The production of exopolysaccharides by *Lactobacillus delbrueckii* subsp. *bulgaricus*

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The production of exopolysaccharides by *Lactobacillus delbrueckii* subsp. *bulgaricus*

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Stellingen

1. De bewering van Ranganathan *et al.* dat "ropiness" bij de fermentatie van melk als ongewenst wordt beschouwd, is niet terecht gezien de talloze onderzoeken die sindsdien zijn gedaan om dit fenomeen te verklaren en te verhogen.

   [**Ranganathan B., Chander H., Tiwari M. P.** 1974. Studies on factors affecting ropiness in *Streptococcus lactis*. Milchwissenschaft **34**: 333-334]

2. Een afname in de hoeveelheid geproduceerd exopolysaccharide wordt vaak toegeschreven aan de hydrolyse van EPS door de activiteit van glycohydrolase enzymen. Dit is echter bij melkzuurbacteriën nooit onomstotelijk bewezen.

   [**Cerning J., Bouillanne C., Desmazeaud M. J., Landon M.** 1988. Exocellular polysaccharide production by *Streptococcus thermophilus*. Biotechnol. Lett. **10**: 255-260]

   [**Macura D., Townsley P. M.** 1984. Scandinavian ropy milk - identification and characterization of endogenous ropy lactic streptococci and their extracellular excretion. J. Dairy Sci. **67**: 735-744]

3. De suikersamenstelling van EPS geproduceerd door *Streptococcus thermophilus* is sterk afhankelijk van de activiteiten van biosynthetische enzymen van suikernucleotiden. Bij *Lactobacillus delbrueckii* subsp. *bulgaricus* wordt de suikersamenstelling van EPS sterker bepaald door de activiteiten van enzymen betrokken bij de productie van de repeterende eenheid en de polymerisatie van EPS.

   [Dit proefschrift]

   [**Petit C., Grill J.P., Maazouzi N., Marczak R.** 1991. Regulation of polysaccharide formation by *Streptococcus thermophilus* in batch and fed-batch cultures. Appl. Microbiol. Biotechnol. **36**: 216-221]

4. De suggestie van Qian *et al.* en Sjöberg en Hahn-Hägerdal dat ß-glucose 1-fosfaat een rol speelt bij de synthese van polysacchariden is voorbarig, zolang dit niet nader is bestudeerd.

   [**Qian N., Stanley G.A., Hahn-Hägerdal B., Rådström P.** 1994. Purification and characterization of two phosphoglucomutases from *Lactococcus lactis* subsp. *lactis* and their regulation in maltose- and glucose-utilizing cells. J. Bacteriol. **176**: 5304-5311]

   [**Sjöberg A., Hahn-Hägerdal B.** 1989. ß-Glucose-1-phosphate, a possible mediator for polysaccharide formation in maltose-assimilating *Lactococcus lactis*. Appl. Environ. Microbiol. **55**: 1549-1554]

5. Om in een wetenschappelijk tijdschrift te publiceren is niet alleen de kwaliteit van het werk van belang, maar moet men ook een gelukkige hand hebben in de keuze van de editor.

6. Verkeerspleinen waarbij naderende voertuigen voorrang hebben op verkeer dat zich op het verkeersplein bevindt, dienen te worden afgeschaft.

7. Volgzaamheid beperkt het eigen denkvermogen.

8. Bij de bouw van voetbalstadions verliest men vaak uit het oog dat het belangrijkste onderdeel van een stadion de grasmat is.

9. De monopoliepositie van de Nederlandse Spoorwegen als commercieel bedrijf is misplaatst.

10. Dat de top van veel sportorganisaties steeds meer gaat lijken op bejaardenverenigingen, is de sport onwaardig.

11. In onze rechtshandige samenleving worden linkshandigen gedwongen veelzijdig te zijn.

Stellingen behorende bij het proefschrift, getiteld "The production of exopolysaccharides by *Lactobacillus delbrueckii* subsp. *bulgaricus*". Gert Grobben, Wageningen, vrijdag 22 mei 1998.

Contents

1. General introduction

1. Polysaccharides

Polysaccharides are macromolecules or a part of macromolecules which have a skeleton built up of units of sugars or sugar derivatives. There is an enormous number of varieties of sources, forms, structures and functions of polysaccharides. The structures of higher plants are dominated by polysaccharides such as cellulose and xylans (in cell walls), starch, pectin and alginate. Chitin is a significant structural component of the insect cuticle and it is present in cell walls of several groups of fungi. Pectins are components of higher plants, berries and fruits. Starch is the predominant storage polysaccharide in plants. Other plants produce fructans like levans or inulin in addition to, or as an alternative to starch as storage material.
Polysaccharides are produced in different forms [8]:

1. Structural polysaccharides, i. e. starch and cell wall material in the form of peptidoglycan, cellulose, pectin or teichoic acid,
2. Polysaccharides as a part of lipopolysaccharides in Gram-negative bacteria,
3. Polysaccharides as a part of glycoproteins,
4. Pure polysaccharides located intracellular or outside the cell wall as extracellular polysaccharides.

The latter form will be discussed in more detail in this chapter.

1.1 Bacterial exopolysaccharides

As do many eukaryotic organisms like higher plants or fungi, many bacteria produce extracellular polysaccharides. Two forms of these extracellular polysaccharides are known, attached to the bacterial cell wall (capsule) or unattached as extracellular slime. The names *extracellular polysaccharides*, *exopolysaccharides* and *EPS* are generally used for all forms of polysaccharides excreted in the environment or found outside the cell wall [141]. There is a great heterogeneity in amount, sugar composition, degree of branching and structure of EPS produced by bacteria. In general, exopolysaccharides may be composed of only one specific type of monosaccharide (homopolysaccharides) or of several types of monosaccharides (heteropolysaccharides). Both types of polysaccharides can exist in either a linear or a branched form, depending on the micro-organism producing them. Sutherland [144] placed the extracellular polysaccharides in four categories, depending on their structure and the mechanism of their biosynthesis:

1. Homopolysaccharides which are produced extracellularly by glycosyltransferase-like enzyme reactions,
2. Homopolysaccharides produced intracellularly by other enzyme reactions than glycosyl-transferases,
3. Heteropolysaccharides composed of identical repeating units,
4. Heteropolysaccharides with an irregular structure.

Dextran is the most important commercial homopolysaccharide produced outside the cell wall by dextran sucrase using sucrose as the sole substrate. Extracellular heteropolysaccharides which

have been studied extensively are xanthan from *Xanthomonas campestris*, succinoglycan from *Rhizobium* and other species, gellan and related polysaccharides from *Pseudomonas elodea* and *Sphingomonas paucimobilis* and bacterial alginate produced by *Pseudomonas* and *Azotobacter* species [97; 145]. A large variety of structurally different capsular polysaccharides produced by *Klebsiella* species is presented by Isaac [66].

1.2 Function of exopolysaccharides

The role of exopolysaccharides produced by bacteria is not completely understood yet. It is suggested that EPS play a role in protecting the cell against desiccation, phagocytosis and attacks by bacteriophages, as well as in the uptake of nutrients and metal ions. Furthermore it is assumed that EPS is involved in the interactions between bacteria and other organisms like plants and other bacteria or surface particles [14; 15]. For instance, oral streptococci like *Streptococcus mutans* and *S. salivarius* producing glucans are able to adhere to the surfaces of teeth to form dental plaque [14; 84]. It was found that the exopolysaccharides normally do not function as a carbon and energy source, since slime-producing bacteria do not have enzyme systems to degrade the polysaccharides they produce. The production of extracellular polysaccharides is in general not essential for bacterial cell growth and viability. Non-mucoid mutant strains, unable to produce EPS, showed an identical growth rate and metabolism as the EPS producing wild type strains and they could often be isolated in a relatively easy way [141]. Furthermore, it was shown that the removal of capsular polysaccharides by enzymes causes no loss of viability of decapsulated cells *in vitro*, although the presence of extracellular polysaccharides may have survival advantages in natural environments, specially under highly competitive conditions [26; 33].

2. Biosynthesis of extracellular heteropolysaccharides

The production of bacterial extracellular heteropolysaccharides starts in the glycolysis. The first step is the conversion of the carbohydrate substrate to sugar nucleotides which act as the glycoside donors for cell wall material and EPS. Hereafter, the sugar nucleotides are transferred to a membrane-bound lipid phosphate carrier to form the EPS repeating unit and the repeating units are transported across the membrane. At the outside of the cell membrane, the repeating units are coupled to form the polymer.

Most of the knowledge on the mechanisms of the synthesis of EPS in bacteria is focused on Gram-negative bacteria. For instance, *Xanthomonas campestris* produces xanthan gum, a heteropolysaccharide which is composed of pentameric repeating units containing D-glucose, D-glucuronic acid and D-mannose in a ratio of 2 : 1 : 2 decorated with acetyl and pyruvyl groups [67]. In the production of xanthan gum the sugar nucleotides UDP-D-glucose, UDP-D-glucuronic acid and GDP-D-mannose are involved and a biosynthetic pathway of these sugar nucleotides is proposed when the bacterium is grown on glucose, fructose or mannose, see Fig. 1.1. These sugar nucleotides could be detected in cell extracts of *X. campestris* [56]. It was found that a membrane-bound polyprenol-P lipid carrier is involved in the biosynthesis of the EPS repeating unit [65]. A schematic mechanism of the synthesis of the repeating unit of xanthan gum is given in Fig. 1.2.

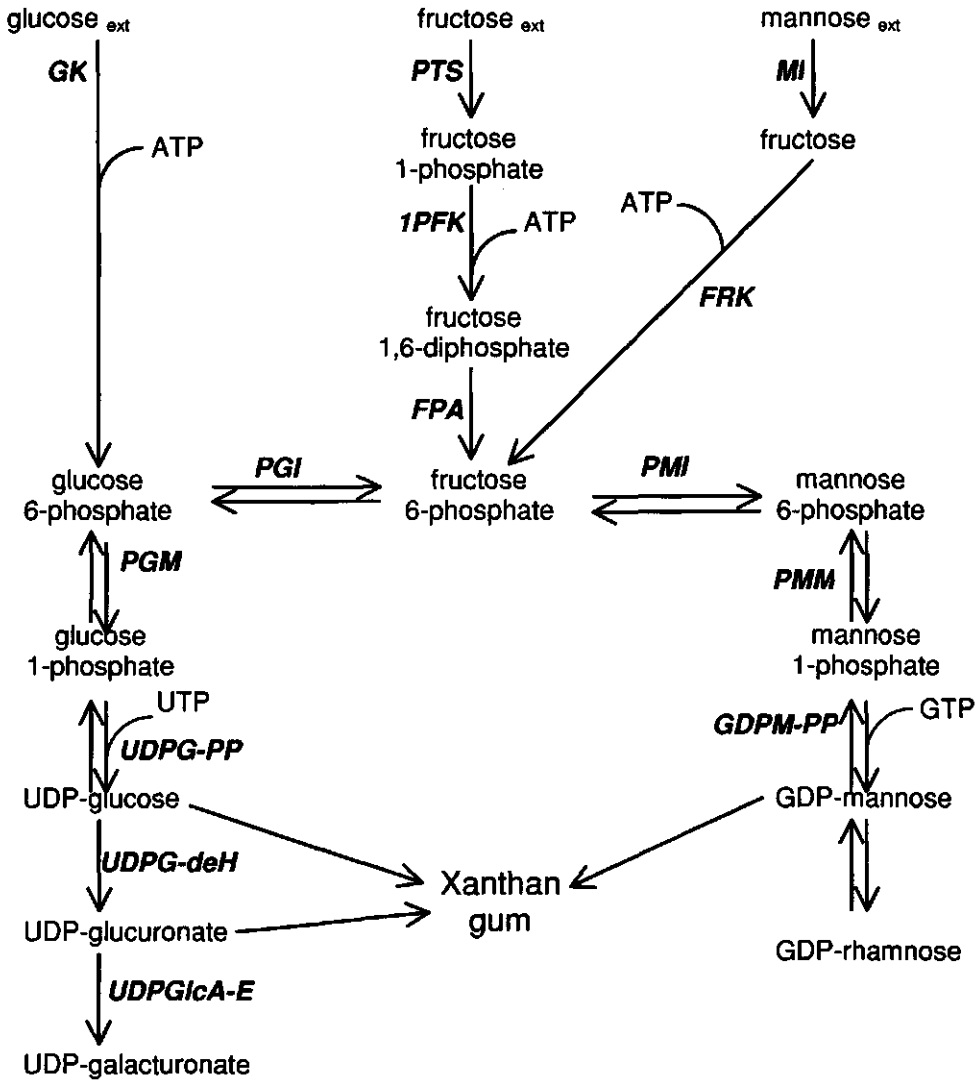


Fig. 1.1: Proposed biosynthesis and intraconversion of sugar nucleotide precursors of xanthan gum (from: Harding et al. [56]). Abbreviations of the enzymes involved: *GK*, glucokinase; *PTS*, phosphoenolpyruvate-dependent sugar phosphotransferase; *1PFK*, 1-phosphofructokinase; *FPA*, fructose 1,6-diphosphatase; *MI*, mannose isomerase; *FRK*, fructokinase; *PGI*, phosphoglucose isomerase; *PMI*, phosphomannose isomerase; *PGM*, phosphoglucose mutase; *UDPG-PP*, UDP-glucose pyrophosphorylase; *UDPG-deH*, UDP-glucose dehydrogenase; *UDPGlcA-E*, UDP-glucuronate epimerase; *PMM*, phosphomannose mutase; *GDPM-PP*, GDP-mannose pyrophosphorylase.

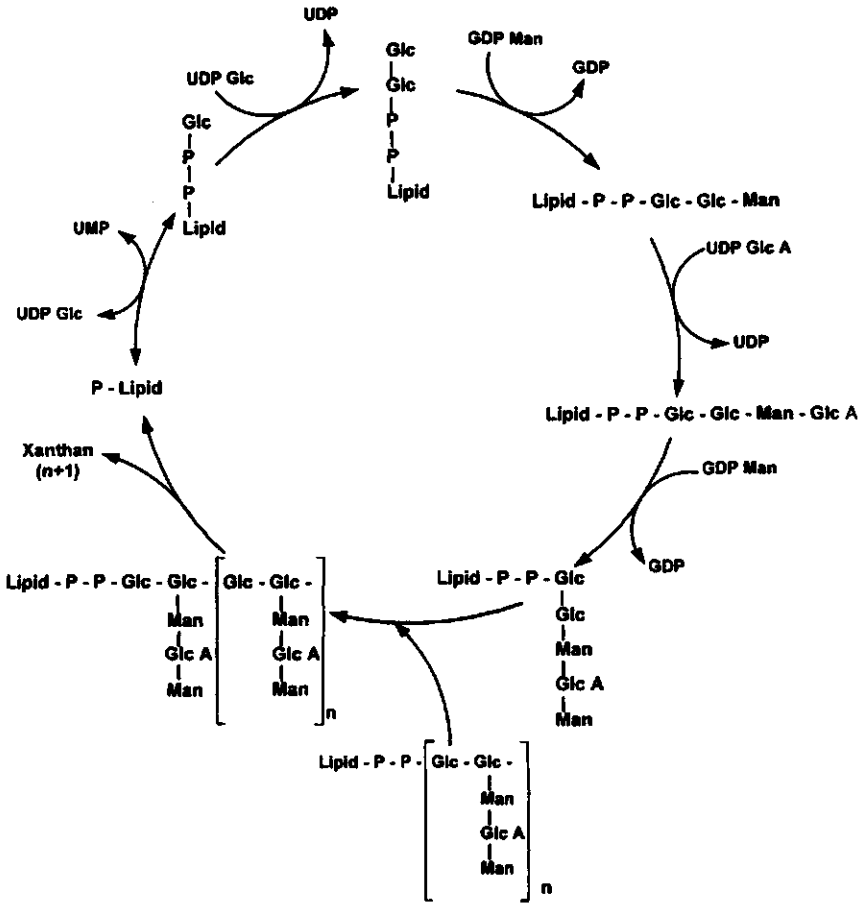


Fig. 1.2: Schematic mechanism of the proposed pathway for the biosynthesis of the exopolysaccharide xanthan by *Xanthomonas campestris* (Ielpi et al., 1993 [65]).

From a UDP-glucose molecule, glucose 1-phosphate is transferred to the monophosphorylated carrier at the release of UMP, catalyzed by a glucosyl transferase. Thereafter, glucose, mannose, glucuronic acid and again mannose are transferred by transferases to the glucose-PP-polyprenol carrier from UDP-glucose, GDP-mannose, UDP-galacturonic acid and mannose at the release of UDP, GDP, UDP and GDP, respectively. Finally, acetate and pyruvate are added to the pentameric repeating unit from acetyl-CoA and phosphoenolpyruvate, respectively [62; 63; 64; 143]. The lipid-associated pentameric oligosaccharide is transported to the outer layer of the cytoplasmic membrane and reacts with another lipid carrier which already consists of multiple repeating units. The new repeating unit is added at the reducing end of the growing xanthan chain [65]. After this reaction, the polymer is extended with one repeat and it is linked to the lipid carrier involved in the assembly of the last repeating unit. A diphosphorylated lipid carrier is released and after a phosphatase reaction, a monophosphorylated polyprenol carrier is again available for another polymerization reaction. The mechanisms of the transport of the repeating unit across the cytoplasmic membrane, the release of the Xanthan polymer into the culture medium and the

regeneration of the monophosphorylated polyprenol carrier are not completely understood yet [65].

2.1 Genetics of polysaccharide production

Recently, the genetic control of exopolysaccharide production in various bacteria has been studied extensively. In *X. campestris*, a 16 kb DNA fragment named *xpsI* or *gum* was found containing a gene cluster with 12 open reading frames, containing genes for glycosyltransferases, acetyltransferases, a ketal pyruvate transferase and enzymes possibly involved in the polymerization and export of the polymer [13; 71]. In *Rhizobium meliloti*, a cluster of *exo* genes, involved in the synthesis of succinoglycan polysaccharide was isolated from a megaplasmid and a model for the biosynthesis and processing of the polymer was proposed, including the genes involved in the specific steps of the biosynthesis, polymerization and export [46; 81].

Genetics and regulation of exopolysaccharide production in other bacteria have been reviewed by Sutherland [144]. The amount of genetic material involved in the synthesis and export of polysaccharides have shown to be fairly similar, and varying from 12 to 17 kb in size. Genes and functions of enzymes involved in the synthesis of the EPS repeating unit have often been clearly characterized, but less is known about the genes of enzymes involved in the polymerization and transport of the polymer. Although these genes are most probably present in the isolated gene clusters, their exact role in the EPS biosynthesis is not completely understood and many researchers only speculate about the involvement of such genes.

2.2 Effect of environmental conditions on polysaccharide production

Most mucoid bacteria are capable of producing EPS independently of the growth conditions, but some growth conditions may influence the amount and structure of the EPS produced. Sutherland [141] stated that in aerobes and facultatively anaerobes, polysaccharide production is normally highest when oxygen is present in non-limiting amounts and as a result of this, polysaccharide production is higher on solid media than in liquid media, at comparable amounts of cells. Furthermore, in *Klebsiella aerogenes* and *Escherichia coli*, exopolysaccharide production was stimulated by an excess of carbohydrate in combination with a nutrient limitation, such as nitrogen, phosphorous or sulfur. Limitation of the carbon source often results in a minimal polymer production [162]. In *Propionibacterium acidi-propionici*, a high carbon:nitrogen ratio enhanced the polysaccharide production [116]. Production of homopolysaccharides, such as dextrans and levans, requires specific carbon substrates like sucrose, since highly specific enzymes are involved in the production of these polymers.

3. Utilization of lactic acid bacteria

3.1 Starter cultures

Lactic acid bacteria derive their name from the production of lactic acid, which is the most important product of the fermentation of carbohydrates. They are of major importance for the food industry, since they play an important role in the production of fermented food products like cheese and yogurt, sour dough, fermented vegetables and fermented meat products. Most of the lactic acid bacteria used in the food industry belong to the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*, but much more genera have already been described [91]. The classification, physiology and the application of lactic acid bacteria have been reviewed recently by several researchers [24; 123; 125].

The starter cultures of lactic acid bacteria applied in the dairy industry can be divided into mesophilic and thermophilic lactic acid bacteria, depending on their optimum growth temperature. Most mesophilic starter cultures are composed of *Lactococcus lactis* strains or *Leuconostoc* strains and several combinations of single or mixed starter cultures are used in for instance the production of cheese, buttermilk and Scandinavian fermented milk products. Their optimum growth temperature is usually < 30 °C. Thermophilic starter cultures are composed of *Streptococcus thermophilus* and/or lactobacilli (*Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. helveticus*, *Lb delbrueckii* subsp. *lactis*, *Lb. acidophilus*) having an optimal growth temperature of approximately 45 °C or more. They are used for the manufacture of yogurt and various types of cheeses, like Emmental, Gruyère, several kinds of Swiss cheese, Gorgonzola or Grana [5; 94].

3.2 Growth of lactic acid bacteria

Lactic acid bacteria are auxotrophic for many metabolites, so they require complex growth media. They require carbohydrates as a carbon and energy source, specific peptides and amino acids for protein biosynthesis, vitamins as precursor or catalyst for several enzyme activities, mineral salts and nucleic acid bases [23; 38; 68; 79; 96]. Since they do not possess a respiratory chain, they can grow anaerobically, although many strains are aerotolerant. Therefore, lactic acid bacteria do not grow in water or soil, but they grow well in carbon- and nitrogen-rich habitats, such as milk, milk products, vegetables and meat. In these habitats, lactic acid bacteria are often superior to other microorganisms, because of their ability to grow at low pH levels, caused by the fermentation of carbohydrates to organic acids.

3.3 The role of starter cultures

The role of lactic acid bacteria in the manufacture of fermented food products is diverse. They produce lactate from carbohydrates, which causes not only an acidification of the product, but has also an antimicrobial effect on spoilage micro-organisms [59; 164]. Furthermore, they produce substances, such as acetoin, diacetyl, acetaldehyde and other volatile compounds, which contribute to the aroma and flavor of fermentation products and have antimicrobial properties [91; 154]. Several lactic acid bacteria are able to produce bacteriocins, which are small proteins with antimicrobial activities [73; 74]. Therefore, lactic acid bacteria have been used in the keepability of fermented food products for a long time and nowadays the preserving ability of lactic acid bacteria is still very important in places where no refrigerating capacity is available, like developing countries [25].

