*).

Most of the genetic alteration approaches have focused on the genes coding for the glycosyl-transferases. The genes coding for such enzyme undergo non-polar disruption. Moreover, the *epsD* gene of *L. lactis* NIZO B40 failed to produce EPS (*111*). However, both homologous and heterologous insertion approaches of genes coding for priming galactosyltransferases resulted in the failure to produce EPS, possibly because the transferase activities involved in the synthesis of oligosaccharide repeat units were restricted to specific lipid-bound acceptor sugars.

The heterologous gene insertion from Gram-negative to Gram-positive bacteria has also been carried out. A complete gene cluster from *S. thermophilus* Sfi6 has been expressed into a non-EPS producer *L. lactis* MG1363, which did not result in an adequate production of EPS (*112*). Importantly, the structure of the produced EPS varied from that of *S. thermophilus* Sfi6 EPS, since the α(1,6)-linked α-D-Gal*p* in the main chain of α-D-galactose replaced the α-D-*N*-acetylgalactosamine. The synthesis of a 'designer' polysaccharide has also been developed, where the *aceP* gene was inactivated in the EPS-producing strain. This gene codes for a glucosyltransferase required in the construction of a branching side chain. The EPSs synthesised by the genetically modified bacteria have a truncated branch (*113*). The major problem faced by the researchers in this field is the instability of expression, which inhibits the industrial application.

L. mesenteroides B-742CB dextransucrase (168.6 kDa) gene *dsrB742* was inserted into a pRSET expression vector. The extracellular activity of such enzyme increased about 6.7-fold after the expression in *E. coli* BL21(DE3) pLysS, compared to native strain. The purified enzyme showed similar biochemical properties, but the ability to synthesize α(1,3) branching decreased in comparison with *L. mesenteroides* B-742CB dextransucrase (*114*). The *L. reuteri* levansucrase His_6-(pRBBm15) and Strep-tagged (pRBBm13) variants were coproduced extracellularly in *B. megaterium* host. Levansucrase secretion of the host was mediated by signal peptide of the extracellular esterase LipA, which was further increased with the coexpression of the signal peptidase gene *sipM*. Fused affinity tags allowed the efficient one-step purification of the levansucrase which synthesized new fructosyloligosaccharides from the novel donor substrates D-Gal-Fru, D-Xyl-Fru, D-Man-Fru, and D-Fuc-Fru (*115*).

Ecological Significance of Probiotic Exopolysaccharides

The ecological significance of EPS production was found to be linked mainly to the biofilm formation, stress resistance and sucrose utilization in LAB strains. The occurrence of HoPS- and HePS-producing LAB strains in the oral cavity and intestinal ecosystems confirms the significance of EPS formation for the survival of these strains in such type of adverse conditions. EPS produced by intestinal lactobacilli may participate in similar functions (*116*). EPSs produced by probiotics support the host strain to coaggregate and adhere to intestinal epithelial cells and also to interfere with pathogenic bacterial adhesion to human intestinal mucus (*117*). This indicates that EPSs may play a role in the gut that is mainly related to the colonization of probiotics in these competitive surroundings. It has also been suggested that EPSs play a role in the sequestering of essential cations (*118*), in adhesion and in biofilm formation (*15,119*).

Applications

EPSs exhibit immense diversity in composition, so they can find numerous applications in food, cosmetic, agronomy and pharmaceutical industries. EPS production by diverse probiotic hosts could be significant for applications in the food industry provided they give sufficient yield. The production process should be economically feasible, which could be possible by employing cheaper substrates and cost-effective fermentation conditions. Probiotic strains which are facultative anaerobes would be more effective in producing energy and could overcome the problem of low oxygen level, which occurs during EPS production by obligate aerobes, *viz. X. campestris*.

Firmness and creaminess are the most significant sensory attributes of dairy products popular among consumers. The addition of EPSs as texturizers and stabilizers increases the viscosity of the product, and they interact with proteins and micelles by binding water, hence supporting the rigidity of the casein network. However, EPSs can decrease syneresis and improve product stability. EPSs synthesized from few strains of lactic acid bacteria (LAB) can improve textural properties and decrease susceptibility to syneresis in fermented dairy products (*2*). A better perception of the structure-function relationship of EPSs in a dairy food matrix remains a challenge in order to improve their applications to meet the consumers' demand for delicious and also healthier products. The use of dextran in panettone and other types of bread is known; likewise, the addition of non--bacterial hydrocolloids is adopted traditionally in industrial baking process (*120*). Numerous studies revealed the initiative of substitution of these additives by probiotic

EPS (*viz*. levan), which offer data for dough and bread parameters, *viz.* textural factors, water retention, moisture and specific bread volume (*121*).