4. Types of exopolysaccharides produced by lactic acid bacteria.

Several lactic acid bacteria are able to produce exopolysaccharides [14; 15; 135]. EPS are of great interest in the food industry as thickeners, stabilizers or gelling agents, because of their rheological behavior and viscofying properties. In contrast to organisms like the xanthan producing plant-pathogenic micro-organism *Xanthomonas campestris*, lactic acid bacteria are normally food-grade organisms, since they have the GRAS status (Generally Recognized As Safe). Xanthan is only permitted as food additive when no living bacteria are present in the purified polymer. Since there is a increasing demand for 100% natural food without additions, lactic acid bacteria producing EPS may act as an acceptable alternative for thickening agents. The in situ utilization of lactic acid bacteria producing EPS in the manufacture of fermented food products offers a good opportunity. Particularly in the dairy industry, EPS producing lactic acid bacteria are used in an extensive way; they are involved in the manufacture of products which have a slimy texture, such as yogurt, Nordic ropy milk products like viili, and kefir. In the dairy industry, it is thought that the use of

slime-producing thermophilic lactic acid bacteria in the manufacture of yogurt will not only increase the viscosity of the fermentation product, but also decrease the susceptibility to syneresis [14; 128; 159]. Other investigators claim that lactic acid bacteria and/or the exopolysaccharides produced by lactic acid bacteria may have beneficial effects, such as anti-tumor activities [103; 110]. Nakajima et al. [106] reported that the milk fermented by the slime producing *Lactococcus lactis* subsp. *cremoris* SBT 0495 had a cholesterol-lowering effect. The relationship between EPS production and viscosity and texture of fermented milk products was shown earlier [14; 128; 147], but it was found that ropy and non-ropy strains applied for the production of Dutch stirred yogurt can produce identical amounts of exopolysaccharides, so that the relationship between the viscosity of the fermented milk and the role of EPS is much more complicated [137]. In general, the magnitude of the viscofying effect may be affected by not only the amount of polymer produced, but also by the molecular weight, the sugar composition, the degree of branching of the EPS and the interaction between the EPS and milk proteins. Therefore, the exopolysaccharides produced by the starter cultures may contribute to the ropy character of the fermentation product, but ropiness and exopolysaccharide production are not principally equivalent [135].

Based on their chemical composition, the EPS produced by lactic acid bacteria can be classified in homopolysaccharides and heteropolysaccharides. Both types will be discussed below with some examples.

4.1 Homopolysaccharides

Several genera and species of lactic acid bacteria producing extracellular homopolysaccharides have been investigated, such as *Leuconostoc*, *Pediococcus* and oral streptococci. An example of a homopolysaccharide producing lactic acid bacterium is *Leuconostoc mesenteroides* that is able to produce high amounts of dextran [72]. These polymers are produced outside the cell, when sucrose is used as the carbohydrate and energy source by these organisms. The polymerization reaction is carried out by extracellular glycosyltransferases, which hydrolyze sucrose molecules and transfer the D-glucose residues to growing glucan chains. These chains can be linear or branched, depending on the glycosyltransferase involved. The polysaccharide dextran, produced by *Ln. mesenteroides*, consist of glucose units with mainly α-(1→6) linkages (up to 95 %) with some branch points at positions (1→2), (1→3) and/or (1→4). The structure of dextran can be comblike, laminated or ramified [72]. The polymerization reaction of the main dextran chain is catalyzed by the enzyme dextran sucrase: it cleaves the sucrose disaccharide, releasing D-fructose and transferring the α-D-glucose to an acceptor molecule, which is bound to the enzyme. The reaction catalyzed is as follows:

$$[\alpha\text{-}(1\rightarrow6)\text{-D-glucose}]_n + \text{sucrose} \rightarrow [\alpha\text{-}(1\rightarrow6)\text{-D-glucose}]_{n+1} + \text{D-fructose}$$

The energy released from the hydrolysis of sucrose (23 kJ mol^{-1}) enables this reaction, since the glucosidic bond in dextran has a lower free energy (12-17 kJ) [144]. *Ln. mesenteroides* B-742 appeared to produce two dextrans, a fraction "L" containing α-(1→6)-glucose linkages and α-(1→4)-glucosyl branching points and a fraction "S" composed of a α-(1→6)-linked glucose chain with branching points at α-(1→3) [132; 133; 134].

The streptococcal homopolysaccharide mutan is produced in a similar way by the oral lactic acid bacterium *Streptococcus mutans*. Mutan is a polymer which differs from dextran in containing a high percentage of α-(1→3) linkages and this polymer is often insoluble in water. Some *Streptococcus salivarius* strains are able to produce levan, a fructan homopolysaccharide composed of β-(2→6) fructofuranoside residues with branch points at position 1 [14].

Another type of homopolysaccharide is polygalactan, produced by *Lactococcus lactis* subsp. *cremoris* H414 [53]. This exopolysaccharide is composed of structurally identical pentameric repeating units of three β-(1→3), one β-(1→4) and one α-(1→4) linked D-galactosyl units. Although this polygalactan is a homopolysaccharide, the biosynthesis of this polymer is completely different from the biosynthesis of dextran mentioned above and it is expected that this polymer is synthesized in a similar way as was found for the biosynthesis of heteropolysaccharides.

4.2 Heteropolysaccharides

A broad range of lactic acid bacteria produce extracellular heteropolysaccharides. Compared to the homopolysaccharides, which are normally produced in relatively high amounts, the production of heteropolysaccharides by lactic acid bacteria is much lower. The molecular weight, degree of branching and the sugar composition of the EPS vary depending on the strain and the culture conditions. The viscofying properties and the rheological behavior of the polysaccharides are strongly dependent on the chain length, the amount of side chains and the monomeric composition. The exopolysaccharides produced by lactic acid bacteria are generally composed of identical repeating units varying from 2 to 8 monosaccharide residues and the chain lengths vary depending on the bacterial strain. The exopolysaccharides produced by lactic acid bacteria contain mostly D-glucose and D-galactose residues in varying amounts together with other sugars. The biosynthesis of polysaccharides is intracellular and located at the cytoplasmic membrane. They are all synthesized starting at the conversion of sugars of the carbon source into sugar nucleotides as precursors for the synthesis of the repeating units of the EPS.

5. Lactic acid bacteria producing heteropolysaccharides

In the following sections some extracellular heteropolysaccharide-producing strains of *Lactobacillus*, *Streptococcus thermophilus* and *Lactococcus lactis* will be discussed, including EPS-compositions and/or -structures.

5.1 *Lactobacillus* sp.

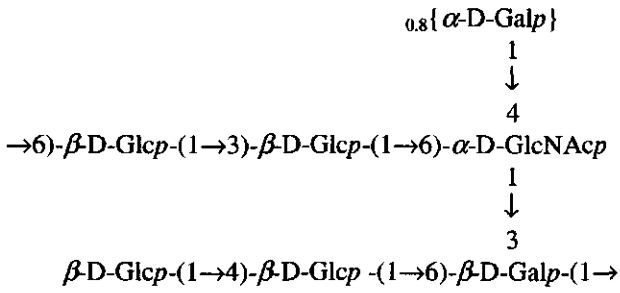
Among lactobacilli, there are many extracellular heteropolysaccharide producing species, like *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus*, *Lb. casei* and *Lb. sake*. *Lb. delbrueckii* subsp. *bulgaricus* strains producing exopolysaccharides will be discussed later in this chapter.

Among *Lb. helveticus* strains producing exopolysaccharides, there are many different polymers isolated with repeating units mostly containing glucose and galactose. Also *N*-acetyl-D-glucosamine was found in the polymer produced by one of the strains investigated. As shown in Fig. 1.3a-d, the repeating units of four *Lb. helveticus* have many variations and they have most probably different rheological and chemical behaviors.

Lb. helveticus strain TY1-2 produced an exopolysaccharide which had a molecular weight of 1.6×10^6 and was composed of a branched heptameric repeating unit containing *N*-acetylglucosamine, galactose and glucose [166] (Fig. 1.3a). A presumed spontaneous mutant of strain TY1-2, *Lb. helveticus* TN-4, produced an exopolysaccharide which showed to have a viscosity property which was different from that of the wild type strain TY1-2. The molecular weight was slightly higher (1.8×10^6) and it was composed of a hexameric repeating unit of galactose and glucose monomers in the ratio 3:3 [167] (Fig. 1.3b). An identical structure was found for the repeating unit of the polymer produced by *Lb. helveticus* Lh59. Only the molecular weight appeared to be 2.0×10^6, which was higher than the molecular weight of the polymer of

strain TN-4 [140]. Staaf et al. [138] isolated and characterized EPS produced by another *Lb. helveticus* strain and found that the repeating unit had a structure different from the strains mentioned before, with glucose and galactose monomers in the ratio 2:4 (Fig. 1.3c). *Lb. helveticus* strain 766 produced EPS composed of a hexameric repeating unit with a chain of 4 glucose and 1 galactose monomers, with one side chain, composed of a terminally linked galactose [121] (Fig. 1.3d).

A.



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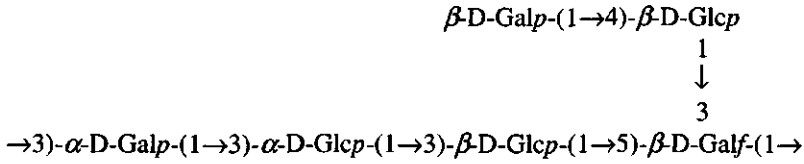

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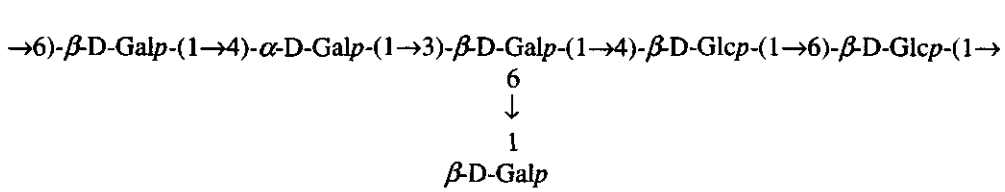

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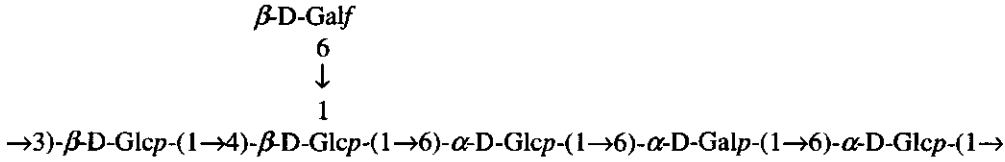

Fig. 1.3: Structure of the repeating unit of exopolysaccharides, produced by four different *Lactobacillus helveticus* strains. A. strain TY1-2 [166], B. strains TN-4 [167] and Lh59 [140]. C. strain characterized by Staaf et al. [138], and D. strain 766 [121].

Strains of *Lb. casei* subsp. *rhamnosus* produced a capsular polysaccharide, consisting of rhamnose, glucose and galactose in a molar ratio of approximately 2:1:1. The composition of this polymer was similar to the polysaccharide components normally present in the cell wall of this

organism and since the cell wall of the encapsulated strain lacked the rhamnose-containing polysaccharide, it was suggested that the capsule represented polysaccharide which had failed to become attached to the cell wall mucopeptide [75]. *Lb. casei* CG11 grown on glucose produces 130 mg l^{-1} exopolysaccharide which was mainly composed of glucose (75 %) and rhamnose (15%), galactose was present in a very low amount [76]. When lactose was used as the carbon source, EPS production was significantly lower (35 mg l^{-1}) and the EPS was composed of glucose (63%), rhamnose (21%), galactose (13%) and mannose (7%) [21]. Non-mucoid variants of this strain produced a low amount of EPS and when grown on glucose, the EPS had a similar composition as the parental strain, but when grown on lactose a polymer was produced with a high amount of galactose (48%), glucose (38%) and mannose (9%) and no rhamnose residues [21]. *Lb. casei* CRL 87 produced EPS which was mainly composed of rhamnose (47%) and galactose (41%) with a low amount of xylose (7%). Molecular weight analysis of the polymer showed the presence of two polymers with molecular weights of 8×10^5 and 5×10^5, respectively and with an identical chemical composition. It was concluded that the second polymer was probably a degradation product of the first [101].

The structure of the repeating unit of the exopolysaccharide produced by *Lb. sake* 0-1 was elucidated by Robijn et al. [120]. The molecular weight appeared to be 6×10^6 and it was composed of 3 glucose, 2 rhamnose and 1 *sn*-glycerol 3-phosphate residues.

5.2 *Streptococcus thermophilus*

S. thermophilus is, together with *Lb. delbrueckii* subsp. *bulgaricus*, involved in the fermentation of milk to form yogurt and there is a growing interest in the utilization of slime-producing strains to obtain a product with a high consistency. Not only slime-producing *Lb. delbrueckii* subsp. *bulgaricus* strains have been investigated extensively, but also several *S. thermophilus* strains producing EPS were isolated and their exopolysaccharides were studied. The EPS produced by *S. thermophilus* strains mostly contain glucose and galactose monomers (Table 1.1).

Two *S. thermophilus* strains, CNRZ 389 and CNRZ 1068 produced EPS consisting mainly of galactose and glucose monomers in differing amounts, the amount of galactose in the polymer was higher in most cases. The EPS produced by strain CNRZ 389 also contained a low amount of mannose monomers [18]. Doco et al. [29] isolated EPS from the ropy strains CNCMI 733, CNCMI 734 and CNCMI 735 and found that the EPS produced was composed of linear tetrameric repeating units composed of D-galactose, D-glucose and *N*-acetyl-D-galactosamine in the ratio 2:1:1. It was not clear whether all three strains tested produced this same polysaccharide. The molecular weights were identical (1.0×10^6), but the monomeric sugar composition of the EPS produced by the three strains varied slightly, as was reported later [30].

Recently, Stingele et al. [139] found that the repeating unit of the EPS produced by *S. thermophilus* Sfi6 had an identical structure, and the polymer had a molecular weight of 8×10^5. On the other hand, *S. thermophilus* strains Sfi12 and Sfi39 produced EPS with different sugar compositions and structures: strain Sfi12 produced a polymer with a branched hexameric repeating unit containing D-galactose, L-rhamnose and D-glucose in the ratio 3:2:1 and the polymer produced by strain Sfi39 had a branched tetrameric repeating unit with 2 D-galactose and 2 D-glucose residues [83].

Table 1.1: Production, composition and molecular weights of exopolysaccharides, produced by *Streptococcus thermophilus* strains.

strain	amount mg l^{-1}	molecular weight	repeating unit	ratio sugar composition[a] Glc	Gal	others	references
OR 901	19	1.1×10^6	heptamer		5	2 Rha	4; 12
CNRZ 389	57	-	-	4.8	11	1 Rha, 3 Man	18
CNRZ 1068	166	-	-	1	1.4		18
-		1.0×10^6	tetramer	1	2	1 GalNAc	29
CNCMI 733	40	1.0×10^6	tetramer	1	2.2	0.9 GalNAc	29; 30
CNCMI 734	67.3	1.0×10^6	tetramer	1	1.9	0.8 GalNAc	29; 30
CNCMI 735	53	1.0×10^6	tetramer	1	2.2	0.8 GalNAc	29; 30
Sfi12	105	$>2.0 \times 10^6$	tetramer	1	3	2 Rha	83
Sfi39	350	$>2.0 \times 10^6$	tetramer	1	1		83
-	2500	-	-	1	1.7	3.0 Man, 3.6 Uro	113
-	3000	-	-	1	2.8	1.1 Man, 0.7 Man	113
Sfi6	175	7.9×10^5	tetramer	1	2	1 GalNAc	139

[a] Abbreviations: Glc, glucose; Gal, galactose; Man, mannose; Rha, rhamnose; GalNAc, *N*-acetylgalactosamine; Uro, uronic acid.

Ariga et al. [4] isolated EPS from strain OR 901 and found that the polymer had a sugar composition of D-galactose and L-rhamnose in the ratio 1.5:1 and the repeating unit had probably a branched form. Recently, the structure of the repeating unit of this polysaccharide was clarified as a branched heptameric repeating unit of D-galactose and L-rhamnose residues in the molar ratio 5:2 [12]. A *S. thermophilus* strain investigated by Petit et al. [113] produced an extracellular polysaccharide and the composition of the polymer was dependent on the fermentation rate of lactose. In batch cultures, a polymer was produced containing mainly mannose and uronic acids, with lower amounts of glucose and galactose. When grown in a fed-batch culture, the composition of the polymer was dependent on the flow rate of lactose. A flow rate of 2.6 mM h^{-1} resulted in the production of a polysaccharide with almost equal amounts of glucose, galactose, mannose and uronic acids. On the other hand, when the lactose flow rate was limited to 1.5 mM h^{-1}, galactose became the major polysaccharide component (49 %) and the other compounds were present in much lower amounts. Schellhaass [127] found that four *S. thermophilus* strains (SF, yp, DEN-B3 and 410) produced EPS which contained glucose and galactose in the ratio 1:2. Sugars like mannose, arabinose and xylose were not found and other monosaccharides were not determined.

5.3 Mixed starters of *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*

The fermentation of milk to the formation of yogurt is a result of the associative growth of *Lb. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. In this so called proto-cooperation, each strain produces substances absent in the initial growth medium, but stimulatory for the other strain. *Lb. delbrueckii* subsp. *bulgaricus* is more proteolytic than *S. thermophilus*, producing a combination of extracellular proteinases and peptidases [6] to provide small peptides and amino acids available for growth of both strains, while *S. thermophilus* produces formic acid, necessary for purine biosynthesis in *Lb. delbrueckii* subsp. *bulgaricus*, and also carbon dioxide [31; 57].

Some *S. thermophilus* strains are able to ferment the galactose, excreted by *Lb. delbrueckii* subsp. *bulgaricus*, using the Leloir pathway when lactose is present in limiting concentrations [60].

Stingele et al. [139] stated that in a milk fermentation of short duration with a mixed culture of slime-producing strains of *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, the EPS produced by the latter is predominant. Cerning et al. [19] found that the ropy strain *S. thermophilus* CNRZ 1066 grown in non-fat dry milk produced 337 mg l^{-1} EPS whereas the non-ropy *Lb. delbrueckii* subsp. *bulgaricus* strain CNRZ 398 produced little if any EPS. However, when the two strains were grown simultaneously, exopolysaccharide production strongly increased to almost 800 mg l^{-1}. When a slime-forming *S. thermophilus* strain was grown in association with a non-slime-forming *Lb. delbrueckii* subsp. *bulgaricus* strain, the exopolysaccharide produced was composed of glucose (56%) and galactose (41%) and only trace amounts of mannose and pentose were detected [15]. Tamime and Robinson found that the yogurt starter culture RR (*Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) produced an exopolysaccharide which contained only glucose and the polymer was characterized as β-glucan [146]. The "yogurt" polymer described by Marshall [91] was a polysaccharide containing galactose, glucose, xylose and uronic acids. Trace amounts of protein were also detected.

5.4 *Lactococcus lactis*

Strains of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* have been used in the manufacture of Nordic ropy sour milk products like 'Viili' and 'Långfil', very popular in the Scandinavian countries. The thick consistence of these products is caused by slime-forming variants of these bacteria in the starter cultures [10; 37].

The slime material produced by ropy *Lc. lactis* subsp. *cremoris* SBT 0495 contained carbohydrates and proteins. The polysaccharide, named viilian, was composed of D-galactose, D-glucose and L-rhamnose in the ratio 2:2:1. The repeating unit was pentameric and branched and contained not only five monosaccharides but also a phosphate group [104; 105]. An exopolysaccharide with identical repeating units was found in the slime-producing *Lc. lactis* subsp. *cremoris* strain NIZO-B40 [77]. As mentioned earlier in this chapter, *Lc. lactis* subsp. *cremoris* strain H414 produced polygalactan, a branched homopolysaccharide with repeating units composed of D-galactose monomers [53]. *Lc. lactis* subsp. *cremoris* strain LC330 appeared to produce two different extracellular polysaccharides [92]. One polymer was neutral and had a molecular mass of at least 1×10^6 Da and the other was charged, contained phosphorous and had a lower molecular mass of 1×10^4 Da. The neutral polysaccharide was composed of galactose, glucose and glucosamine in an approximate ratio of 6:3:2 and it contained branched repeating units. The smaller polysaccharide had a more complex structure with glucose, rhamnose, galactose and glucosamine in a ratio of approximately 6:5:4:1 and it had a high phosphorous content.

6. Biosynthesis of exopolysaccharides in lactic acid bacteria

A stepwise biosynthetic pathway of the repeating unit of the exopolysaccharide "viilian", produced by *Lc. lactis* subsp. *cremoris* SBT 0495 is proposed by Oba et al. [109]. The membrane-bound lipid carrier, functioning as a sugar-acceptor was identified as undecaprenol. Oligosaccharide intermediates, bound to the undecaprenol carrier were identified and after comparison with the primary structure of the repeating unit of the EPS [104; 105], a biosynthetic pathway was postulated.

The amount and ratio of the different sugar nucleotides produced by lactic acid bacteria may influence the amount and composition of the exopolysaccharides produced. As already mentioned

before in this chapter, it was observed that the sugar composition of the EPS produced by the *S. thermophilus* strain investigated by Petit et al. [113] was dependent on the fermentation rate of lactose. The *S. thermophilus* strain grown on lactose fermented both the glucose and the galactose monomer simultaneously, indicating the presence of the Leloir pathway for galactose fermentation [150; 151]. At high fermentation rates with an excess of lactose (batch cultures), relatively more mannose was found in the EPS, whereas in fed-batch cultures with low lactose fermentation rates, relatively more galactose monomer was present in the polysaccharide. The fermentation rate only influenced the production rate of the EPS, but not the total amount of polymer produced. The pathways for glucose and galactose fermentation and the synthesis of sugar nucleotides is shown in Fig. 1.4.

At increasing lactose fermentation rates, the ratio GlcK/GalK activity increased, which means a lower galactose fermentation rate and a lower production rate of galactose 1-phosphate and UDP-galactose. On the other hand, the ratio PGI/PGM activity strongly increased at high fermentation rates and there was also a slight enhancement of the GMP/UGP activity ratio, resulting in a stronger carbon flow towards the production of GDP-mannose compared to the production of UDP-glucose. Therefore, Petit et al. concluded that the activities of enzymes leading to the production of UDP-glucose, UDP-galactose and GDP-mannose is influenced by the fermentation rate of lactose, resulting in a change of the composition of the EPS produced by *S. thermophilus*. Unfortunately, the authors did not investigate the influence of the lactose fermentation rate on the production of precursors of uronic acids in the EPS on the level of enzyme activities, although the amount of uronic acids in the EPS was strongly dependent on the lactose fermentation rate [113].

In *Lactococcus lactis* grown on maltose, it was observed that β-glucose 1-phosphate did not participate in the energy metabolism of the cell and therefore it was speculated that it might be a mediator for the synthesis of polysaccharides [136]. When grown on glucose, *Lc. lactis* showed no β-phosphoglucose mutase or UDP-glucose pyrophosphorylase activity with β-glucose 1-phosphate, so it was stated that the synthesis of both enzymes required maltose in the growth medium and was repressed by glucose [114]. However, no investigations were published on the EPS production by the *Lc. lactis* strain on either glucose or maltose, so that these suggestions might be premature. In other lactic acid bacteria, this mechanism of EPS production is most probably not active, since many strains are not able to ferment maltose and since nothing is known about any EPS producing activity with β-glucose 1-phosphate involved.

6.1 Effect of the medium composition and growth conditions on the EPS production

Many investigations on the production of exopolysaccharides by lactic acid bacteria used in the manufacture of fermented milk products have been carried out with milk. However, it was observed that the amount and composition of exopolysaccharides produced can be affected by varying the medium composition, the growth conditions and/or interactions between different bacterial strains involved in starter cultures [16]. Production of EPS has been investigated in milk ultrafiltrate [19; 20], whey permeates [43; 44] and complex synthetic media like MRS [42; 43; 88], Elliker's broth [27; 34; 43] and M17 [27; 107; 148], but it was often observed that such complex media strongly interfere with the isolation procedure of exopolysaccharides. Therefore, chemically defined media have been used for growth and EPS production by lactic acid bacteria [21; 53; 76; 92]. Compared to milk, whey and complex synthetic media, defined media are very useful in the investigation of the influence of individual medium components on the growth and EPS production.

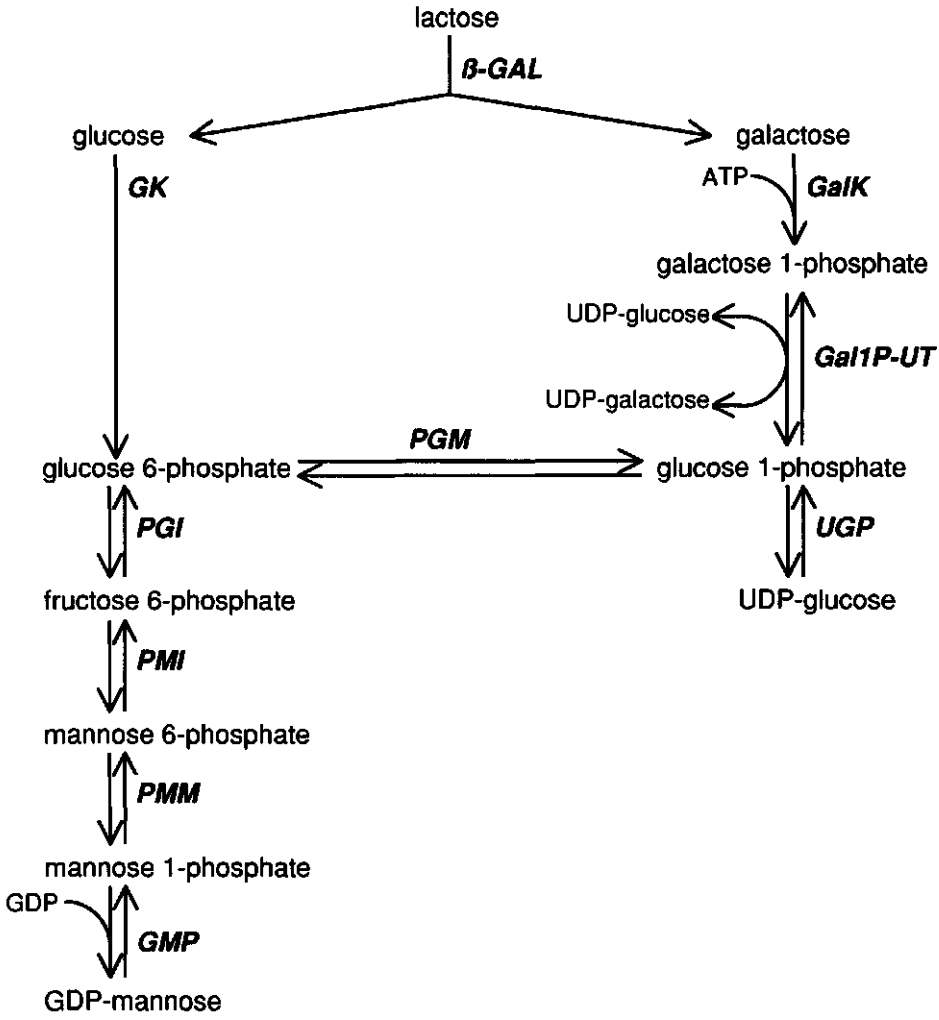

Fig. 1.4: Pathways for production of neutral sugar nucleotides as precursors for exopolysaccharide synthesis by *Streptococcus thermophilus* grown on lactose (from: Petit et al., 1991 [113]). Abbreviations of the enzymes involved: *β-Gal*, β-galactosidase; *GK*, glucokinase; *GalK*, galactose kinase; *Gal1P-UT*, galactose 1-phosphate uridyl transferase; *PGM*, phosphoglucose mutase; *PGI*, phosphoglucose isomerase; *PMI*, phosphomannose isomerase; *PMM*, phosphomannose mutase; *PGM*, phosphoglucose mutase; *GMP*, GDP-mannose pyrophosphorylase; *UGP*, UDP-glucose pyrophosphorylase [36; 56; 150].

Lb. delbrueckii subsp. *bulgaricus* strain CNRZ 416 produced 60 mg l^{-1} EPS in non-fat dry milk. When 1% casein was added, growth was not affected, but the production of the polymer was significantly higher (285 mg l^{-1}). When grown on MRS broth or other synthetic media, strain CNRZ 416 did not produce EPS at all. On the other hand, the addition of casein did not stimulate the EPS production by *S. thermophilus* strains. Exopolysaccharide production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 was stimulated by the addition of hydrolyzed casein or sodium

formate, but only when the strain was grown in skim milk [42]. When grown in MRS broth, hydrolyzed casein and sodium formate had no influence on the polymer production. Schellhaass [127] stated that neither growth nor EPS production was affected by casein or whey proteins in mesophilic and thermophilic lactic acid bacteria. *Lb. casei* CG11 was shown to grow in a chemically defined medium supplemented with a fermentable sugar and the amount of EPS produced was dependent on the carbohydrate source [21; 76]. When grown on glucose, a higher amount of EPS was produced than when grown on lactose, galactose or sucrose. On the other hand, the production of EPS by *Lb. delbrueckii* subsp. *bulgaricus* CRL 420 was shown to be independent of the carbon source [89]. *Lb. casei* CRL 87 grown in a synthetic APT medium produced the highest amount of exopolysaccharide (56 mg l^{-1}) with galactose as the carbon source and the lowest amount when grown with lactose (31 mg l^{-1}). When Mn^{2+} or Ca^{2+} ions were added to the growth medium containing galactose, this strain produced significantly higher amounts of EPS (118 and 83 mg l^{-1}, respectively). An addition of both Mn^{2+} and Ca^{2+} resulted in a further enhancement of the exopolysaccharide production to 124 mg l^{-1} [100]. On the other hand, when grown on glucose as the carbohydrate source, Mn^{2+} stimulated growth but not the EPS production related to cell growth, but the addition of citrate caused a significant enhancement of the specific EPS production [99]. In a *S. (Lactococcus) lactis* strain, a linear relationship was observed between the amount of calcium ions added and the ropiness of the cultures. It was stated that calcium ions in milk increases the viscosity due to formation of colloidal casein precipitate which may stimulate ropiness [118].

EPS production has often been shown to be dependent on the growth temperature. In most cases, the yield of EPS was higher at lower incubation temperatures. Sutherland [142] postulated that when bacteria are growing slowly, cell wall biosynthesis will also be slow. This makes more isoprenoid carrier available for extracellular polymer synthesis. A similar phenomenon was found for *Lb. delbrueckii* subsp. *bulgaricus* CRL 870 and RR [98; 127], *Lb. casei* CG11 [76], *S. thermophilus* CRL 807 and yp [127], and *S. cremoris* (*Lactococcus lactis* subsp. *cremoris*) 351 [127]. Cerning et al. [20] found that EPS production from mesophilic lactic acid bacteria is almost 50% higher when grown at 25 °C instead of 30 °C. On the other hand, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 produced more polymer at higher incubation temperatures [42]. *S. thermophilus* strain S22 produced EPS in the stationary phase of growth. A reciprocal ratio was found between EPS production and growth of the strain: temperatures and carbohydrate sources which were unfavorable for growth increased the polymer synthesis [41].

It was found that in *Lb. casei* EPS production under controlled pH was significantly higher than in acidifying batch cultures [102]. Higher exopolysaccharide production obtained by maintaining the pH of the culture medium at a constant value was also reported for other bacteria, such as *Xanthomonas campestris* [95] and *Pseudomonas* sp. EPS-5028 [90]. Schellhaass [127] found higher growth and slime production by several lactic acid bacteria when the initial pH of the medium was adjusted to 6.0; at lower initial pH levels, growth and slime formation were much lower. However, it was unknown if the specific EPS production was influenced by the initial pH in these experiments. Recently, it was observed that *Lb. delbrueckii* subsp. *bulgaricus* strain RR produced high amounts of EPS when grown in a continuous fermenter on partially deproteinized sweet whey supplemented with lactose, casamino acids and ammonium chloride. The highest growth yield and EPS production were observed at pH 6.5 (up to 2.13 g EPS per liter), at lower pH levels, both growth yield and EPS production decreased [45]. Exopolysaccharide production in strain RR was higher when the culture pH was periodically adjusted to 6.2 during the fermentation than when there was no pH control [44].

6.2 Genetics of EPS production in lactic acid bacteria

Several investigators found that the EPS producing ability was unstable in mesophilic and thermophilic lactic acid bacteria, specially at higher incubation temperatures or when frequently subcultured [87]. In mesophilic bacteria, it was found that ropy (mucoid) phenotype was related to the presence of plasmids and the instability has been attributed to the loss of these plasmids [155; 157]. In *Lc. lactis* strains the ropy phenotype was linked to a single plasmid [107; 155; 165]. In *Lb. casei*, a correlation between the mucoid phenotype and the presence of a 4.5 MDa (about 7 kb) plasmid was suggested [157]. A 30 kb plasmid in *Lb. casei* CG11 contained genes which are involved in the EPS biosynthesis or its regulation: when this plasmid was absent, the strain showed no mucoid character [76]. On the other hand, in the slime-producing *Lb. casei* CRL 87 no plasmids could be detected and the production of EPS in this strain was suggested to be stable [101]. It appeared that *Lb. delbrueckii* subsp. *bulgaricus* strain 2ol was plasmid-free, so that it was suggested that the mucoid character was located on the chromosome [157]. It was generally stated that the EPS producing ability of thermophilic lactic acid bacteria is chromosomally located [14; 15]. A decrease in the amount of EPS produced is also often explained as a result of EPS hydrolysis by glucohydrolase activities [18; 87], but until now this has not been clearly demonstrated.

Recently, a 40 kb plasmid pNZ4000 encoding exopolysaccharide production was isolated from *Lc. lactis* subsp. *cremoris* NIZO-B40 and characterized [77; 78]. On the plasmid, a 12 kb gene cluster containing 14 genes was found which was essential for the EPS biosynthesis. A hypothesis was made that the genes code for EPS biosynthetic enzymes such as glycosyltransferases and enzymes involved in the polymerization, export and regulation. The *epsD* gene was clearly demonstrated to be involved in the first step of the biosynthetic pathway: the glycosyltransferase activity resulting in the linkage of glucose from UDP-glucose to a membrane-linked lipid carrier at the release of UMP. A model was suggested for the biosynthetic pathway of the repeating unit of the NIZO-B40 EPS and the genes involved in the different steps. Stingele et al. [139] reported the identification and characterization of the *eps* gene cluster of *S. thermophilus* Sfi6 required for the synthesis of EPS. This strain was shown to have a stable ropy phenotype. A stable non-ropy mutant in which genes involved in EPS biosynthesis had been inactivated was made using Tn*916* transposon mutagenesis. Sequence analysis of gene regions of a genomic library of strain Sfi6 revealed a 15.25-kb region containing 15 open reading frames, the first and the last were predicted not to be involved in EPS synthesis. In this region, a 14.52-kb fragment was found encoding for 13 genes which were thought to be responsible for the biosynthesis, regulation, polymerization and excretion of EPS. The sequences of the genes were analyzed and on the basis of homologies with related genes of other micro-organisms, the biochemical role of 10 genes was identified. The gene cluster was cloned into a lactococcal vector to form the recombinant plasmid pFS101. The non-EPS-producing strain *Lc. lactis* MG1363 was transformed with this plasmid and the transformant appeared to produce EPS: this proved that the *eps* gene cluster was responsible for EPS production. Unfortunately, it is still unknown whether the exopolysaccharide produced by this transformed strain is identical to the EPS produced by the parental *S. thermophilus* strain. Four genes on the pNZ4000 plasmid that encoded for enzymes involved in EPS synthesis by *Lc. lactis* NIZO-B40 appeared to have considerable similarity with genes on the chromosomal *eps* gene cluster of *S. thermophilus* Sfi6. The organization, transcriptional direction and presumed functions of the genes of the different gene clusters are highly conserved [158].

A 4074-bp fragment of chromosomal DNA of another *S. thermophilus* strain (NCFB 2393) containing genes for EPS production was identified and cloned. Five open reading frames were found and showed homology with a *cps* cluster involved in EPS synthesis in *S. pneumoniae* [47]. The functions of the *cpsA*, *cpsC*, *cpsD* and *cpsE* gene were probably identical to those of the *epsA*, *epsC*, *epsD* and *epsE* genes found in the *eps* gene cluster of *S. thermophilus* Sfi6 [139]. The *cpsE*

gene is likely to encode for the phosphate-prenyl glycosyl 1-phosphate transferase, catalyzing the first step in polysaccharide biosynthesis.

7. Production of exopolysaccharides by *Lactobacillus delbrueckii* subsp. *bulgaricus*.

In the manufacture of yogurt, the utilization of exopolysaccharide producing lactic acid bacteria, in order to increase the viscosity and texture of the product and to prevent syneresis, have gained much attention in recent years. There is not only an increasing demand for 100% natural food products without additions, but also is the addition of stabilizers and other texture-promoting agents to natural yogurts not permitted in France and The Netherlands, so that slime-producing strains of the thermophilic lactic acid bacteria involved in the manufacture of yogurt, *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, have gained much attention in these countries in particular and they have been studied extensively [11; 17; 18; 19; 54]. In this thesis, the influence of the medium composition on the production of exopolysaccharides by a *Lactobacillus delbrueckii* subsp. *bulgaricus* strain grown in a chemically defined medium is described, and therefore some characteristics of this bacterium, including taxonomy, growth and exopolysaccharide production are discussed.

7.1 Taxonomy of *Lactobacillus delbrueckii* subsp. *bulgaricus*

The genus *Lactobacillus* was proposed by Beijerinck [7], based on the morphological and physiological properties. Lactobacilli are Gram-positive, non-sporeforming rods, chemo-organotrophic and they grow only in rich media containing amino acids, peptides, vitamins, nucleic acid derivatives, mineral salts, fatty acids or fatty acid esters and fermentable carbohydrates as an energy source [70; 129]. They are obligatory fermentative and most strains are aerotolerant, although they grow best under anaerobic or microaerophilic conditions. When oxygen is present in high concentrations, lactic acid bacteria can produce large amounts of hydrogen peroxide, which is toxic for the organisms since they often do not have catalase activity [170]. The optimal initial pH is slightly acidic, between 4.5 and 6.4. An increased level of carbon dioxide may stimulate growth of some species.

Lactobacillus delbrueckii subsp. *bulgaricus* was isolated from yogurt and cheese and described by Orla-Jensen [111] as *Thermobacterium bulgaricum* because of the ability to grow at 40 °C and named *Lactobacillus bulgaricus* by Rogosa and Hansen [122]. Weiss *et al.* [160] renamed the strain, together with *Lb. delbrueckii*, *Lb. lactis* and *Lb. leichmanii* as members of one genospecies, *Lactobacillus delbrueckii*, described by Leichmann [80], because of the high DNA-DNA homologies of 90 - 100% among each other and the great number of basic characteristics. *Lb. delbrueckii* subsp. *bulgaricus* is a thermophilic, Gram-positive, rod-shaped (length rods 2 - 9 µm) aerotolerant bacterium. The G+C content of the DNA was close to 50 mol %. The optimum growth temperature ranges from 43 to 46 °C [117].

7.2 Carbohydrate metabolism of *Lb. delbrueckii* subsp. *bulgaricus*

In contrast to many other lactic acid bacteria, which can generally grow on many carbohydrates, like hexoses, disaccharides or pentoses [6; 70], *Lb. delbrueckii* subsp. *bulgaricus* is only able to ferment glucose, lactose and fructose and some strains can also ferment mannose [122]. Galactose is normally not a fermentable substrate for the organism. *Lb. delbrueckii* subsp. *bulgaricus* shows a homofermentative metabolism of carbohydrates: the only fermentation product is D-lactate.

Therefore, *Lb. delbrueckii* subsp. *bulgaricus* is classified in the Group I obligatory homofermentative lactobacilli [69; 70].

Glucose is transported into the cell via a phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS), resulting in the formation of intracellular glucose 6-phosphate, whereas the uptake of lactose occurs via a permease [58]. Once inside the cell, lactose is cleaved into glucose and galactose by β-galactosidase. Glucose is metabolized via the Embden-Meyerhof-Parnas (EMP)-pathway, and galactose is excreted, probably via a lactose-galactose antiport system [61; 170]. Fructose is taken up and phosphorylated by a PEP: fructose PTS. Theoretically, per mole carbohydrate fermented, 2 moles of D-lactate and 2 moles of ATP are generated. In practice, a lower value is commonly obtained, and sugar carbon is also involved in the formation of for instance biomass [6] or other products like exopolysaccharides.

7.3 Exopolysaccharide production by *Lb. delbrueckii* subsp. *bulgaricus*

Several strains of *Lb. delbrueckii* subsp. *bulgaricus* have been described which are able to produce exopolysaccharides. Gruter et al. [54] analyzed the structure of the EPS produced by strain rr and found that the polymer was composed of a branched heptameric repeating unit containing glucose, galactose and rhamnose in the ratio 1:5:1 (Fig. 1.5).

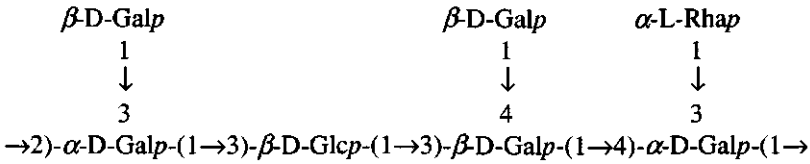

Fig. 1.5: Repeating unit of the exopolysaccharide, produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* rr (Gruter et al., 1993[54]).

Like *S. thermophilus*, most exopolysaccharides produced by *Lb. delbrueckii* subsp. *bulgaricus* strains are composed of galactose and glucose in varying amounts and sometimes also containing rhamnose or mannose (Table 1.2). Groux [52] found that the EPS produced by a *Lb. delbrueckii* subsp. *bulgaricus* strain was composed of galactose, glucose, arabinose and mannose, but it was found that the ratio of these sugar monomers was dependent on the isolation procedure, so the obtained results give qualitative rather than quantitative information. The only polymer having a sugar composition completely different from the others was produced by strain CRL 420 and appeared to contain glucose and fructose in a molar ratio of 1:2 [89]. The EPS produced by strain CRL 420 had a molecular weight of 2.0×10^5, which was less than half of the molecular weight of the EPS produced by strain CNRZ 416a (Table 1.2). It was found that *Lb. delbrueckii* subsp. *bulgaricus* strain CNRZ 737 produced the highest amount of EPS, when grown in fat free milk or milk ultrafiltrate, to which 1% casein was added [19]. Garcia-Garibay and Marshall [42] found that strain NCFB 2772 grown in skim milk produced 126 mg l^{-1} of an extracellular polymer which was composed of carbohydrates and protein in the ratio 1.2:1.

Table 1.2: Production, composition and molecular weights of exopolysaccharides, produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* strains.

strain	amount mg l^{-1}	molecular weight	repeating unit	ratio sugar composition[a] Glc	Gal	others	references
-	-	-	-	1	3.9	1.2 Ara, 1.4 Man	52
CRL 870	121	-	-	-	-	-	98
CRL 420	-	2.0×10^5	-	1		2 Frc	89
CNRZ 416a	285	4.9×10^5		1	4	1 Rha	17; 19
CNRZ 737	424	-	-	1	4	1 Rha	17; 19
CNRZ 1187	-	-	-	9	14	1 Man	11
rr	-	-	heptamer	1	5	1 Rha	54
RR	-	-		1	2		127
RR	2130	-	-	-	-	-	45
NCFB 2772	126	-	-	-	-	-	42

[a] Abbreviations: Glc, glucose; Gal, galactose; Ara, arabinose; Man, mannose; Frc, fructose; Rha, rhamnose.

8 Outline of this thesis

The production of EPS by several *Lb. delbrueckii* subsp. *bulgaricus* strains had been investigated by several researchers, but these investigations were carried out with milk, whey permeate or MRS broth as the growth media. Therefore, knowledge on the influence of medium components on the production of EPS is limited when such media are used instead of chemically defined media. In this thesis, variations in the medium composition and physiological factors were shown to significantly affect the growth and EPS production of *Lb. delbrueckii* subsp. *bulgaricus* strain NCFB 2772.

In **Chapter 2**, a chemically defined medium was described in which *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 was able to grow and to produce EPS. It was shown that growth and EPS production were strongly correlated and that no EPS was produced when cell growth had ceased.

Chapter 3 describes the influence of variations in concentration of several nutrients on the growth and production of exopolysaccharides by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 in both batch and continuous cultures.

In **Chapter 4**, the effect of omissions of medium components on the EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 is described. As a result of a multiple vitamin omission from the growth medium, the strain produced considerably more EPS.

Chapter 5 shows the effect of the carbohydrate source on the production of EPS and the activities of enzymes leading to the biosynthesis of sugar nucleotides. When grown on fructose, the amount of EPS produced was much lower than when grown on glucose. No activities of enzymes leading to the synthesis of rhamnose nucleotide could be detected in fructose-grown cells.

In **Chapter 6**, the exopolysaccharides produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture on fructose or glucose were analyzed. It was found that glucose-grown cultured produced EPS which was composed of 2 fractions in almost equal amounts with different molecular weights. When grown on glucose, mainly a low molecular weight fraction was produced.

2.