The benefits of EPSs are enormous, which encourages their explorations. The commercial exploitation of probiotic EPSs would certainly develop novel applications (*120,122*). In this context, considerable progress has been made in discovering and developing new microbial EPSs that possess novel industrial importance (*123*). The consumption of EPSs with health benefits has been achieved (*124*). In addition, there are reports on the positive effect of EPSs produced by *Lactobacillus* on gut health, *viz.* an anti-tumor effect (*125*), a cholesterol-lowering effect by the EPSs from fermented milk viili (a type of Finnish yoghurt) (*126*) and immunomodulatory effects of EPS from *Bifidobacterium adolescentis* M101-4 (*127*). EPS synthesized by bifidobacteria from dairy and intestinal origins can modulate the intestinal microbiota *in vitro*, promoting changes in some numerically and metabolically relevant microbial populations and shifts in the production of short chain fatty acids (*62*).

There is not a single report available on EPS application in fermented meat products, sauerkraut or vinegar in order to understand their effect on them. *In situ* production appears to be less effective in traditional wheat and rye dough systems due to strain-dependent acid formation, which may be required but counteracts positive EPS effects. Forthcoming chances of EPS application may therefore lie within special applications as in gluten-free bread, where both reduced pH and EPS should have synergistic positive effects on the structure.

EPS from *Bacillus licheniformis* impaired HSV-2 replication in human peripheral blood mononuclear cells (PBMC) and exhibited antiviral and immunomodulatory effects (*128*). Levan (HoPS) finds application in cosmetic, pharmaceutical and chemical industries. It is also being used as a food or feed additive with prebiotics and has been reported to exhibit hypocholesterolemic effects (*129*). Alginates can be used as thickening agents (*130*). Xanthan is widely used as a viscosifer, thickener, emulsifier or stabilizer in the food industry (*131*). Several other biological roles of EPSs have been suggested. These include a selective advantage in the natural habitat of these microbes, such as protection against dehydration, phagocytosis and protozoan predation, bacteriophage attack, antibiotics, or toxic compounds (*119*). The major limitation for the growth of EPSs as food ingredients on industrial scale is their low yield during fermentation. A reduction in the acetic to propionic acid ratio has been proposed as an indicator of a potential hypolipidemic effect of prebiotics (*132*). EPSs from bifidobacteria exhibited such acid ratio (*62*).

L. lactis survival during the gut transition is not influenced by EPS production. They can be found either attached to the cell or in suspension that offers improved tolerance against copper and nisin (positively charged bacteriocin). Moreover, cell-associated EPSs also protect the bacteria against bacteriophages and lysozymes. EPSs do not show positive influence on bacterial survival when exposed to increased temperature, freezing, freeze drying and antibiotics (*118*). The bifidobacterial cell wall components in cell culture extract exhibited intense effects on cell proliferation and cytokine production but EPSs neither stimulated lymphocyte proliferation nor induced cytokine secretion (*73*).

Conclusion

The investigation in order to obtain healthy nutraceuticals with valuable bioactive properties has drawn attention to probiotic exopolysaccharides. Functional foodstuffs which assert health-related benefits beyond their dietetic constituents are growing steadily in the food industry worldwide. The evidence of the encouraging health properties of probiotic exopolysaccharides is well accredited. Probiotic bacteria could serve as live bio-factories for the production of various polysaccharides for novel application in food processing industry. Carbon metabolism influences the sugar nucleotide alteration but nitrogen source was not found to be directly correlated with EPS production. The development of probiotic strains that produce EPSs at commercial level cannot be achieved until mutual approaches of fermentation studies and genetics are employed prior to synergistic manipulations for elevated microbial activities or physiology.

The knowledge about EPS production in probiotic bacteria has developed greatly over the last three decades. Studies dealing with EPS production have led to the identification and characterization of interesting housekeeping genes. The encoded enzymes are essential for designing metabolic engineering strategies to increase EPS production level by probiotic organisms. However, until today this approach has been hampered by the lack of EPS biosynthesis. The development of models will add a correct correlation to metabolic engineering approaches. Studies based on modelling must be developed to predict the behaviour of EPS and their several crucial molecular properties like molecular mass or chain length, polymer stiffness or monomer linkage type in EPS backbone that determine their effect on a product property like viscosity. Such models must be validated further prior to accomplishing a perfect approach to predict the exact properties of a specific EPS. EPS research is needed to construct an improved and altered probiotic strains that could produce designer EPSs at an increased level.

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