Production of extracellular polysaccharides by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in a chemically defined medium

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Abstract

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 2772 produced an extracellular polysaccharide when grown in a chemically defined medium with glucose or lactose as the substrate carbohydrate. The isolated extracellular polysaccharide had a sugar composition of glucose, galactose and rhamnose in a ratio of 1 : 6.8 : 0.7. The production of extracellular polysaccharides increased at higher temperatures, but the bacterium rapidly lost its polysaccharide producing ability at 47°C. Production of polysaccharides was growth-related: no polysaccharide production was found after growth had ceased. An excess carbohydrate did not result in increased polysaccharide production.

Introduction

Extracellular polysaccharides (EPS) produced and excreted by lactic acid bacteria give a thicker texture and higher viscosity of fermented milk products like yogurt. Slime producing strains are used extensively in France and The Netherlands, because the addition of stabilizers of plant or animal origin to unfruited yogurts is prohibited in these countries [17; 127]. Furthermore, there is an increased popularity for "100% natural" food products without additions [128].

Lb. delbrueckii subsp. *bulgaricus* NCFB 2772 produces a polymer when grown in skim milk. The production of polymer increased at higher temperatures or when sodium formate or hydrolyzed casein were added [42]. However, conflicting results have been reported for different strains grown in various media. When grown in skim milk, sodium formate and hydrolyzed casein had a stimulating effect on the polymer production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772, but not when grown on MRS broth [42]. Schellhaass [127] found a higher slime production in *Lb. delbrueckii* subsp. *bulgaricus* at lower incubation temperatures. There was also a variation in the sugar composition of the EPS of different *Lb. delbrueckii* subsp. *bulgaricus* strains. Galactose was the major component of the EPS in most strains, with glucose, rhamnose, mannose and pentoses as other components [14; 15; 42]. Manca de Nadra *et al.* [89] isolated an EPS from

Lb. delbrueckii subsp. *bulgaricus* CRL 420 with a sugar composition of glucose and fructose in a ratio of 1:2.

To investigate the physiological background of EPS production and the effect of the medium composition, a chemically defined medium is required. However, the knowledge on the effect of culture conditions on both the production and the sugar composition of EPS produced by lactic acid bacteria grown in defined media is almost completely lacking. It was found that EPS was produced by *Lb. casei* CG11, isolated from cheese, when grown in a chemically defined medium [76]. *Lb. delbrueckii* subsp. *bulgaricus* CRL 420 produced EPS when grown in a semi-synthetic medium [89].

In the present work, we have investigated the effects of growth parameters on the production and on the sugar composition of EPS from *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772. These experiments were performed in a chemically defined medium.

Materials and methods

Bacterial strain and culture medium

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 2772 was obtained from the National Collection of Food Bacteria (Reading, UK). Cultures were grown in a MRS medium [88] and stored at -80°C until required. Further cultivations were performed in a chemically defined medium which contained per liter distilled water: 1.0 g KH_2PO_4, 2.5 g $Na_2HPO_4.2H_2O$, 0.9 g citric acid.H_2O, 6.75 g sodium acetate, 1.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 0.05 g $MnCl_2.4H_2O$, 1 ml Tween-80, 10 mg adenine, 10 mg guanine, 10 mg uracil, 10 mg xanthine, 0.3 g L-cysteine, 0.1 g of the following (L-)amino acids: alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine hydrochloride, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine, 1 ml trace element solution, 1 ml vitamin solution and 25 g glucose monohydrate unless otherwise indicated. The trace element solution contained per liter distilled water: 10 ml 25% HCl, 1.5 g $FeCl_2.4H_2O$, 190 mg $CoCl_2.6H_2O$, 70 mg $ZnCl_2$, 6 mg H_3BO_3, 36 mg $Na_2MoO_4.2H_2O$, 24 mg $NiCl_2$, 2 mg $CuCl_2.2H_2O$. The vitamin solution contained per liter distilled water: 100 mg 4-aminobenzoate, 50 mg folic acid, 50 mg lipoic acid, 100 mg riboflavine, 20 mg biotin, 100 mg Ca-pantothenate, 200 mg nicotinic acid, 500 mg pyridoxamine, 50 mg pyridoxine, 200 mg thiamine, 100 mg vitamin B_{12}. The medium was sterilized by passing through a 0.2 mm sterile filter (Schleicher & Schuell or Gelman Sciences).

Growth experiments

Batch cultivations were carried out in static, N_2-flushed sealed bottles with 50 ml medium. After 48 h at 37°C, growth was monitored by optical density measurement at 600 nm or by cell dry weight measurement, using a standard curve of OD at 600 nm against cell dry weight. The turbidity values at 600 nm were a consequence of the number of cells with some contribution (approx. 5 per cent) from the EPS production. Continuous culture and pH-controlled batch culture experiments were performed using a glass fermenter (Applikon, the Netherlands) with a working volume of 1 l at 40°C, controlled pH of 6.0, 50 rpm and anaerobically under a N_2 atmosphere, unless otherwise stated.

Isolation of extracellular polysaccharides
The EPS was isolated according to the method of Garcia-Garibay and Marshall [42]. After the second precipitation step, the EPS was centrifuged (30 min, 27,000 g, 4°C) and resuspended in distilled water adjusted to pH 4.0. The EPS solution was dialyzed against 5 l distilled water (24 h, 4°C) and lyophilized.

Analyses
Total carbohydrate was determined using the method of Dubois *et al.* [32]. Polysaccharides were hydrolyzed in 1 N trifluoroacetic acid for 1 h at 120°C. Samples were dried under a stream of N_2 gas at 70°C and resuspended in distilled water. Sugars, lactic acid and EPS hydrolysates were analyzed by HPLC using an Interaction ION-300 organic acids column (LC-Service, Emmen, the Netherlands) at 70°C, 0.4 ml min^{-1} and with 5 mM H_2SO_4 as eluent, and detected by a refractive index detector.

Results

Growth and EPS production on different substrates
Lb. delbrueckii subsp. *bulgaricus* grew well and produced EPS in acidifying batch cultures with defined medium supplemented with glucose or lactose as the carbohydrate substrate. When grown on lactose, it was found that galactose accumulated in the growth medium. The organism grew less well when either fructose or mannose were used and EPS production was substantially lower. In all cases, *Lb. delbrueckii* subsp. *bulgaricus* showed a homofermentative metabolism (Table 2.1). No growth was found either in defined medium or in MRS broth medium supplemented with galactose, maltose, rhamnose or sucrose. Stimulating factors like sodium formate and hydrolyzed casein [42] did not affect the amount of EPS produced in defined medium. The optimal pH for both growth and EPS production was near 6.0. The pH had no effect on the EPS production in both batch and continuous cultures.

Table 2.1: Growth and production of EPS by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 in defined medium on different substrates. The organism was grown in static acidifying batch cultures containing 20 g l^{-1} substrate at 37°C. Growth, EPS and metabolic end products were measured after 48 h of incubation.

Substrate	Biomass dry wt (g l^{-1})	Substrate remaining (mM)	Products: galactose (mM)	lactate (mM)	EPS (mg l^{-1})	Carbon recovery (%)
Glucose (111 mM)	1.15	57.2	0	85.5	36.8	96
Lactose (56 mM)	1.20	13.6	46.1	85.5	32.3	111
Fructose (111 mM)	0.65	71.6	0	56.6	8.0	93
Mannose (111 mM)	0.35	99.9	0	23.3	5.4	102

Effect of temperature on the growth and EPS production

Lb. delbrueckii subsp. *bulgaricus* was grown in a continuous culture with defined medium supplemented with 167 mM glucose at pH 6.0 and dilution rate 0.075 h^{-1}. Samples were taken after at least 5 volume changes and growth was monitored by measuring the OD at 600 nm . Cell dry weight was calculated from the OD using a standard curve. An OD reading of 1.0 was equivalent to 0.75 mg ml^{-1} dry weight of cells. The specific EPS production was calculated by dividing the amount of EPS produced by the cell dry weight measurement (Fig. 2.1). The specific EPS production increased with increasing temperature. However, it was found that above 45°C, *Lb. delbrueckii* subsp. *bulgaricus* quickly lost its EPS-producing ability. For further experiments, 40°C was chosen as the incubation temperature.

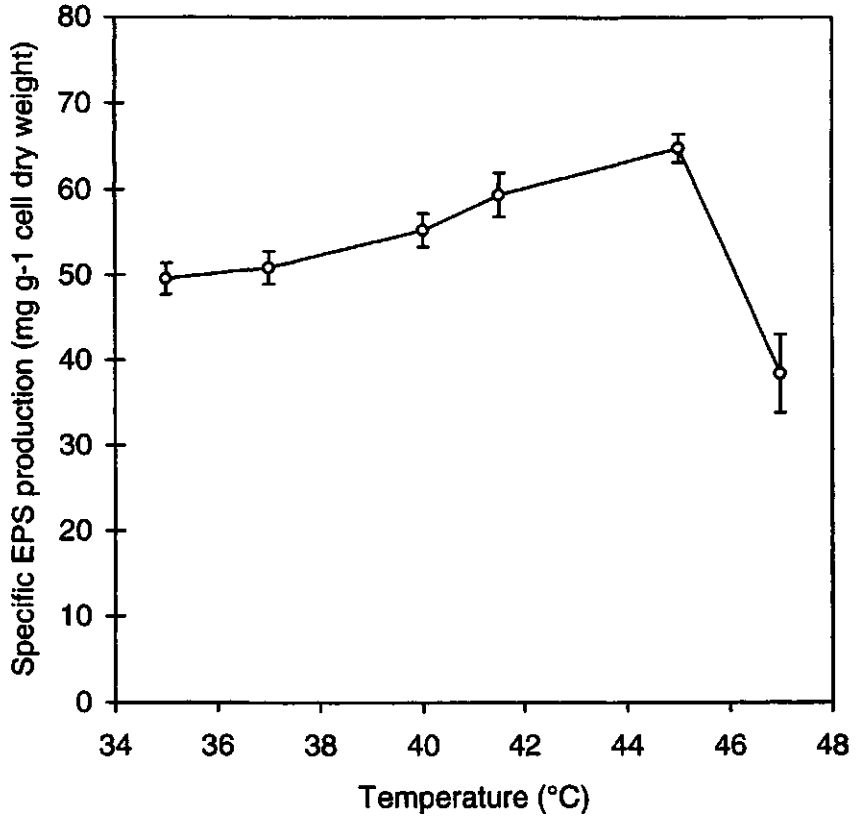

Fig. 2.1: Effect of the incubation temperature on the specific EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus*. The organism was grown in continuous culture in defined medium supplemented with 167 mM glucose at pH 6.0 and at a dilution rate of 0.075 h^{-1}. Each value represents the average of duplicate measurements

Growth curve and initial glucose concentration

Lb. delbrueckii subsp. *bulgaricus* was grown in a pH-controlled batch culture at 40°C, pH 6.0 and with 200 mM glucose. Fig. 2.2 shows that growth, glucose utilization, lactate fermentation and EPS production occurred simultaneously for 20 h. After this period, growth stopped, lysis of the culture was observed and EPS was not further produced. However, the remaining glucose was homofermentatively converted to lactate.

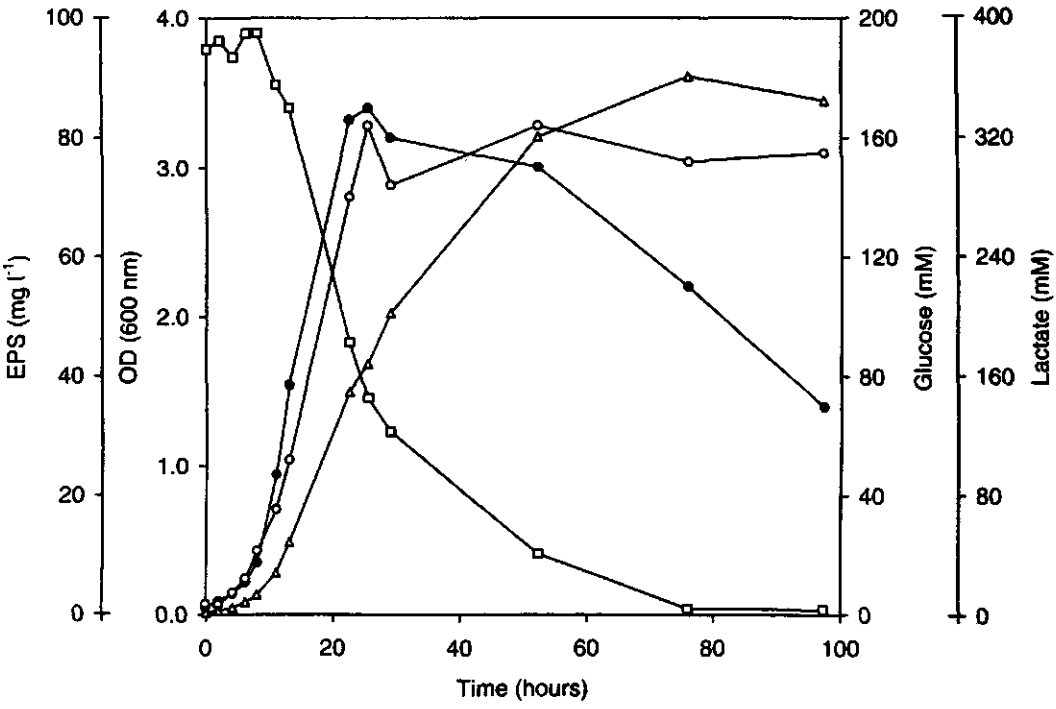

Fig. 2.2: Growth curve and EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus*. Cells were grown in a pH-controlled batch culture in defined medium and with initially 200 mM glucose at 40°C and pH 6.0. Each value represents the average of duplicate measurements. ●: OD (600 nm), ○: EPS, Δ: lactate, □: glucose

The effect of the initial glucose concentration on the growth and EPS formation is shown in Fig. 2.3. *Lb. delbrueckii* subsp. *bulgaricus* was grown in batch culture at 40°C and pH 6.0. Growth, glucose and lactate concentrations and EPS production were measured after 48 h of incubation. It was found that glucose is the limiting growth substrate up to 100 mM. Above 100 mM, no further increase in growth and EPS production took place, while glucose remained in the medium. The carbon recovery was between 88 and 104 per cent.

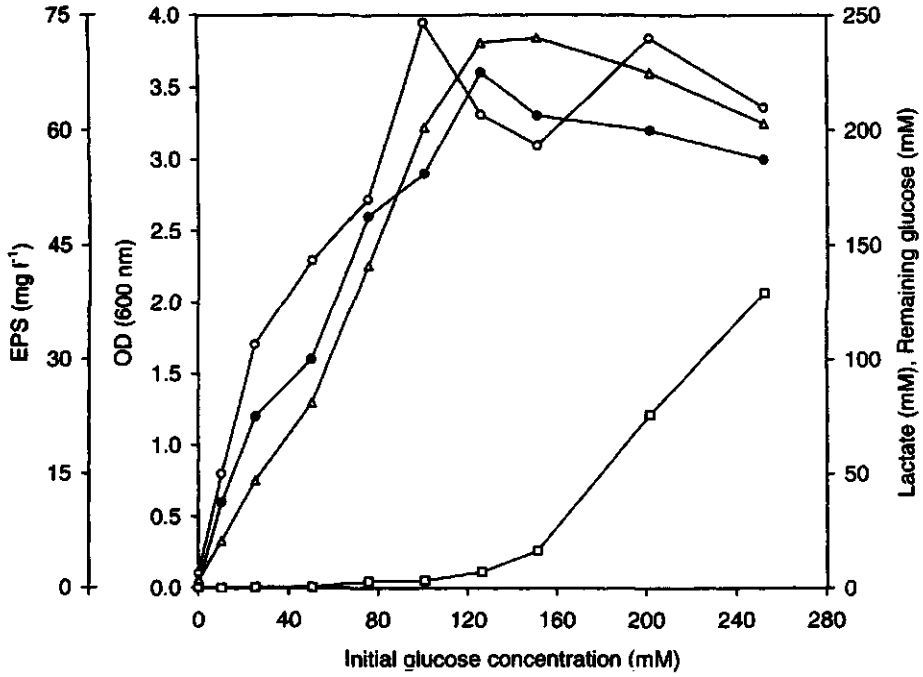

Fig. 2.3: Effect of initial glucose concentration on growth and EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus*. Cells were grown for 48 h in a pH-controlled batch culture with defined medium at pH 6.0 and 40°C. Each value represents an average of duplicate measurements. ●: OD (600 nm), ○: EPS, Δ: lactate, □: glucose

Sugar composition of the EPS

The EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* in the experiments mentioned above were hydrolyzed and its sugar composition was analyzed. Both glucose-grown and lactose-grown batch cultures gave an EPS composed of glucose, galactose and rhamnose in an average ratio of 1 : 6.8 : 0.7. At an early growth phase (OD below 0.4), the EPS had relatively more glucose compared to rhamnose. The sugar composition of the EPS was not influenced by both temperature and pH.

Discussion

When grown in skimmed milk, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 produced EPS. However, a more defined medium is required to investigate the physiological aspects and the effect of medium composition on the EPS production and its sugar composition. Extracellular polymer was found when the organism was grown in MRS broth, but the MRS broth strongly interfered with the isolation procedure of the EPS [42]. Therefore, in this study we used a chemically defined medium.

It was found that the EPS producing ability of thermophilic lactic acid bacteria like *Lb. delbrueckii* subsp. *bulgaricus* is lost due to subculturing or to high temperatures [14; 42]. In mesophilic lactic acid bacteria, the EPS producing ability is plasmid-linked [155], but it is still

unclear how this phenotype is regulated in thermophilic lactic acid bacteria. To prevent the effect of the loss of the EPS production, we used a new frozen stock culture for each experiment.

Lb. delbrueckii subsp. *bulgaricus* NCFB 2772 produced EPS in defined medium with either glucose or lactose. When grown in batch cultures on lactose, the amount of EPS was comparable with glucose-grown cultures. It was found that, when grown on lactose, glucose was used for growth and galactose accumulated in the growth medium. Zourari *et al.* [170] suggested that in *Lb. delbrueckii* subsp. *bulgaricus* lactose uptake takes place via a lactose permease. Inside the cell, lactose is cleaved by b-galactosidase to form glucose and galactose. The latter is exchanged with lactose via a lactose - galactose antiport system.

The EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 had a sugar composition which is comparable to the sugar composition found in the *Lb. delbrueckii* subsp. *bulgaricus* strains CNRZ 398 and rr [17; 54] grown in skim milk. The polymer of *Lb. delbrueckii* subsp. *bulgaricus* CRL 420 grown in milk had a monomer composition of glucose and fructose in a ratio of 1:2 and the composition was independent of the carbon source [89]. In our strain, residual galactose was apparently not used for the production of EPS because the EPS of glucose-grown and lactose-grown cultures had a similar monomer composition. When grown on a mixture of glucose and galactose, there was also no utilization of galactose for the production of EPS (data not shown). An investigation of the biosynthetic pathway of EPS and its regulation is required to understand this phenomenon.

The EPS production increased with increasing temperatures, which was also found when *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 was grown in skimmed milk [42]. This is in contrast to the results obtained by Schellhaass [127] who found higher productions of slime at lower incubation temperatures in *Lb. delbrueckii* subsp. *bulgaricus*, *S. cremoris* and *S. thermophilus* strains grown in whey protein concentrate solution. In *Lb. casei* it was found that the yield of EPS was lower at 30°C than at 25°C [76].

A direct relationship between growth, lactate fermentation and EPS production was found in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772. After growth had ceased, no further polymer production was found and the remaining carbohydrate appeared to be fermented to lactate. It was found in other EPS-producing lactic acid bacteria that an excess of carbohydrate, in combination with a nutrient limitation such as nitrogen or phosphorous, stimulates the production of EPS [14]. *Lb. casei* and *Lb. delbrueckii* subsp. *bulgaricus* CRL 420 continued producing EPS beyond the stationary growth phase [76; 89].

It is clear that further work will be needed to elucidate the effects of growth conditions and of different substrates on the amount and sugar composition of EPS. More understanding of this topic can most probably be achieved with studies on the activities and regulations of the enzymes involved in both metabolic and EPS production pathways. The defined medium described in this study should simplify these studies.

3.

Influence of ions on growth and production of exopolysaccharides by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in batch and continuous culture

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Submitted for publication.

Abstract

The influence of varying concentrations of ions on the growth and exopolysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in batch and continuous culture in a defined medium was investigated. At growth-limiting concentrations of either manganese or phosphate, the specific exopolysaccharide production decreased and the polymer contained a lower amount of rhamnose residues. Higher manganese or phosphate concentrations did not affect the amount of polymer produced. Low concentrations of phosphate in the growth medium slightly affected the sugar composition of the exopolysaccharide. A high citrate concentration decreased growth of the strain, but had no influence on the specific exopolysaccharide production.

Introduction

Several lactic acid bacteria produce exopolysaccharides (EPS), either attached to the cell wall or excreted into the environment as slime material. EPS play an important role in the improvement of the texture and stability of fermented milk products like yogurt, viili and kefir and the prevention of syneresis [14; 104]. EPS producing strains of yogurt starter cultures, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* have been investigated particularly in France and The Netherlands, since the addition of texture-promoting agents to natural yogurts is prohibited.

The amount and composition of the EPS produced by lactic acid bacteria is dependent on a number of parameters, like temperature, initial pH, carbon source or the availability of minerals, vitamins and other medium components. For the investigation of the nutritional requirements and the effect of medium components and other chemicals on EPS production, chemically defined media have been shown to be very useful. Furthermore, defined media are very important when

studying the qualitative and quantitative EPS production, since they do not interfere with the EPS isolation procedure, as was observed when complex synthetic media like MRS were used [42].

In previous work it was shown that *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 produces approximately four times more EPS when grown in a chemically defined medium supplemented with glucose or lactose, than when fructose was supplied as the carbohydrate source, although cell densities were comparable. It was found that the EPS produced on glucose were composed of glucose, galactose and rhamnose in the ratio 1:6.8:0.7, whereas the EPS produced on fructose contained mainly galactose and glucose residues [48; 49]. In a simplified defined medium, from which several vitamins and trace elements were omitted, the EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 significantly increased, although growth of the strain was reduced.

EPS may contribute in the uptake of nutrients and metal ions when these are present in very low concentrations [14] and it was also suggested that limiting amounts of nutrients in the growth medium in combination with a carbon excess may stimulate the formation of EPS [141]. On the other hand, some ions may be essential for the activities of enzymes involved in EPS production. However, most investigations on the role of ions on the production of EPS revealed that increasing ion concentrations stimulate the formation of EPS. Gamar et al. [40] stated Mg^{2+}, Mn^{2+} and Fe^{2+} to be essential for growth of the EPS-producing *Lb rhamnosus* strain C83. Mozzi et al. [99] found that growth and EPS production by *Lb casei* grown in APTgl broth was stimulated by the addition of $MnSO_4$ or triammonium citrate, but the addition of Mg^{2+} or K^+ only stimulated growth and not the specific production of EPS. In *Lactobacillus* sp. KPB-167B, the addition of 5 mM $CaCl_2$ to MRSL medium resulted in an enhancement of the polysaccharide kefiran [168]. A stimulating effect of increasing phosphate levels on the EPS production by *Propionibacterium acidi-propionici* was observed [116].

In this work, the influence of the omission or the addition in low concentrations of several ions on the growth and EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in batch and continuous culture on defined medium was investigated.

Materials and methods

Microorganism

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 2772 was obtained from The National Collection of Food Bacteria (Reading, UK). The strain was stored at -80 °C in MRS broth medium containing 15% glycerol and reactivated in MRS broth medium at 37 °C for 16 hours.

Growth experiments

Batch cultures were grown in static, nitrogen-flushed, sealed glass bottles (volume 115 ml) containing 50 ml defined medium which was composed as described before [48] at 37 °C and initial pH 6.0. 83.3 mM Glucose was used as the carbon and energy source, unless described otherwise. The medium was sterilized by passing through a 0.2 µm sterile filter. Growth was monitored by optical density (OD) measurement at 600 nm or by cell dry weight measurement, using a standard curve of the OD against cell dry weight [48]. Duplicate 10 ml culture samples were centrifuged (15 min, 20,000 *g*, 4 °C), the cells were washed two times with distilled water and dried at 105 °C for 16 hours. Sugars and lactic acid were determined by high-performance liquid chromatography (HPLC) as described before [48]. To evaluate the requirements for nutrients, the single omission technique was used, and growth experiments were performed as described before [51]. All tests were performed at least in duplicate.

Continuous culture experiments
Continuous culture experiments were performed in a glass fermenter (Applikon, Schiedam, The Netherlands) with a working volume of 600 ml. The pH of the cultures was maintained at 6.0 ± 0.1 by titration with NaOH, the growth temperature was 40 °C, agitation rate 100 rev min^{-1} and the dilution rate 0.075 h^{-1}. The cultures were kept under anaerobic conditions by flushing the fermenter with nitrogen gas. Glucose was added as the carbohydrate source at 83.3 mM, which appeared to be growth-limiting when all nutrients were added in the standard concentrations [48]. The effect of limiting concentrations of other nutrients was investigated by reducing the amount of these nutrients in the growth medium. The concentrations of these nutrients were regarded as growth-limiting when residual glucose was detected in the cultures. Steady state conditions were re-established after at least five volume changes.

Isolation and sugar composition of the EPS
Cultures were treated with 17 % (vol/vol) of a 80% (w/vol) trichloroacetic acid solution and centrifuged (20 min, 27000 g, 4 °C) [42]. The EPS were isolated by ethanol precipitation [48], the total carbohydrate content of the EPS was determined using the phenol/sulfuric acid procedure of Dubois et al. [32]. For the determination of the monomeric sugar composition, the EPS were hydrolyzed 1h at 121 °C in 1 M trifluoroacetic acid, dried under a stream of N_2 gas at 70 °C and resuspended in distilled water. The solution containing the hydrolysates were analyzed by HPLC measurement [48].

Results and discussion

Influence of manganese ions
The effect of decreased Mn^{2+} concentrations on growth and EPS production by *Lb delbrueckii* subsp. *bulgaricus* NCFB 2772 was studied in batch cultures and growth and EPS production were determined after 48 hours of incubation. Mn^{2+} was shown to be essential for growth of the strain. Table 3.1 shows that at very low Mn^{2+} concentrations, a decrease in the specific EPS production was observed. At Mn^{2+} concentrations of 1.5 µM and higher, the specific EPS production did not differ from the value found at standard conditions (252 µM $MnCl_2$), a further enhancement of the Mn^{2+} concentration (up to 500 µM) had no effect. Compared with the EPS produced under standard conditions, only the EPS produced at 2.5 µM Mn^{2+} contained a slightly lower amount of rhamnose monomer. The results were not in agreement with those obtained with *Lb casei* CRL 87, in which the addition of Mn^{2+}, alone or in combination with citrate, Ca^{2+} or SO_4^{2-}, strongly stimulated EPS production [99; 100]. *Rhizobium meliloti* JJ-1 produced four times more EPS in media containing 500 µM Mn^{2+} than in media without Mn^{2+} addition [2]. It was stated that manganese is important for the activity of several enzymes in the Embden-Meyerhof-Parnas pathway, like hexokinase, phosphofructokinase, 3-phosphoglycerate kinase, enolase and pyruvate kinase [115]. In our strain, however, Mn^{2+} apparently has less effect on the activity of enzymes leading to production of EPS.

Table 3.1: Effect of initial $MnCl_2$ concentration on growth and EPS production by *Lb delbrueckii* subsp. *bulgaricus* NCFB 2772. Cells were grown in unshaken, N_2-flushed sealed bottles with defined medium supplemented with 83 mM glucose at 37 °C and initial pH 6.0. OD and EPS measurements were performed after 48 hours of incubation. Each value represents the mean of duplicate measurements.

$MnCl_2$ (µM)	OD (600 nm)	EPS (mg l^{-1})	Specific EPS production[a] (mg EPS mg^{-1} cell dry weight)
0.5	0.1	ND	ND
1.5	0.5	5.8	15.5
2.5	1.2	24.2	26.9
5.0	1.3	25.0	25.6
10.0	1.2	28.2	31.3
30.0	1.5	30.5	27.1
40.0	1.4	31.0	29.5
90.0	1.7	34.8	27.3
252.0	1.9	42.3	29.7

[a] The specific EPS production was calculated using a standard curve of OD at 600 nm against cell dry weight [48].
ND: not determined.

Effect of phosphate ions

Lb delbrueckii subsp. *bulgaricus* NCFB 2772 was grown in continuous culture (40 °C, pH 6.0 ± 0.2, D 0.075 h^{-1}) and the influence of decreased concentrations of phosphate on the growth and EPS production was investigated. Phosphate ions were replaced by chloride ions, in order to balance Na^+ and K^+ concentrations. It was found that phosphate at concentrations lower than 1.0 mM became the growth-limiting substrate. The specific EPS production appeared to be slightly lower when phosphate was the growth-limiting substrate. With increasing phosphate concentrations, growth and specific EPS production were comparable with those at standard growth conditions (21.5 mM phosphate) of the strain. The EPS produced at phosphate limiting conditions had a lower amount of rhamnose, whereas the amount of galactose residues slightly increased at phosphate concentrations of 1 - 2 mM (Table 3.2). It was already found that growth of lactic acid bacteria was only possible in the presence of phosphate [86]. In *Propionibacterium acidi-propionici*, extracellular polysaccharide production increased in the presence of phosphate [116]. *Pseudomonas* sp. NCIB 11264 produced less EPS in media containing small amounts of phosphate, but this was mainly caused by the low buffering capacity of the growth medium. In a buffered medium, the amount of EPS was slightly affected by the phosphate concentration [163]. Until now, no effect of phosphate on the EPS production by lactic acid bacteria has been reported.

Table 3.2: Effect of initial phosphate concentration on growth and EPS production by *Lb delbrueckii* subsp. *bulgaricus* NCFB 2772. Cells were grown in continuous culture with defined medium supplemented with 83 mM glucose (40 °C, pH 6.0, D 0.075 h^{-1}). Each value represents the mean of duplicate measurements.

Phosphate (mM)	OD (600 nm)	EPS (mg l^{-1})	Specific EPS production[a] (mg EPS mg^{-1} cell dry weight)	EPS composition[b] Glc : Gal : Rha
0	0.04	ND	ND	1.0 : 7.0 : 0.3
0.2	0.5	10.3	30.5	1.0 : 6.3 : 0.3
0.5	0.7	18.2	34.7	1.0 : 5.9 : 0.3
1.5	2.1	98.8	62.7	1.0 : 7.8 : 0.4
2.0	2.3	117.6	68.2	1.0 : 9.0 : 0.5
5.0	2.6	115.4	59.2	1.0 : 6.5 : 0.6
10.0	2.3	103.7	60.1	1.0 : 5.9 : 0.6
21.5	2.5	110.8	59.1	1.0 : 6.0 : 0.7

[a] The specific EPS production was calculated using a standard curve of OD at 600 nm against cell dry weight [48].
[b] The amount of galactose and rhamnose is related to the amount of glucose. Glc, glucose; Gal, galactose; Rha, rhamnose.
ND: not determined.

Other nutrients

It was found that the omission of Fe^{2+}, Zn^{2+} or NH_4^+ ions had no effect on the growth of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772, and the EPS production and EPS sugar composition were not affected by variations in the concentration of these components. Conflicting results have been reported for the iron requirements of lactic acid bacteria. Pandey et al. [112] investigated 23 strains of lactic acid bacteria, including several lactobacilli, and stated that none of these strains needed iron for growth. Also, *Lb. plantarum* grew well in the absence of iron [3]. On the other hand, the *Lactobacillus* strains investigated by Ledesma et al. [79] all required Fe^{2+}.

Furthermore, we showed that an increased citrate concentration inhibited growth of *Lb delbrueckii* subsp. *bulgaricus* NCFB 2772 in batch culture. When 100 mM citrate was present, the final OD of the culture was only 0.9. Neither the specific EPS production nor the sugar composition of the EPS were affected by a high citrate concentration. In previous work, it was found that a complete omission of citrate from the growth medium resulted in a slightly lower OD and EPS production [51]. The inhibitory effect of high concentrations of citrate on the growth of lactic acid bacteria was explained by the fact that citrate ions form complexes with bivalent metallic ions like Mg^{2+} and Mn^{2+}, and these complexes have shown not to be available for growth. The addition of higher concentrations of Mg^{2+} and Mn^{2+} can overcome the toxic effects of citrate [86]. In contrast to *Lb casei*, no stimulatory effect of citrate addition on the EPS production was observed [99].

Conclusion

The addition of nutrients like phosphate or manganese in growth-limiting amounts had only influence on the production of EPS at very low concentrations. At these concentrations, the specific EPS production decreased and a lower amount of rhamnose in the EPS was found. In previous work it was found that at low optical densities, for instance in the early logarithmic growth phase, the EPS contained a lower amount of rhamnose related to the amount of glucose monomers [48]. Recently, we found that *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 produces two types of EPS concurrently, which differ in molecular weight (mol. wt.) and monomeric sugar composition [50]. The EPS fraction with a low mol. wt. (4×10^4) contained less rhamnose residues than the high-mol. wt. fraction (1.7×10^6) and the production of this fraction was independent of the growth substrate. It seems that at low cell densities, the production of this low-mol. wt. fraction is preferred to the production of the high-mol. wt. fraction and limiting amounts of ions only have an indirect influence on the EPS production by this strain. Further research on the EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown at low optical densities will be needed to clarify these findings.

4.

Enhancement of exopolysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 with a simplified defined medium

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Abstract

The aim of this work was to investigate the medium requirements for growth and production of exopolysaccharides by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772. The strain was grown in batch cultures on a chemically defined medium and the technique of single omission of medium components was applied in order to determine the nutritional requirements. The omission of aspartic acid, glutamic acid and glycine affected growth only slightly, and the omission of glutamine, asparagine or threonine resulted in a stronger reduction of the growth. All other amino acids were essential. Multiple omissions of amino acids caused an almost complete loss of growth. *Lb. delbrueckii* subsp. *bulgaricus* only required riboflavin, Ca-pantothenate and nicotinic acid as individual vitamins. Very surprisingly, when only these vitamins were present in the medium and other vitamins were not, less growth was observed compared to the complete medium, but the amount of exopolysaccharides produced was significantly higher. These observations were studied in more detail in a simplified defined medium in which *Lb. delbrueckii* subsp. *bulgaricus* was able to grow and produce exopolysaccharides. Although the final optical density in the simplified medium was lower, the production of exopolysaccharides was about two-fold higher than in the complete medium.

Introduction

The thermophilic lactic acid bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are important in the dairy industry, since they are applied in the fermentation of milk to form yogurt. Several strains have been isolated and analyzed which were capable of formation of exopolysaccharides (EPS), that give a higher viscosity and a thicker texture to the product [14; 17; 18; 42; 89; 127; 159]. Since the addition of stabilizers of animal or plant origin to natural yogurts is prohibited in France and The Netherlands, and since there is a

growing popularity for food products without additions, the utilization of EPS producing lactic acid bacteria has gained more popularity.

For optimal growth, lactic acid bacteria require very complex media like milk, whey ultrafiltrate or complex synthetic media such as MRS broth [88] and M17 broth [148]. However, for the investigation of the physiological background of EPS production in lactic acid bacteria, a chemically defined medium is required and the exact nutritional requirements of the lactic acid bacteria remain unknown when complex media are used. A chemically defined medium containing a carbohydrate source, mineral salts, amino acids, vitamins and nucleic acid bases therefore is more suitable to investigate the influence of nutrients on the growth, the metabolic pathways and the synthesis of EPS in these bacteria. Recently, for some lactic acid bacteria a simple synthetic growth medium was composed. These bacteria belong to the genera *Lactococcus* [23; 68], *Streptococcus* [108] and *Leuconostoc* [38]. However, for *Lb. delbrueckii* subsp. *bulgaricus*, the minimal requirements are still unknown, and also the effect of the medium composition other than the carbohydrate source on the production of EPS has not yet been elucidated. A synthetic culture medium was composed in which several lactobacilli were able to grow [79], but growth requirements of *Lb. delbrueckii* subsp. *bulgaricus* were only partially investigated.

Chemically defined media are particularly important when studying the quantitative and qualitative production of EPS by lactic acid bacteria and also the regulation of EPS synthesis. Previously we used a chemically defined medium which contained a carbohydrate source, mineral salts, amino acids, nucleic acid bases and vitamins [48]. In this work we have simplified and optimized this medium for growth and EPS production using the technique of the omission of a single medium component, which is generally used to investigate the requirements for medium components [119]. By using this technique, we composed a simplified chemically defined medium in which *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 was able to produce increased levels of EPS. Previously, it has only been possible to study the effect of the carbohydrate source. The strain metabolized only four carbohydrates: glucose, lactose, fructose and mannose and the production of EPS was dependent on the carbohydrate source. When grown on glucose or lactose, the strain produced considerably higher amounts of EPS than when grown on fructose to equal cell densities. Compared with the other carbohydrate sources, both growth and EPS production on mannose were much lower [48; 49].

A defined medium is also very useful in the investigation of the composition of the EPS produced. It was found that complex media like MRS strongly interfere with the isolation procedure of macromolecules like EPS [42] and that the exact amount and composition of the EPS produced are unclear when these complex media are used. In the chemically defined medium used in our previous work, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown on glucose produced two EPS fractions concurrently and in almost equal amounts, which differed in molecular weight and composition of the EPS repeating unit. The high molecular weight fraction (molecular weight 1.7×10^6) contained glucose, galactose and rhamnose in the ratio 1:5:1 and the low molecular weight fraction (molecular weight 4×10^4) was composed of glucose, galactose and rhamnose in the ratio 1:11:0.4. When fructose was used as the sole carbohydrate source, mainly a low molecular weight EPS fraction was produced [50]. It is possible that not only the carbohydrate source, but also other medium components may affect the composition of the EPS.

In this work, the effect of the omission of medium components on the growth and EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 was investigated and a simplified defined medium was composed in which the strain produced a considerably higher amount of EPS.

Materials and methods

Bacterial strain and culture medium

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 2772 was obtained from the National Collection of Food Bacteria (Reading, UK). Cultures were stored at -80 °C in MRS medium [88] containing 15% glycerol until required, and reactivated in MRS medium at 37 °C and initial pH 6.0 for 16 hours. Growth experiments were performed in a chemically defined medium in which growth and production of EPS was shown before [48], containing: 7.4 mM KH_2PO_4, 14.1 mM Na_2HPO_4, 4.3 mM citric acid, 82.3 mM sodium acetate, 18.7 mM NH_4Cl, 0.8 mM $MgSO_4.7H_2O$, 0.3 mM $MnCl_2.4H_2O$, 1 ml l^{-1} Tween-80, 0.07 mM adenine, 0.07 mM guanine, 0.09 mM uracil, 0.07 mM xanthine, 1.1 mM L-alanine, 0.5 mM L-arginine, 0.7 mM L-asparagine, 0.8 mM L-aspartic acid, 2.5 mM L-cysteine, 0.7 mM L-glutamine, 0.7 mM L-glutamic acid, 1.3 mM glycine, 0.6 mM L-histidine, 0.8 mM L-isoleucine, 0.8 mM L-leucine, 0.6 mM L-lysine hydrochloride, 0.7 mM L-methionine, 0.6 mM L-phenylalanine, 0.9 mM L-proline, 1.0 mM L-serine, 0.8 mM L-threonine, 0.5 mM L-tryptophan, 0.6 mM L-tyrosine and 0.9 mM L-valine, 1 ml l^{-1} of a trace element solution containing 81.1 mM HCl, 0.8 mM $CoCl_2.6H_2O$, 0.01 mM $CuCl_2.2H_2O$, 7.5 mM $FeCl_2.4H_2O$, 0.1 mM H_3BO_3, 0.2 mM $Na_2MoO_4.2H_2O$, 0.1 mM $NiCl_2$ and 0.5 mM $ZnCl_2$, 1 ml l^{-1} of a vitamin solution containing: 0.7 mM *p*-amino benzoate, 0.08 mM biotin, 0.1 mM folic acid, 0.24 mM lipoic acid, 1.6 mM nicotinic acid, 0.4 mM Ca-pantothenate, 2.1 mM pyridoxamine, 0.3 mM pyridoxine, 0.27 mM riboflavin, 0.6 mM thiamine and 0.07 mM vitamin B_{12}. Glucose was used as the carbohydrate source at a initial concentration of 111 mM, unless described otherwise. The defined medium was sterilized by passing through a 0.2 µm sterile filter (Schleicher & Schuell, Dassel, Germany or Gelman Sciences, Ann Arbor, Mich.).

Growth, EPS isolation and EPS characterization

Batch cultivations were performed in unshaken, nitrogen-flushed sealed bottles (volume 115 ml) containing 50 ml of defined medium at 37°C and initial pH 6.0, unless otherwise stated. Growth was monitored after 48 hours by OD measurement at 600 nm. EPS were isolated as described before [48], the total carbohydrate content of the isolated EPS was measured using the phenol/sulfuric acid method of Dubois et al. [32] and the sugar composition of the EPS was determined after hydrolysis by HPLC measurement [48]. Molecular weight of the EPS was measured using High Performance Size Exclusion Chromatography (HPSEC) as described before [50].

Growth experiments to evaluate nutritional requirements

In order to identify the nutritional requirements of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772, the strain was grown in batch cultures with defined medium and the omission technique of one of the medium components was used. Before being used for growth experiments, cells grown in defined medium were centrifuged (15,000 *g*, 10 min, 4°C), washed twice in phosphate buffer (20 mM, pH 6.0), resuspended in the same buffer and finally added to the medium, from which one or more of the medium components was omitted. In these test media, the strain was subcultured three times in succession, by adding 0.5 ml of a culture incubated for 48 hours to 50 ml of fresh medium. After this, the optical densities of the third subcultures were compared with tho OD of a positive control without nutrient omissions. These experiments were performed in triplicate.

Simplified defined medium for growth and EPS production
Based on the results of the analyses of the nutritional requirements of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772, a simplified medium was composed, containing all medium components necessary for growth and EPS production by the strain. The growth and EPS production were investigated in a pH-controlled batch culture, using a glass fermenter (Applikon, Schiedam, The Netherlands) with a working volume of 1.5 liter at 40°C, pH 6.1 ± 0.1, 50 rev min^{-1} and under a N_2 atmosphere. Glucose was used as the carbohydrate source at a initial concentration of 166 mM. Growth and EPS production by *Lb. delbrueckii* subsp. *bulgaricus* in the simplified medium was compared with growth and EPS production under the same conditions in the complete medium.

Results

Gas requirement
Growth of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 was not significantly affected by the composition of the gas phase. Growth and EPS production under a nitrogen atmosphere, a N_2/CO_2 atmosphere with varying ratios and under air were comparable. Growth in the presence of 100% O_2 in the gas phase was performed in shaken sealed bottles (volume 115 ml) with 25 ml medium in a horizontally shaking water bath at 37°C and resulted in less growth of the strain with a final OD of 0.9; however, glucose fermentation pattern and EPS production related to growth were not affected.

Nutritional requirements for growth
It appeared that most amino acids were essential for growth of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772. The single omission of aspartic acid, glutamic acid or glycine affected growth only slightly, but significantly less than the control, whereas the omission of asparagine, glutamine or threonine resulted in a stronger reduction of the growth (Table 4.1). No growth was observed when any of the other amino acids was omitted from the growth medium. The strain did not grow when only the essential amino acids were present. When a growth medium was used without aspartic acid, glutamic acid and glycine, almost no growth was observed after 48 hours, but after 96 hours, the OD of the culture increased to 0.9. Omissions of single or multiple amino acids neither affected the amount of EPS relative to cell density, nor the sugar composition of the EPS (data not shown). The same was observed when guanine or xanthine was omitted from the growth medium. On the other hand, the omission of uracil prevented growth completely, whereas the omission of adenine resulted in a reduction of the OD and EPS production by approx. 50 per cent.

The omission of ammonium chloride from the growth medium had only little effect on the growth of *Lb. delbrueckii* subsp. *bulgaricus*. The strain grew well when the trace elements ($FeCl_2$, $CoCl_2$, $ZnCl_2$, H_3BO_3, Na_2MoO_4, $NiCl_2$ and $CuCl_2$) were omitted individually or all at once. Sodium acetate, $MgSO_4$ and $MnCl_2$ appeared to be essential for growth. The omission of Tween-80 or citric acid decreased growth of the strain. Addition of $CaCl_2$ (10 µM) to the growth medium had no effect. In all these omission experiments, growth and EPS production were proportional and no changes in the EPS sugar composition were observed. The addition of 20 mM morpholine-propanesulfonic acid (MOPS) buffer together with a tenfold lower amount of phosphate did not affect growth and EPS production of the strain significantly. When the vitamins were omitted individually from the growth medium, only riboflavin, Ca-pantothenate and nicotinic acid appeared to be essential for growth, whereas a slightly lower OD was found when vitamin B_{12} was omitted.

Table 4.1: Nutrient requirements of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 in defined medium investigated by omission of a single medium component. OD measurements were performed after 48 hours of incubation. Values are the mean of triplicate measurements.

Omitted medium component	OD (600 nm)	Omitted medium component	OD (600 nm)
none	2.0 ± 0.18	L-isoleucine	0.3 ± 0.10
phosphate	0.0 ± 0.02	L-leucine	0.0 ± 0.01
citric acid	1.3 ± 0.21	L-lysine	0.0 ± 0.02
Na-acetate	0.1 ± 0.03	L-methionine	0.3 ± 0.07
NH_4Cl	1.8 ± 0.18	L-phenylalanine	0.3 ± 0.08
$MgSO_4$	0.0 ± 0.01	L-proline	0.0 ± 0.00
$MnCl_2$	0.2 ± 0.04	L-serine	0.0 ± 0.01
Tween-80	0.7 ± 0.02	L-threonine	0.8 ± 0.06
trace elements	1.9 ± 0.26	L-tryptophan	0.2 ± 0.03
		L-tyrosine	0.3 ± 0.05
adenine	0.9 ± 0.09	L-valine	0.0 ± 0.00
guanine	2.1 ± 0.21		
uracil	0.0 ± 0.02	p-amino benzoic acid	2.1 ± 0.22
xanthine	2.1 ± 0.17	biotin	2.2 ± 0.12
		folic acid	1.9 ± 0.15
L-alanine	0.0 ± 0.00	lipoic acid	1.9 ± 0.22
L-arginine	0.0 ± 0.02	nicotinic acid	0.0 ± 0.00
L-asparagine	0.9 ± 0.04	Ca-pantothenate	0.4 ± 0.10
L-aspartic acid	1.5 ± 0.17	pyridoxamine	2.2 ± 0.06
L-cysteine	0.4 ± 0.08	pyridoxine	2.3 ± 0.15
L-glutamic acid	1.6 ± 0.17	riboflavin	0.6 ± 0.10
L-glutamine	1.3 ± 0.11	thiamine	2.3 ± 0.24
glycine	1.6 ± 0.10	vitamin B_{12}	1.6 ± 0.12
L-histidine	0.1 ± 0.02		

Enhanced EPS production by multiple vitamin omission

A single omission of the non-essential vitamins did not change the specific EPS production, but when *Lb. delbrueckii* subsp. *bulgaricus* was grown in a medium containing no vitamins except for riboflavin, Ca-pantothenate and nicotinic acid, the OD of the cultures was much lower after 48 hours, whereas the specific EPS production increased significantly (Table 4.2). The single omission of vitamins did not change the monomeric sugar composition of the EPS. A multiple omission of vitamins resulted in EPS containing a slightly lower content of galactose monomer.

Table 4.2: Effect of the individual and multiple omission of non-essential vitamins on the growth and EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 in defined medium. OD measurements were performed after 48 hours of incubation. Values are the mean of at least triplicate measurements.

Omission	OD (600 nm)	EPS (mg l^{-1})	specific EPS production (EPS mg^{-1} cell dry weight)[a]
none	2.0 ± 0.18	43.3 ± 1.0	28.9
p-amino benzoate	2.1 ± 0.22	35.2 ± 3.4	22.3
biotin	2.2 ± 0.12	39.3 ± 2.6	23.8
folic acid	1.9 ± 0.15	40.0 ± 3.9	28.1
lipoic acid	1.9 ± 0.22	32.9 ± 2.6	23.1
pyridoxamine	2.2 ± 0.06	34.4 ± 2.7	20.8
pyridoxine	2.3 ± 0.15	55.4 ± 3.4	32.1
thiamine	2.3 ± 0.24	39.1 ± 2.9	22.7
vitamin B_{12}	1.6 ± 0.12	40.0 ± 3.5	33.3
all non-essential vitamins[b]	1.1 ± 0.10	41.1 ± 3.7	49.8

[a] The specific EPS production was calculated using a standard curve of OD at 600 nm against cell dry weight [48].
[b] All non-essential vitamins: *p*-amino benzoate, biotin, folic acid, lipoic acid, pyridoxamine, pyridoxine, thiamine and vitamin B_{12}.

In view of the nutritional requirements, a simplified medium was composed for growth and EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772. Compared to the complete growth medium, the simplified medium contained no NH_4Cl, trace elements, guanine, xanthine and vitamins except for riboflavin, Ca-pantothenate and nicotinic acid. Since multiple omissions of amino acids strongly reduced the growth of the strain, all amino acids were included. A pH controlled batch culture experiment was performed to compare growth and EPS production by strain NCFB 2772 in the complete medium and in the simplified medium. In the simplified medium, strain NCFB 2772 was able to grow with a maximal growth rate of 0.18 h^{-1} and a final OD of 1.4 and it produced 250 mg l^{-1} EPS when grown in a pH-controlled batch culture. On the other hand, in the complete medium the growth rate was 0.23 h^{-1} and the final OD 2.3, while the EPS production was only 130 mg l^{-1} (Fig. 4.1). It was observed that both in the complete medium and in the simplified medium EPS production continued after growth had ceased, but beyond the stationary growth phase significantly more EPS was produced in the simpified medium than in the complete medium. In the simplified medium, the EPS produced had a monomeric sugar composition of glucose, galactose and rhamnose in a ratio of 1 : 5.7 : 0.8, whereas in the complete medium this ratio was 1 : 6.3 : 0.8. Molecular weight analysis of the isolated EPS produced in the simplified medium showed the presence of two fractions with molecular weights of 1.4×10^6 and 7.0×10^4. In both the EPS produced in the complete medium and the EPS produced in the simplified medium, the high molecular weight fraction was present in a two-fold higher concentration than the low molecular weight fraction.

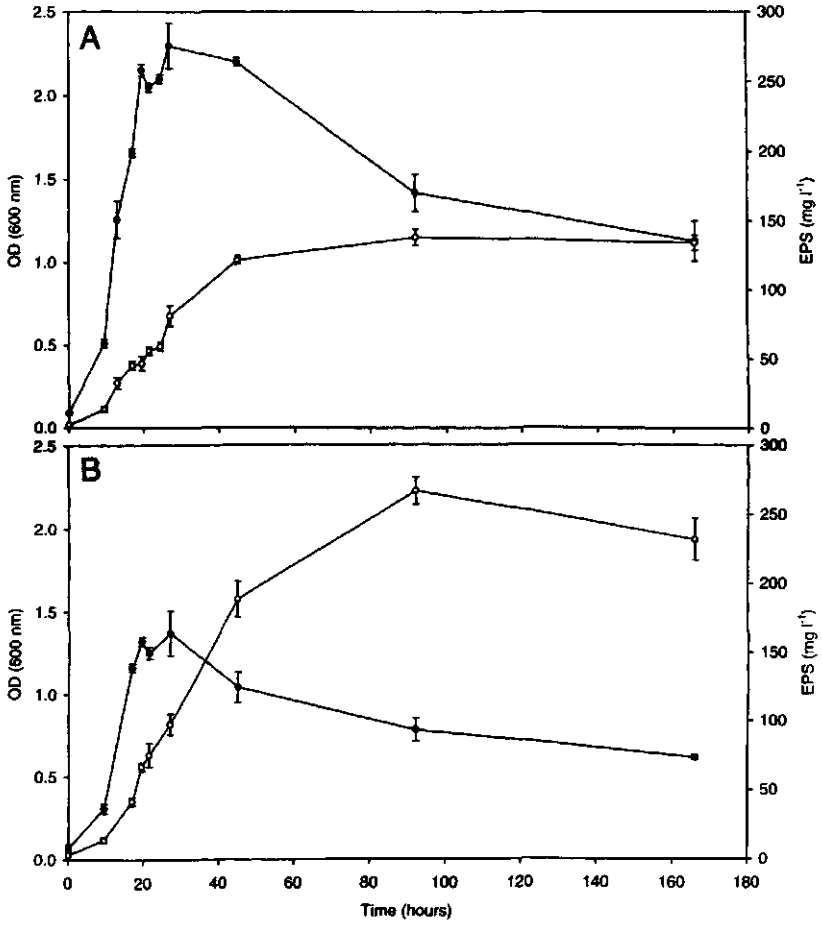

Fig. 4.1: Growth and exopolysaccharide (EPS) production by *Lb. delbrueckii* subsp. *bulgaricus* in pH- controlled batch cultures at 40 °C and pH 6.0 ± 0.1 in the complete defined medium (A) and the simplified medium (B). Each value represents the average of duplicate measurements and varied from the mean by not more than 10 per cent. ●, OD at 600 nm; ○, EPS (mg l^{-1}).

Discussion

Lb. delbrueckii subsp. *bulgaricus* NCFB 2772 produces EPS when grown in a defined medium supplemented with a carbohydrate source. The amount of the EPS produced was thought to be affected only by the growth temperature or the carbohydrate source and it was believed that EPS production is strictly coupled to growing cells [48; 49]. In this work, the nutritional requirements of the strain were investigated to determine whether other nutrients can affect the growth and EPS production by the strain.

The technique of single omission gives us a clear indication of the requirement for nutrients for growth of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772. Nevertheless, growth of the strain was not possible if all components which were individually not required were omitted. For instance, a single omission of either aspartic acid, glutamic acid or glycine resulted in a good growth of the culture, whereas growth was poor when these three amino acids were all absent. Since the nitrogen metabolism in this strain is not well understood yet, it is difficult to predict the combined effect of amino acids in the growth medium. Compared to other lactic acid bacteria such as *Lactobacillus plantarum* [124], *Lactococcus lactis* [68] and *Streptococcus thermophilus* [108], *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 required more amino acids. In contrast to the findings of Ledesma et al. [79], who proposed that a requirement for glutamic acid, valine and leucine is a taxonomic criterion for the genus *Lactobacillus*, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grew well in the absence of glutamic acid. The single or multiple omission of amino acids had no effect on the production of EPS relative to cell growth. Regarding the vitamin requirements, it appeared that only nicotinic acid, Ca-pantothenate and riboflavin were essential for growth. Ledesma et al. [79] found that *Lb. delbrueckii* subsp. *bulgaricus* strain ATCC 9224 required the vitamins nicotinic acid and Ca-pantothenate for growth, since these vitamins are involved in co-enzyme biosynthesis of lactobacilli. Riboflavin, a component of flavin coenzymes, appears to be essential for growth of lactic acid bacteria [23].

Multiple omission of all non-essential vitamins reduced growth of the strain, but surprisingly and in contrast to the results obtained for the amino acids, multiple vitamin omission affected the production of EPS strongly. For the first time, it was observed in a lactic acid bacterium that the EPS production was affected by a growth factor other than the carbohydrate source, temperature or pH and that the regulation of the EPS production beyond the stationary growth phase was influenced by the medium composition. Up to now, nothing was known about the relationship between vitamin requirement and EPS production in lactic acid bacteria. Cerning et al. [21] and Kojic et al. [76] found that *Lb. casei* CG11 produces EPS when grown in a basal minimal medium [96] only containing only folic acid, nicotinic acid, Ca-pantothenate, pyridoxine and riboflavin and no other vitamins. The EPS production by *Lb. casei* CG11 continued beyond the stationary growth phase. *Lb. delbrueckii* subsp. *bulgaricus* CRL 420 also produced some EPS after growth had ceased [89], but this organism was grown in a complex medium, so that the influence of vitamins on the EPS production by this strain is unknown. We demonstrated that only the total amount of EPS produced was affected by the multiple omission of vitamins and not the ratio of high molecular weight fraction and low molecular weight fraction of the EPS, as was found when fructose instead of glucose was used as the carbohydrate source [50]. This means that the multiple omission of vitamins affected the production of both the high molecular weight fraction and the low molecular weight fraction, this in contrast to the carbohydrate source, which had mainly an effect on the production of the high molecular weight EPS fraction [50].

It was found that EPS production under controlled pH was significantly higher than in acidifying batch cultures (Table 4.2, Fig. 4.1). This was not only observed earlier in our previous work [48], but *Lb. casei* also produced considerably more EPS when grown under constant pH values than without pH control [102]. Higher exopolysaccharide production obtained by maintaining the pH of the culture medium at a constant value was also reported for *Xanthomonas campestris* [95] and *Pseudomonas* sp. strain EPS-5028 [90].

In conclusion, a simplified defined medium was composed in which *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grew less well than in the complete defined medium, but the production of EPS appeared to be about twofold higher and continued strongly after cell growth had ceased. The single omission technique appeared to be indirectly successful. Using this technique, the nutritional requirements were found, and multiple omission of vitamins resulted in the enhanced EPS production, although it is unclear which factor is responsible for this enhancement. More

investigations on the physiological effects of vitamins on the production of EPS will be necessary for a better understanding of the mechanisms of EPS production.

5.

Influence of fructose and glucose on the production of exopolysaccharides and the activities of enzymes involved in the sugar metabolism and the synthesis of sugar nucleotides in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772

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Abstract

The effect of fructose and glucose on the growth, production of exopolysaccharides and the activities of enzymes involved in the synthesis of sugar nucleotides in *Lactobacillus delbrueckii* subsp. *bulgaricus* grown in continuous culture was investigated. When grown on fructose, the strain produced 25 mg l^{-1} exopolysaccharide composed of glucose and galactose in the ratio 1:2.4. When the carbohydrate source was switched to a mixture of fructose and glucose, the exopolysaccharide production increased to 80 mg l^{-1}, while the sugar composition of the exopolysaccharide changed to glucose, galactose and rhamnose in a ratio of 1:7.0:0.8. A switch to glucose as the sole carbohydrate source had no further effect. Analysis of the enzymes involved in the synthesis of sugar nucleotides indicates that in cell-free extracts of glucose-grown cells the activity of UDP-glucose pyrophosphorylase was higher than in cell-free extracts of fructose-grown cells. The activities of dTDP-glucose pyrophosphorylase and the rhamnose synthetic enzyme system were very low in glucose-grown cultures but could not be detected in fructose-grown cultures. Cells grown on a mixture of fructose and glucose showed similar enzyme activities as cells grown on glucose. Analysis of the intracellular level of sugar nucleotides in glucose-grown cultures of *Lb. delbrueckii* subsp. *bulgaricus* showed the presence of UDP-glucose and UDP-galactose in a ratio of 3.3 : 1, respectively, a similar ratio and slightly lower concentrations were found in fructose-grown cultures. The lower production of exopolysaccharides in cultures grown on fructose may be caused by the more complex pathway involved in the synthesis of sugar nucleotides. The absence of activities of enzymes leading to the synthesis of rhamnose nucleotide in fructose-grown cultures appeared to result in the absence of rhamnose monomer in the exopolysaccharides produced on fructose.

Introduction

In the dairy industry, lactic acid bacteria producing and excreting exopolysaccharides (EPS) are often used in the manufacture of fermented milk products. These polymers are thought to play a role in the viscosity and texture of the fermentation products and in the prevention of syneresis. The viscofying effect in yogurt is not only influenced by the amount of EPS, but also by its structure. Slime-forming strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are used as starter cultures in the manufacture of yogurt. A number of EPS producing lactic acid bacteria have been investigated [9; 14; 17; 19; 29; 113], but there is still a lack of knowledge on the physiological aspects of EPS production by lactic acid bacteria.

It was reported that *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 produces EPS when grown in skimmed milk [42] or in a defined medium supplemented with glucose or lactose [48]. The EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* strains CNRZ 416 [17] and rr [54] contained glucose, galactose and rhamnose in a ratio of approximately 1 : 5 : 1. When grown in a chemically defined medium with glucose or lactose, strain NCFB 2772 produced EPS with a monomeric sugar composition of glucose, galactose and rhamnose in a ratio of 1, 6.8, 0.7, respectively. When grown on fructose, the amount of EPS produced was substantially lower.

Lb. delbrueckii subsp. *bulgaricus* shows a homofermentative metabolism of glucose, fructose and lactose [69; 152]. When grown on lactose as the sole carbohydrate source, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 only used the glucose monomer, while galactose accumulated in the medium. The sugar composition of the EPS produced in lactose-grown cultures was not affected by the residual galactose [48]. Therefore, in the experiments performed here, glucose is used as the carbohydrate source instead of lactose. It was observed that growth of *L bulgaricus* NCFB 2772, sugar consumption and EPS production were strongly related [48]. It is still not clear in what way the carbohydrate source affects the amount and sugar composition of the EPS produced and how the production of EPS is regulated in *Lb. delbrueckii* subsp. *bulgaricus*. More knowledge of the biosynthetic pathway of EPS and the regulation of the carbon flow between the Embden-Meyerhof-Parnas (EMP) pathway and the synthesis of EPS in cultures grown on different carbohydrate sources is needed to elucidate its regulation.

In this work we report the EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in a continuous culture on a defined medium supplemented with fructose, fructose plus glucose and glucose as fermentable carbohydrates. In order to investigate the differences in EPS production and sugar composition between fructose-grown and glucose-grown cultures of *Lb. delbrueckii* subsp. *bulgaricus*, the activities of some of the key enzymes involved in the EMP pathway and the biosynthesis of sugar nucleotides as the precursors for EPS synthesis [82] were determined. Furthermore, the intracellular levels of sugar nucleotides of fructose-grown and glucose-grown *Lb. delbrueckii* subsp. *bulgaricus* were measured.

Materials and methods

Bacterial strain, growth conditions and EPS isolation and analysis

Lb. delbrueckii subsp. *bulgaricus* NCFB 2772 was obtained from the National Collection of Food Bacteria (Reading, UK). Growth experiments were performed in a defined medium as described by Grobben et al. [48]. The EPS were isolated by precipitation in ethanol according to the method of Garcia-Garibay and Marshall [42], total carbohydrate was determined using the phenol-sulfuric acid method of Dubois et al. [32]. Sugars, lactic acid and the monomeric sugar composition of the EPS after hydrolysis were determined by HPLC measurement [48].

Preparation of permeabilized cells and cell-free extracts

Lb. delbrueckii subsp. *bulgaricus* cultures were harvested by centrifugation at 20,000 *g* for 10 min at 4 °C. The cells were washed twice with cold 0.01 M potassium phosphate buffer (pH 6.8) containing 5 mM $MgCl_2$. For the preparation of cell-free extract, cells were resuspended in the same buffer and disrupted by X-Press. Unbroken cells and cell debris were removed by centrifugation at 20,000 *g* for 20 min at 4 °C and the supernatant was used as cell-free extract. For the assay of phosphoenolpyruvate:sugar phosphotransferase 1 ml of a cell suspension was permeabilized with 50 µl of a toluene: acetone solution (1:9 vol/vol) and vortexed for 5 min at room temperature. The toluene-treated cells were prepared immediately before the sugar phosphotransferase assays [22].

Enzyme assays (see Fig. 5.2)

All *in vitro* enzyme assays were performed in a volume of 1.0 ml in 1-ml glass cuvettes at 37 °C in a Perkin-Elmer 550A spectrophotometer with freshly prepared cell-free extracts or toluene-treated cells, unless described otherwise. In all assays, the reaction velocity was linearly proportional to the amount of cell-free extract or permeabilized cells. The formation or disappearance of NAD(P)H was monitored by measuring the absorbance at 340 nm. The activities of the enzymes described below were expressed in nmol min^{-1} mg $protein^{-1}$. The enzyme activities were calculated by subtracting the values of the endogenous activities. The protein contents of the cell free extracts and permeabilized cells were determined using the method of Lowry et al. [85]. The protein content of cell suspensions was determined after extraction with 1 volume 1 M NaOH at 100 °C for 30 min.

For the phosphoenolpyruvate(PEP) - glucose-phosphotransferase system and PEP - fructose phosphotransferase system spectrophotometric assay [58], the reaction mixture contained 50 µmol potassium phosphate pH 6.8, 5 µmol $MgCl_2$, 5 µmol PEP, 0.15 µmol NADH, 50 µg of lactate dehydrogenase and toluene-treated cells. The reaction was started by adding glucose or fructose to a final concentration of 1 mM.

The phosphoglucose isomerase reverse reaction contained 50 µmol potassium phosphate pH 6.8, 5 µmol $MgCl_2$, 0.4 µmol NADP, 0.01 ml of glucose 6-phosphate dehydrogenase (180 U ml^{-1}) and cell-free extract. The reaction was started by adding 2.5 µmol fructose 6-phosphate [131].

The α-phosphoglucose mutase reaction mixture contained 50 µmol triethanolamine pH 7.0, 5 µmol $MgCl_2$, 0.4 µmol NADP, 0.05 µmol glucose 1,6-diphosphate, 0.01 ml of glucose 6-phosphate dehydrogenase (180 U ml^{-1}) and cell-free extract. The reaction was started with 1 µmol α-glucose 1-phosphate [114].

The reaction mixtures for the 1-phosphofructokinase and 6-phosphofructokinase assays contained 50 µmol TRIS/HCl pH 7.5, 5 µmol $MgCl_2$, 50 µmol KCl, 1.25 µmol ATP, 0.15 µmol NADH, 50 µg aldolase, 20 µg triose phosphate isomerase/ glycerol phosphate dehydrogenase and cell-free extract. The reaction was started by adding 1 µmol fructose 1-phosphate (1-phosphofructokinase assay) or 1 µmol fructose 6-phosphate (6-phosphofructokinase assay) [28].

The fructose 1,6-diphosphatase reaction mixture contained 50 µmol glycylglycine pH 8.5, 5 µmol $MgCl_2$, 0.4 µmol NADP, 25 µg of phosphoglucose isomerase, 25 µg of glucose 6-phosphate dehydrogenase and cell-free extract. The reaction was started by adding 2 µmol fructose 1,6-diphosphate [28].

The uridine diphosphate (UDP)-glucose pyrophosphorylase forward-reaction mixture contained 50 µmol TRIS/HCl pH 7.5, 8 µmol $MgCl_2$, 1.58 mg cysteine hydrochloride (pH 7.5), 0.5 µmol NAD, 1.25 µmol UTP, 25 µg UDP-glucose dehydrogenase and cell-free extract. The reaction was started by adding 1 µmol α-glucose 1-phosphate [39].

The thymidine diphosphate(dTDP) -glucose pyrophosphorylase forward-reaction mixture contained 50 µmol TRIS/HCl pH 7.5, 8 µmol $MgCl_2$, 1.58 mg cysteine hydrochloride (pH 7.5), 1.25 µmol dTTP and cell-free extract. The reaction was started by adding 1 µmol α-glucose 1-phosphate and stopped by adding 50 µl 1 M HCl (final volume of the reaction mixture: 1000 µl). The samples were assayed by HPLC measurement (see below).

The UDP-galactose 4-epimerase reaction mixture contained 40 µmol glycylglycine-NaOH pH 8.5, 5 µmol $MgCl_2$, 0.6 µmol NAD, 25 µg UDP-glucose dehydrogenase and cell-free extract. The reaction was started with 0.2 µmol UDP-galactose. The activity of the UDP-galactose 4-epimerase was also determined by measuring the concentration of UDP-galactose and UDP-glucose by HPLC measurement. The reaction mixture was similar to the reaction mixture used in the spectrophotometric assay, except that NAD and UDP-glucose dehydrogenase were omitted. The reaction was started with 0.1 µmol UDP-galactose. Incubation occurred at 37 °C and the reaction was stopped by adding 50 µl 1 M HCl.

The dTDP-glucose 4,6-dehydratase reaction (in a volume of 700 µl) contained 50 µmol TRIS/HCl pH 8.0 and cell-free extract. The reaction was started with 0.3 µmol dTDP-glucose. After incubation, the reaction was stopped by adding 300 µl 0.5 M NaOH. After further incubation for 10 min at 37 °C. The formation of dTDP-4-oxo-6-deoxyglucose was determined at 320 nm. The molar absorption coefficient to dTDP-4-oxo-6-deoxyglucose was 6.5 10^3 l mol^{-1} cm^{-1}. [169].

dTDP-rhamnose-synthetic enzyme system [93] included dTDP-glucose 4,6-dehydratase and the complex of dTDP-4-dehydrorhamnose epimerase and dTDP-4-dehydrorhamnose reductase, which converts dTDP-4-oxo-6-deoxyglucose into dTDP-rhamnose. The assay was performed as described for the dTDP-glucose 4,6-dehydratase, except that 0.5 µmol NADPH was added. The formation of dTDP-rhamnose from dTDP-glucose was assayed at 340 nm.

Analysis of sugar nucleotides

Sugar nucleotides were isolated from cell cultures using a formic acid treatment as described by Harding et al. [56]. A Shandon 5 mm Hypersil ODS (4.6 x 250 mm) HPLC column (LC-Service, Emmen, The Netherlands) was used for the determination of sugar nucleotides. The compounds were eluted with 40 mM triethylamine-phosphate pH 6.5, with a flow rate of 1.0 ml min^{-1} at room temperature. The compounds were detected at 260 nm.

Results

Effect of the carbohydrate source on growth and EPS production

Lb. delbrueckii subsp. *bulgaricus* was grown in a N_2-flushed continuous culture on 167 mM fructose at 40 °C, pH 6.0, and at a dilution rate of 0.075 h^{-1} and growth was monitored by measuring the absorbance at 600 nm. *Lb. delbrueckii* subsp. *bulgaricus* produced 24 mg l^{-1} EPS with a monomeric sugar composition of glucose and galactose in a ratio of 1 : 2.4. No rhamnose or other sugar monomers were detected. After a steady state had been re-established, the medium was switched to one containing both 83 mM fructose and 83 mM glucose at the same dilution rate. The amount of EPS produced rapidly increased up to 80 mg l^{-1} and hereafter EPS production was related to sugar consumption (Fig. 5.1) and lactate fermentation. Glucose was preferred to fructose as the carbohydrate source, since most fructose remained in the growth medium. The EPS produced had a sugar composition of glucose, galactose and rhamnose in a ratio of 1 : 7.0 : 0.8 respectively. A switch to a medium containing 167 mM glucose as the sole carbohydrate source had no further effect on the growth, EPS production and sugar composition of the EPS.

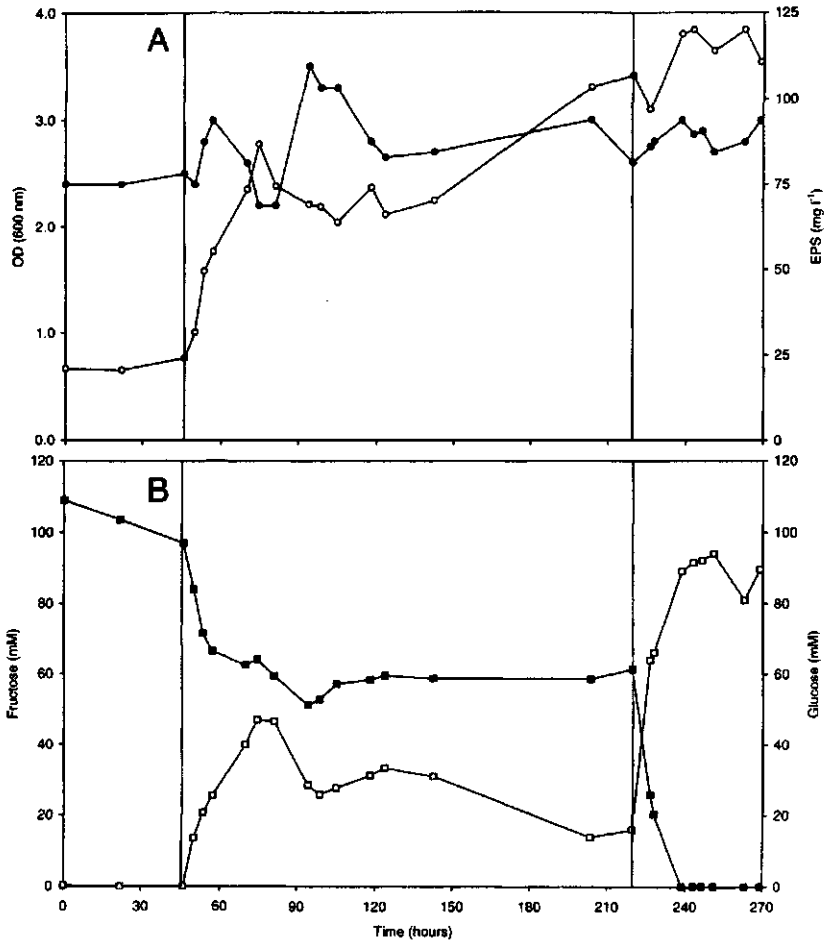

Fig. 5.1: Growth and production of exopolysaccharides (EPS) by *Lb. delbrueckii* subsp. *bulgaricus* in continuous culture in defined medium (40 °C, pH 6.0, D 0.075 h^{-1}) on 167 mM fructose. After 45 hours, the carbohydrate source was switched to 83 mM fructose + 83 mM glucose. After 220 hours, the carbohydrate source was switched to 167 mM glucose. Each value represents the average of duplicate measurements and varied from the mean by not more than 15%. A: ●: absorbance at 600 nm, ○: EPS (mg l^{-1}). B: ■: fructose (mM), □: glucose (mM)

Table 5.1: Activities of enzymes in the Embden-Meyerhof-Parnas pathway and the pathway leading to the biosynthesis of sugar nucleotides in *Lb. delbrueckii* subsp. *bulgaricus* grown in continuous cultures on fructose (167 mM), fructose plus glucose (each 83 mM) and glucose (167 mM). In the phosphoenolpyruvate (PEP)-sugar phosphotransferase (PTS) assays permeabilized cells were used. Each value represents the average of at least 5 measurements. Enzyme activities expressed as nmol min^{-1} mg cell $protein^{-1}$. ND, not determined

Enzyme	Carbon source		
	Fructose	Fructose + Glucose	Glucose
PEP-glucose PTS	82	80	79
PEP-fructose PTS	31	20	16
Phosphoglucose isomerase	603	659	646
1-Phosphofructokinase	907	141	114
6-Phosphofructokinase	2075	1955	2100
Aldolase	585	668	549
Fructose 1,6-diphosphatase	6.8	ND.	7.6
α-Phosphoglucose mutase	323	471	464
UDP-glucose pyrophosphorylase	1.9	7.1	5.2
dTDP-glucose pyrophosphorylase	0	ND	4.4
UDP-galactose 4-epimerase	142	240	206
dTDP-glucose 4,6-dehydratase	2.4	3.8	4.1
dTDP-rhamnose synthesis	0	ND	1.2

Enzyme activities

Lb. delbrueckii subsp. *bulgaricus* was grown in continuous cultures supplied with a medium containing 167 mM fructose, 83 mM fructose + 83 mM glucose or 167 mM glucose. After steady state conditions had been re-established (after at least 5 volume changes), permeabilized cells or cell free extracts were prepared and the activities of enzymes involved in the EMP pathway and the synthesis of sugar nucleotides were measured (Table 5.1, Fig. 5.2).

The activity of PEP-fructose phosphotransferase system was highest in fructose-grown cells. There were no variations in the activities of the PEP-glucose phosphotransferase system. No formation of fructose 6-phosphate from fructose was found by PEP-fructose phosphotransferase activity. No activities were found using 5 μmol ATP instead of PEP (glucokinase). Cell-free extracts of fructose-grown cells showed high 1-phosphofructokinase activity, while the activities of this enzyme in cell-free extracts of glucose-grown and glucose plus fructose-grown cells were lower. The activities of UDP-glucose pyrophosphorylase and dTDP-glucose 4,6-dehydratase were higher in cell-free extracts of glucose-grown cultures than of fructose-grown cultures. Glucose-grown cultures showed very low dTDP-glucose pyrophosphorylase and dTDP-rhamnose-synthesizing activities, whereas neither of these activities was detected in cell-free extracts of fructose-grown cells. Fructose-grown cultures showed slightly lower α-phosphoglucose mutase and UDP-galactose 4-epimerase activities compared to fructose + glucose-grown and glucose-grown cultures.

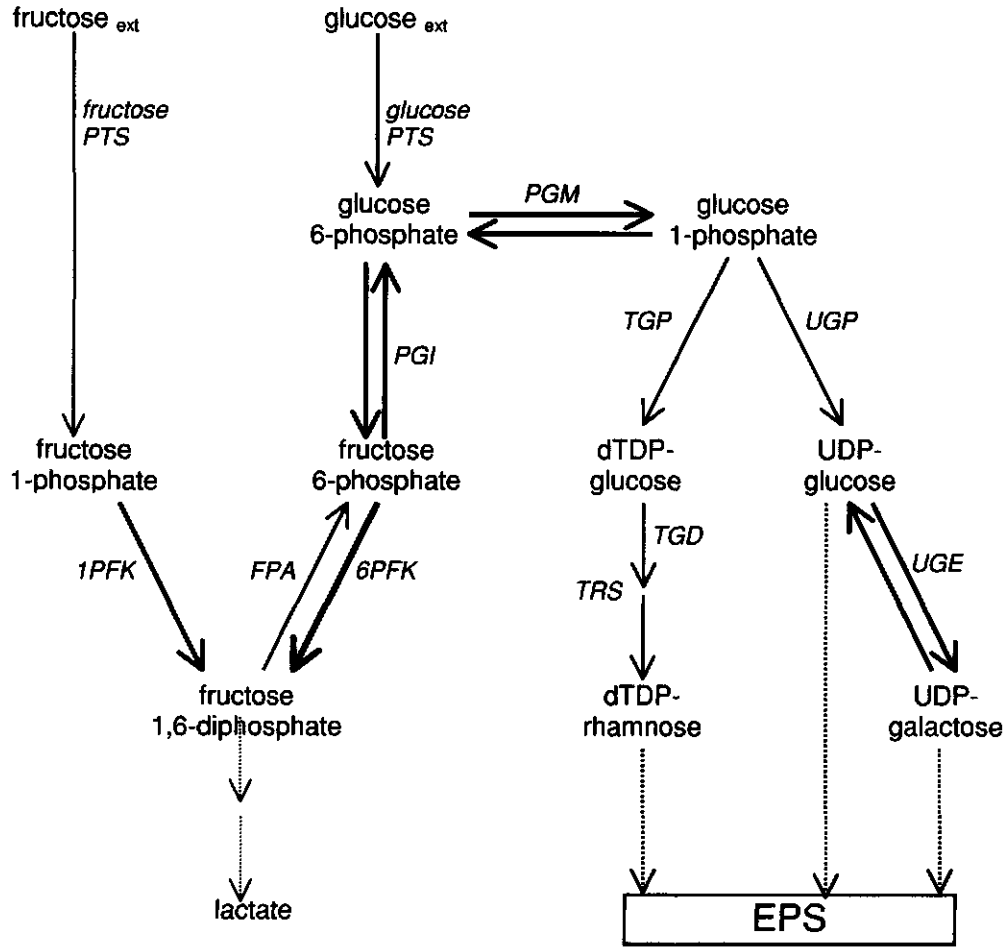

Fig. 5.2: Proposed pathway for fermentation of glucose and fructose and the biosynthesis of sugar nucleotides in *Lb. delbrueckii* subsp. *bulgaricus*. The thickness of the arrows is proportional to the enzyme activities found in permeabilized cells or cell-free extracts of glucose-grown cultures (dashed arrows: not determined). EPS, exopolysaccharides; *FPA*, fructose 1,6-diphosphatase; *1PFK*, 1-phosphofructokinase; *6PFK*, 6-phosphofructokinase; *PGI*, phosphoglucose isomerase; *PGM*, α-phosphoglucose mutase; *PTS*, phosphoenol-pyruvate-sugar phosphotransferase system; *TGD*, dTDP-glucose 4,6-dehydratase; *TGP*, dTDP-D-glucose pyrophosphorylase; *TRS*, dTDP-rhamnose synthetic enzyme system; *UGE*, UDP-galactose 4-epimerase; *UGP*, UDP-glucose pyrophosphorylase

When UDP-galactose 4-epimerase activities in glucose-grown and in fructose-grown *Lb. delbrueckii* subsp. *bulgaricus* were detected using HPLC measurements, it was found that UDP-glucose was formed from UDP-galactose during the assay (Fig. 5.3). The UDP-galactose 4-epimerase activity was 159 nmol min^{-1} mg $protein^{-1}$. Finally, an equilibrium was established with UDP-glucose and UDP-galactose in a ratio of 3 : 1, respectively. No activities were found of phosphofructose mutase, β-phosphoglucose mutase, UDP-glucose pyrophosphorylase using ß-

glucose 1-phosphate, UDP-galactose uridyl transferase, GDP-glucose-pyrophosphorylase and phosphomannose isomerase.

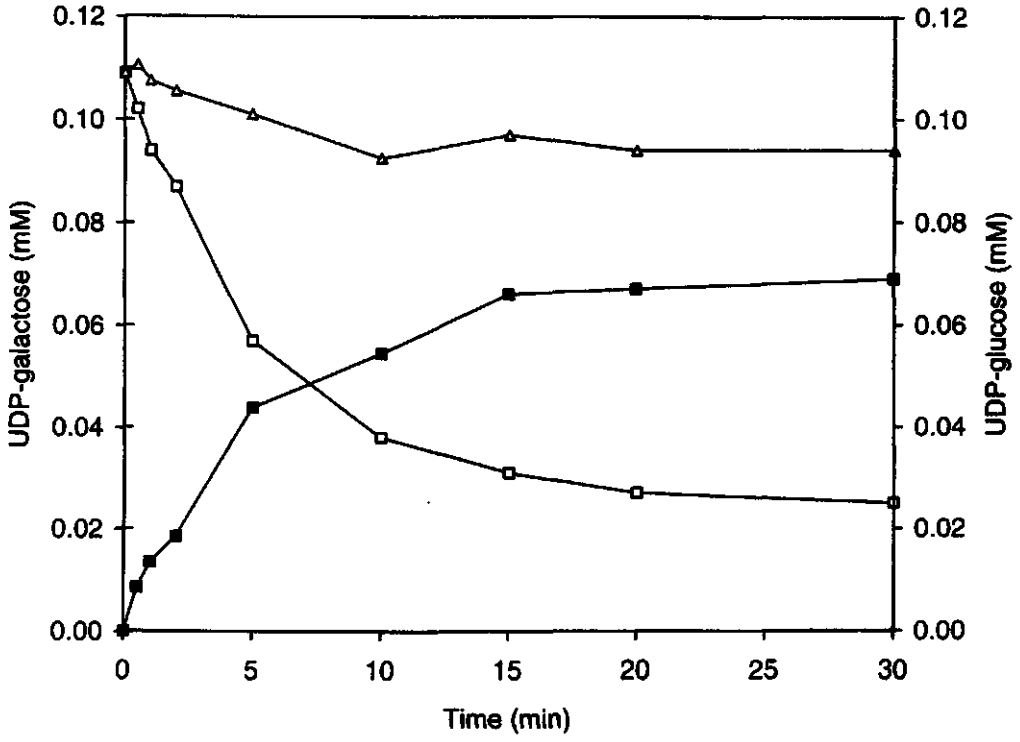

Fig. 5.3: Activity of UDP-galactose 4-epimerase in *Lb. delbrueckii* subsp. *bulgaricus* grown on glucose. The concentrations of UDP-galactose and UDP-glucose were detected by HPLC measurement. Values were determined in triplicate and varied from the mean by less than 10%. □: UDP-galactose, ■: UDP-glucose, Δ: UDP-galactose plus UDP-glucose

Analysis of intracellular sugar nucleotides

Analysis of the intracellular level of sugar nucleotides in glucose-grown cells showed the presence of UDP-glucose and UDP-galactose in a concentration of 53.8 and 16.3 µmol g cell protein^{-1} respectively, which gave a ratio of 3.3 : 1. In cell extracts of fructose-grown *Lb. delbrueckii* subsp. *bulgaricus*, the concentrations of UDP-glucose and UDP-galactose were 35.3 and 11.9 µmol g cell protein^{-1} respectively, so that the ratio of UDP-glucose and UDP-galactose appeared to be 3.0 : 1, respectively. dTDP-glucose could not be detected, while no standard of dTDP-rhamnose was commercially available.

Discussion

It was found that the amount of EPS produced in fructose-grown continuous cultures was substantially lower than in glucose-grown continuous cultures and that the EPS had a different monomeric sugar composition. In a previous paper we have reported that *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 produced less EPS from fructose or mannose than from lactose or glucose [48]. This suggests that the regulation of the biosynthetic pathway of EPS in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 may be dependent on the carbohydrate source. Also Cerning et al. [21] reported that EPS production and its monomeric sugar composition were dependent on the carbon source in *Lb. casei* CG11 grown in a defined medium. On the other hand, the sugar composition of the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* CRL 420 was shown to be independent of the carbohydrate source [89].

The activities of key enzymes involved in the sugar metabolism and potentially involved in the production of precursors for EPS biosynthesis in *Lb. delbrueckii* subsp. *bulgaricus* grown on fructose, fructose plus glucose and glucose were analyzed. Since no fructose 6-phosphate formation from fructose could be measured in fructose-grown cultures, it is proposed that fructose is phosphorylated by the PEP-fructose phosphotransferase system to form fructose 1-phosphate, since also a high 1-phosphofructokinase activity was found in fructose-grown cultures. Thompson [152] proposed a PEP-fructose phosphotransferase system leading to fructose 1-phosphate in lactic acid bacteria. Fructose-grown *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 showed relatively high PEP-fructose phosphotransferase and 1-phosphofructokinase activities, whereas they were lower in glucose-grown cultures. In cell free extracts of cells grown in the presence of both fructose and glucose, the activities of both enzymes were comparable with those found in glucose-grown cultures. This indicates that the two enzymes are repressed by glucose. EPS production from fructose is theoretically possible leading from fructose 1-phosphate via fructose 1,6-diphosphate, fructose 6-phosphate, glucose 6-phosphate, glucose 1-phosphate and UDP-glucose (Fig. 5.2). In this case, fructose 1,6-diphosphatase could be a regulatory enzyme in the synthesis of sugar nucleotides in fructose-grown cultures, since its activity is much lower than the activity of 6-phosphofructokinase, which is in the opposite direction. This could cause a lower amount of EPS produced by fructose-grown cultures. The UDP-glucose pyrophosphorylase activity in *Lb. delbrueckii* subsp. *bulgaricus* grown on glucose was higher than when grown on fructose. The activity of UDP-galactose 4-epimerase was only slightly lower in fructose-grown cultures. Since fructose-grown cultures produced EPS with a lower level of galactose, it appears that UDP-galactose 4-epimerase does not play an important role in the sugar composition of the produced EPS. In cell-free extracts of fructose-grown cells no activities of dTDP-glucose pyrophosphorylase and the dTDP-rhamnose synthetic enzyme system were detected: this may result in the absence of rhamnose in the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* grown on fructose.

Analysis of the sugar nucleotide content of cells indicates that glucose-grown cells contain UDP-glucose and UDP-galactose in a ratio of 3.3 : 1 respectively. This is apparently due to the activity of UDP-galactose 4-epimerase, since also an equilibrium of UDP-glucose and UDP-galactose in a ratio of 3 : 1 was established during this enzyme assay (Fig. 5.3). The concentrations and ratios of UDP-glucose and UDP-galactose in cell extracts of glucose-grown was only slightly higher than in fructose-grown cultures, such that the sugar monomeric composition appeared not only to be dependent on the sugar nucleotide level inside the cell, but probably also dependent on the assembly of the EPS repeating unit [161]. Furthermore, the transport or the polymerization of the EPS can also affect the amount or the sugar composition of the EPS. Therefore, more investigations into the transport system and the assembly of the EPS are required.

In conclusion, the lower amount of EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 from fructose may be caused by the longer pathway leading from fructose to the biosynthesis

of UDP-glucose and UDP-galactose or the low activity of fructose 1,6-diphosphatase, although the differences in the sugar nucleotide levels between extracts of glucose-grown and fructose-grown cells were less than might be expected, regarding the differences in the amount of EPS produced. The differences in the sugar composition appeared not to be caused by the amount of sugar nucleotides. The absence of dTDP-glucose pyrophosphorylase and dTDP-rhamnose-synthetic enzyme system activities in fructose-grown cultures appeared to result in the absence of rhamnose monomers in the EPS produced on fructose. Since the UDP-glucose : UDP-galactose ratios were similar, the differences in the amount of galactose monomer in the EPS is apparently not affected by the activity of UDP-galactose 4-epimerase, but possibly on the level of the assembly of the EPS repeating unit, the transport or the polymerization of the EPS. More investigations on the structure of the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* grown on fructose and glucose will be performed to elucidate the influence of the carbohydrate source.

6.

Analysis of the exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture on glucose and fructose

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Abstract

The exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in defined medium were investigated. At equal cell densities, the strain produced 95 mg l^{-1} exopolysaccharides with glucose and 30 mg l^{-1} with fructose as the carbohydrate source. High-performance size-exclusion chromatography of the exopolysaccharides produced on glucose showed the presence of two fractions with molecular weights (mol. wt.) of 1.7×10^6 and 4×10^4 in almost equal amounts. The exopolysaccharides produced on fructose contained mainly fraction of low mol. wt. of 4×10^4. The high-mol. wt. fraction of the purified exopolysaccharides produced on glucose appeared to have a sugar composition of galactose, glucose and rhamnose in the molar ratio of 5 : 1 : 1, whereas the low-mol. wt. fraction contained galactose, glucose and rhamnose in the molar ratio of approximately 11: 1 : 0.4. The purified exopolysaccharide fractions produced on fructose showed comparable ratios. The high molecular-weight-fractions contained terminally linked galactose, 1,2,3-linked galactose, 1,3,4-linked galactose, 1,3-linked glucose and terminally linked rhamnose. The low-molecular-weight fractions contained mainly 1,3-linked galactose and 1,6-linked galactose and lower amounts of other sugar linkages. The production of the high-mol. wt. fractions appeared to be dependent on the carbohydrate source, whereas the low-mol. wt. fractions were produced more continuously.

Introduction

Certain lactic acid bacteria are capable of formation of exopolysaccharides (EPS) when grown in skim milk, whey ultrafiltrate, MRS broth or chemically defined media [9; 11; 14; 17; 18; 19; 21; 76; 89]. EPS-producing lactic acid bacteria are of major importance for the food industry, since they assumed to play an important role in the rheological behavior and stabilization of fermented milk products like yogurt and also the prevention of syneresis [127; 159]. The utilization of slime-producing strains in the manufacture of yogurt is particularly of interest in France and The Netherlands, since the addition of stabilizers to unfruited yogurt is prohibited in these countries [17]. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are used in the manufacture of yogurt and the utilization of EPS producing strains is presumed to enhance its viscosity and texture. Various EPS-producing *Lb. delbrueckii* subsp. *bulgaricus* strains have previously been studied in detail [17; 42; 89; 98; 159].

It has also been shown that not only is the amount of EPS produced by lactic acid bacteria important for the improvement of the viscosity and texture of fermented milk products, but that the molecular weight, sugar composition and the primary structure of the EPS play also an important role [14]. A large number of EPS produced by various *Lb. delbrueckii* subsp. *bulgaricus* strains have been investigated and several different sugar compositions have been found, mostly containing glucose and galactose in varying ratios and sometimes containing one or more other neutral sugars [14]. Bouzar et al. [11] and Groux [52] found that *Lb. delbrueckii* subsp. *bulgaricus* strains produced EPS mainly composed of galactose and glucose, with lower amounts of mannose and arabinose. In contrast, the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* strain CRL 420 contained glucose and fructose in a ratio of 1 : 2 [89]. Structural studies on the purified EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* strain rr showed the presence of branched repeating units of galactose, glucose and rhamnose in the ratio 5 : 1 : 1 respectively [54].

In previous work we found that *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 produced EPS when grown in batch or continuous cultures on a chemically defined medium with glucose as the carbohydrate source. The EPS had a sugar composition of galactose, glucose and rhamnose in the ratio 6.8:1:0.7. It was found that, when the strain was grown on lactose, the amount and sugar composition of the EPS produced was comparable with values for glucose-grown cultures. Lactose was cleaved to form glucose and galactose and only glucose was metabolized, whereas galactose accumulated in the growth medium and was not used for EPS synthesis [48; 49]. When grown with fructose as the carbohydrate source, the amount of EPS produced was substantially lower and the EPS produced were composed of galactose and glucose in the ratio 2.5:1. No rhamnose residues were detected in these EPS. Analyses of the enzymes involved in the synthesis of sugar nucleotides indicated that, in glucose-grown cultures, the activity of UDP-glucose pyrophosphorylase leading to the synthesis of UDP-glucose and UDP-galactose was higher than that in fructose-grown cultures, whereas in fructose-grown cultures no enzyme activities were found that led to the synthesis of dTDP-rhamnose [49]. This indicated that the sugar composition and the structure of the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 are dependent on the carbon source used. Therefore, further structural characterization of the EPS produced under different growth conditions is required.

In order to clarify the differences between the EPS from fructose-grown and glucose-grown cultures we purified the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* grown in a continuous culture on defined medium with either glucose or fructose as the carbohydrate source. The purified EPS were analyzed to determine the molecular weight, the sugar composition and the presence of different types of sugar linkages.

Materials and methods

Bacterial strain, growth conditions and isolation of the EPS

Lb. delbrueckii subsp. *bulgaricus* NCFB 2772 was obtained from the National Collection of Food Bacteria (Reading, UK). The strain was grown in a N_2-flushed continuous culture at 40 °C, pH 6.0 ± 0.1 and at a dilution rate of 0.075 h^{-1}, using a chemically defined medium as described before [48]. Either glucose or fructose was used as the sole carbohydrate source with a final concentration of 139 mM, which resulted in a carbohydrate excess. After steady state conditions had been re-established (after at least 5 volume changes) cultures were treated with 132 g trichloroacetic acid $liter^{-1}$ and centrifuged (20 min., 27 000 *g*, 4 °C). The pH of the supernatant was corrected to 4.0 with NaOH and three volumes of cold absolute ethanol were added to precipitate the EPS. After 3 hours at 4 °C, the precipitated EPS were collected by centrifugation (30 min., 27 000 *g*, 4 °C), dissolved in distilled water, dialyzed at 4 °C against running tap water (24 hours) and distilled water (24 hours, 3 × 10 liters) and lyophilized.

High-performance size-exclusion chromatography

Freeze-dried EPS were dissolved in 0.4 M sodium acetate pH 3.0 to a concentration of 4.0 g l^{-1}. Insoluble fragments were removed by centrifugation at 13 000 rpm in an Eppendorf centrifuge. High-performance size-exclusion chromatography analysis was performed using a SP 8700 HPLC equipped with 3 Bio-Gel TSK columns in series: 60XL, 40XL and 30XL (each 300 × 7.5 mm, Bio-Rad), together with a TSK XL guard column (40 × 6 mm). The system was eluted with 0.4 M sodium acetate pH 3.0 at a flow rate of 0.8 ml min^{-1} at 30 °C (Verbruggen et al. 1995). Peaks were detected with a combined refractive-index detector and viscometer (Viscotek, model 250; Viscotek, Houston, Tex., USA) and a right-angle laser light-scattering detector (Viscotek, LD 600). Molecular weights and intrinsic viscosities were calculated using the light scattering module of the Trisec software (Viscotek).

Purification of the EPS

Approximately 300 mg isolated EPS were dissolved in 50 mM sodium acetate buffer, pH 5.0, to a concentration of 2.0 g l^{-1} and centrifuged (10 000 *g*, 10 min., 4 °C) to remove insoluble residues. The EPS fractions were purified by size-exclusion chromatography (SEC; Biopilot System, Pharmacia) using a Sephacryl S-500 column (length 1020 mm × internal diameter 100 mm, Pharmacia). The column was eluted with 50 mM sodium acetate buffer pH 5.0 at a flow rate of 37 ml min^{-1} and peaks were detected with a refractive-index detector (Shodex RI-72, Separations Analytical Instruments, Hendrik Ido Ambacht, The Netherlands) and with a UV detector (Pharmacia) at 280 nm. Fractions of 210 ml were collected using a Super-Frac fraction collector (Pharmacia). All isolated fractions were analyzed for their neutral sugar content using the orcinol/sulfuric acid method [153] on a Skalar autoanalyzer (Skalar Analytical, Breda, The Netherlands), with galactose as a standard. Fractions containing a sugar peak were pooled, concentrated at 37 °C using a vacuum-evaporator, dialyzed at 4 °C against running tap water (24 h) and distilled water (48 h, 5 × 10 l) and lyophilized.

Sugar composition

Purified EPS fractions were pre-hydrolyzed with 72% (w/w) H_2SO_4 (1 h, 30 °C) and hydrolyzed with 1M H_2SO_4 (3 h, 100 °C) and the sugars released were converted into alditol acetates [35; 156]. The carboxyl groups were reduced using $NaBH_4$ prior to acetylation. Alditol acetates were separated by gas chromatography on a 15 m × 0.53 mm DB 225 column (J & W Scientific, Folsom, Calif., USA) using a Carlo Erba 4200 GC (Carlo Erba Strumentazione, Rodano, Italy).

The oven temperature was set at 230 °C and the flame ionization detector at 260 °C. Hydrogen was used as carrier gas. Inositol was used as an internal standard. Uronic acid was determined using the *m*-hydroxydiphenyl assay as described by Thibault [149].

Sugar linkage analysis

The relative amount and type of glycosidic linkages in the EPS were detected by methylation analysis. Approximately 5 mg purified EPS fractions were dried for 24 h in vacuo in the presence of P_2O_5, dissolved in 1 ml dimethyl sulfoxide and methylated using 1 ml dimethylsulphinyl carbanion and 1 ml iodomethane according to a modified Hakomori method [55; 126]. The methylated EPS fractions were dissolved in methanol/chloroform 1:1 (vol/vol), dialyzed against running tap water (24 h) and distilled water (48 h, 5 × 10 l) and hydrolyzed in 2 M trifluoroacetic acid (1 h, 121 °C). The partially methylated monomers were neutralized using 0.1 ml 1.5 M NH_3, reduced with 0.1 ml 0.5 M NH_3 containing 150 g l^{-1} $NaBH_4$ and acetylated with 0.45 ml 1-methyl imidazole and 3 ml acetic acid anhydride. The partially methylated alditol acetates were extracted with dichloromethane, washed with distilled water, dried in a stream of air at < 10 °C, washed with acetone and dried again. Finally they were dissolved in ethyl acetate and analyzed by gas/liquid chromatography. Peaks were identified on the basis of their relative retention times using inositol as an internal standard. The identity of the components was confirmed by gas chromatography mass spectrometry (GC-MS) [126; 156].

Results

Growth and EPS production in continuous culture

To determine the effect of fructose and glucose on the amount and composition of the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus*, the strain was grown in a continuous culture with defined medium supplemented with either 139 mM fructose or 139 mM glucose. EPS were isolated from the supernatant of cultures growing under steady state conditions. *Lb. delbrueckii* subsp. *bulgaricus* produced 30 mg l^{-1} EPS when grown on fructose and 95 mg l^{-1} EPS when grown on glucose at an absorbance at 600 nm of 2.8 and 3.0, respectively.

Molecular weight analysis of the EPS

The measurements on the molecular weight (mol. wt.) and intrinsic viscosity of the EPS produced on glucose showed that the EPS were composed of two populations, which appeared to have mol. wt. values of 1.7×10^6 and 4.2×10^4 and intrinsic viscosities of 4.3 and 0.3 dl g^{-1} respectively. The EPS produced on fructose showed the presence of two peaks with mol. wt. = 1.3×10^6 and 3.8×10^4. The intrinsic viscosities of both EPS fractions were slightly lower than those of the EPS fractions of glucose-grown cultures. In the EPS produced on glucose, the high-mol. wt. and the low-mol. wt. fractions were present in almost equal amounts, whereas the EPS produced on fructose were mainly composed of the low-mol. wt. fraction (Table 6.1).

Table 6.1: Molecular weight (mol. wt.) and intrinsic viscosity of the exopolysaccharides (EPS), produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown on either glucose or fructose, obtained by high-performance size-exclusion chromatography measurement. Each value represents the results of duplicate measurements and varied from the mean by not more than 5%.

EPS fraction	Relative concentration (%)	mol. wt.	Intrinsic viscosity (dl g^{-1})
Glucose, high-mol. wt.	51.5	1.7×10^6	4.3
Glucose, low-mol. wt.	48.5	4.2×10^4	0.3
Fructose, high-mol. wt.	13.6	1.3×10^6	3.3
Fructose, low-mol. wt.	86.4	3.8×10^4	0.2

Purification of the different EPS peaks

The EPS produced on glucose and on fructose were dissolved in sodium acetate buffer and insoluble parts were removed by centrifugation prior to applying the supernatants on the SEC column. All fractions containing sugar peaks were pooled and purified. The high-mol. wt. fraction of the EPS produced on glucose showed a low absorption at 280 nm, which points to a low amount of protein. The high-mol. wt. fraction of the EPS produced on fructose showed a slightly higher absorption at 280 nm. Both low-mol. wt. fractions appeared to have no absorption at 280 nm.

Table 6.2: Sugar compositions of the purified EPS fractions from *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture with glucose or fructose as the carbohydrate source. The maximum deviation between duplicate measurements was 2%.

EPS fraction	Composition of EPS (mol %)		
	Galactose	Glucose	Rhamnose
Glucose, high-mol. wt.	70	15	15
Glucose, low-mol. wt.	89	8	3
Fructose, high-mol. wt.	65	20	15
Fructose, low-mol. wt.	84	13	3

Sugar composition

The sugar compositions of the purified EPS fractions were determined after hydrolysis and conversion into alditol acetates. Table 6.2 shows that the high-mol. wt. fraction of the EPS produced on glucose contained galactose, glucose and rhamnose monomers in a molar ratio of approx. 5:1:1 respectively. The low-mol. wt. fraction of the EPS produced on glucose contained mainly galactose and glucose, whereas rhamnose was only present in a very low amount, resulting in the molar ratio of approximately 11:1:0.4. The sugar compositions of the high-mol. wt. fraction

and the low-mol. wt. fraction produced on fructose were comparable to those found in the EPS fractions produced on glucose, although the galactose to glucose ratio varied slightly. Other neutral sugars and uronic acids could not be detected.

Table 6.3: Sugar linkage composition of the purified EPS fractions from *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture on glucose and fructose. Each value represents the average of duplicate measurements. Gal, galactose; Glc, glucose; Rha, rhamnose. The ratio is calculated by dividing the percentage terminally linked sugars by the percentage branched sugars

Linkage type	Composition (mol %)			
	Glucose, high-mol. wt. fraction	Glucose, low-mol. wt. fraction	Fructose, high-mol. wt. fraction	Fructose, low-mol. wt. fraction
(Gal)1→	27.9	8.7	33.7	11.1
→2(Gal)1→	4.5	-	-	-
→3(Gal)1→	6.7	32.3	1.3	27.1
→4(Gal)1→	-	-	1.1	8.7
→6(Gal)1→	1.6	37.8	0.5	26.5
↑3 →2(Gal)1→	9.3	4.6	9.9	5.5
↑3 →4(Gal)1→	22.6	7.4	27.0	8.8
→3(Glc)1→	9.6	4.5	10.8	6.5
(Rha)1→	17.9	4.7	15.7	5.7
Terminally linked (%)	45.8	13.4	49.4	16.8
Branched (%)	31.9	12.0	36.9	14.3
Ratio	1.44	1.12	1.34	1.17

Methylation analysis

The sugar linkage compositions of the purified EPS fractions were determined by methylation analysis. As shown in Table 6.3, the high-mol. wt. EPS fractions produced on either glucose or fructose contained mainly terminally (1-) linked galactose and 1,3,4-linked galactose with lower amounts of 1,2,3-linked galactose, 1,3-linked glucose and terminally linked rhamnose. Trace amounts of linearly linked galactose residues were also detected. The low-mol. wt. EPS fractions contained mainly 1,3-linked galactose and 1,6-linked galactose residues, with a lower amount of terminally linked galactose, 1,2,3-linked galactose, 1,3,4-linked galactose, 1,3-linked glucose and terminally linked rhamnose. In the low-mol. wt. EPS fraction produced in fructose-grown cultures 1,4-linked galactose residues were also found.

Discussion

In previous papers we reported that *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown on fructose produced a lower amount of EPS than grown on glucose or lactose and there were differences in the monomeric sugar composition of the EPS [48; 48]. It appeared that these differences were independent of the growth rates on these substrates (data not shown). A possible explanation of this observation might be that this strain produces more than one type of EPS. Cerning et al. [21] suggested that *Lb. casei* produces different types of EPS depending on the carbon source. Two extracellular polysaccharides with different sugar compositions were isolated from *Serratia marcescens*: one had a relatively high content of rhamnose monomer, whereas the other contained no rhamnose [1].

Molecular weight analysis of the EPS showed the presence of a high-mol. wt. fraction (approx. 1.7×10^6) and a low-mol. wt. fraction (approx. 4×10^4). The production of the high-mol. wt. fractions of the EPS appeared to be dependent on the carbohydrate source, whereas the low-mol. wt. fractions were produced more independently of the growth substrate. Cerning et al. [17] found that *Lb. delbrueckii* subsp. *bulgaricus* strain 416a produced a single polysaccharide with a mol. wt. of approximately 5×10^5. The mol. wt. of the EPS produced by strain CRL 420 appeared to be 2×10^5 [89]. Cerning [14] found that *Streptococcus thermophilus* strains produced 2 different polymers at the same time with molecular mol. wt. = 2×10^6 and 3.5×10^4, the EPS fractions appeared to have an identical sugar composition and the latter was possibly a degradation product. Marshall et al. [92] found that *Lactococcus lactis* subsp. *cremoris* LC330 was able to produce two polysaccharides concurrently, with mol. wt. $> 1 \times 10^6$ and 1×10^4, and with different sugar compositions. In contrast to the large polysaccharide, the production of the smaller polysaccharide by this strain was not affected by changes in growth conditions and it appeared to be associated with the cell wall. The EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 were isolated by a usual procedure for the isolation of extracellular (unattached) polysaccharides. Therefore, it is unlikely that capsular polysaccharides are isolated using this procedure, since capsular polysaccharides have often to be detached from cell walls, although the association of the capsular polysaccharides with cell walls is dependent on the bacterial strain [14; 141]. In *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772, the intrinsic viscosities of the high-mol. wt. EPS fractions were comparable with the intrinsic viscosity of purified EPS of *Lb. delbrueckii* subsp. *bulgaricus*, obtained by Cerning [16]. The low-mol. wt. fractions showed almost no thickening properties. Therefore, it is expected that EPS produced on glucose are probably more viscous than an equal amount of the isolated EPS produced in fructose-grown cultures.

The results of our methylation analyses indicate that the high-mol. wt. fractions of the EPS produced on fructose and glucose have a repeating unit that is composed of terminally linked galactose, 1,2,3-linked galactose, 1,3,4-linked galactose, 1,3-linked glucose and terminally linked rhamnose residues. The repeating unit of the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* rr [54] showed a similar sugar linkage composition and it is very possible that the high-mol. wt. fractions of the EPS of our strain and the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* strain rr have repeating units with identical structures, although we did not perform any measurements to determine the order of the glycosidic residues of the EPS repeating unit. The low-mol. wt. fractions of the EPS produced on glucose and fructose contained high amounts of 1,3-linked galactose and 1,6-linked galactose residues. These were not, or only in trace amounts, found in the high-mol. wt. fractions. The relative amount of branched and terminally linked sugar residues were significantly lower in the low-mol. wt. fractions, so that they appeared to be more linear than the high-mol. wt. EPS fractions. It is concluded that the production of both EPS fractions is regulated in different ways. In all EPS fractions investigated with the methylation analysis, the amount of glucose residues was lower than might be expected in comparison to the neutral sugar

compositions (Table 6.2) and the monomeric sugar compositions found in earlier work [48; 49]. On the other hand, the relative amount of rhamnose was higher than expected. This may be explained by the fact that the results obtained by methylation analysis give more qualitative rather than quantitative information [130]. In contrast to our previous results, it was observed that the amount of galactose residues in the EPS produced on fructose was higher than in the EPS produced on glucose. It is still unclear why this discrepancy occurred. Measurements of the sugar composition of the different EPS fractions purified in this work, using the HPLC method described before [48], gave similar results (Table 6.2) to those obtained using the alditol acetate method (data not shown). Since the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown on fructose were mainly composed of the low-mol. wt. fraction, it is concluded that this is apparently responsible for the low concentration of rhamnose in the total EPS produced on fructose [49].

Analysis of the activities of key enzymes leading to the production of sugar nucleotides indicated that, following growth on fructose, no activity could be detected of enzymes involved in the synthesis of dTDP-rhamnose. Therefore, we concluded that the absence of these activities resulted in the absence of rhamnose in the EPS produced on fructose [49]. In this work, however, we found that *Lb. delbrueckii* subsp. *bulgaricus* grown on fructose produced EPS containing rhamnose monomers. Therefore, the enzymes involved in the production of dTDP-rhamnose must be active when grown on fructose, although their activities may be below the detection level of the enzyme assays described in our previous work [49].

In conclusion, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 is able to produce two types of EPS concurrently, which differ in molecular size, intrinsic viscosity and sugar composition. The production of the large polysaccharide is dependent on the carbohydrate source, whereas the small polysaccharide is probably produced more continuously. It is unclear in what way the production of the two types of EPS is regulated in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772, so further investigations will be needed to clarify the biosynthesis of both EPS fractions and how this biosynthesis is regulated.

7.
Concluding remarks

In this thesis, the exopolysaccharide (EPS) production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 was studied. The investigations were focused on the composition of a chemically defined growth medium and the influence of variations of the composition of the growth medium on the EPS production. It was shown that the carbohydrate source is a very important factor. It had a strong impact on the amount, the monomeric sugar composition and the structure of the exopolysaccharides. Furthermore, it affected the activities of key enzymes involved in the synthesis of sugar nucleotides.

1. Influence of growth conditions on EPS production

1.1 Exopolysaccharide production in a defined medium

Like other lactic acid bacteria, *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 requires very complex growth media. However, for investigations on the influence of the medium composition on growth and EPS production, a chemically defined medium is essential. In this thesis, a chemically defined medium was composed in which the strain was able to grow and produce considerable amounts of EPS (Chapter 2). It was found that growth, lactate fermentation and EPS production were strongly related and that no EPS was produced after cell growth had ceased. A higher polymer production took place at higher incubation temperatures: this was in contrast to most other lactic acid bacteria, which produce more EPS at lower temperatures. As already was stated for other thermophilic lactic acid bacteria, the ability to produce EPS was stable in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772, since the genes involved in the polymer production are chromosomally linked, this in contrast to mesophilic lactic acid bacteria that rapidly lose the EPS producing ability, due to the plasmid-linked genes involved in the production of EPS [155; 157]. It was found that the EPS producing ability of the strain grown in continuous cultures (at 40 °C, pH 6.0 ± 0.1 and D 0.075 h^{-1}) was stable for at least three months (data not shown). Therefore, continuous cultures have shown to be very useful to perform experiments on the influence of growth conditions on the EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772.

1.2 Influence of the medium composition.

a. Carbohydrate source. The production of EPS was highest when *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 was grown in continuous culture or pH-controlled batch culture with non-limiting amounts of glucose or lactose as the carbohydrate source. It was shown that when grown on lactose, only the glucose monomer was utilized, whereas galactose accumulated in the growth medium. The galactose monomers were apparently not used for the production of EPS, since the amount and monomeric sugar composition of the exopolysaccharides produced on lactose and on glucose were identical. When the strain was grown on fructose, the amount of polysaccharide produced was considerably lower than when grown on glucose and lactose, although growth yields were comparable.

b. Vitamins. A multiple omission of all vitamins individually not required for growth of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 resulted in a twofold enhancement of the EPS

production in batch cultures, although a multiple vitamin omission caused a reduction of the growth (Chapter 4). The role of vitamins in the polymer production is unclear. Sutherland [142] postulated that in bacteria growing slowly, cell wall biosynthesis is also slow. In this way, more isoprenoid carrier and sugar nucleotides can be made available for extracellular polysaccharide synthesis. This was found in some bacteria grown under non-optimal conditions, like at low growth temperatures. Therefore, a multiple omission of vitamins might result in a change of the carbon flow in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 more directed to the production of polymer instead of cell wall material. However, such an effect caused by vitamin omissions has never been shown before in polysaccharide producing bacteria. Other growth conditions causing reduction of the growth of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 did not show such effects.

2. Biosynthesis of sugar nucleotides

In *S. thermophilus*, it was found that the sugar composition of the EPS is strongly dependent on the activities of sugar nucleotide synthesizing enzymes [113]. In our strain however, it was found that the amount and ratio of UDP-glucose and UDP-galactose in cell extracts of both fructose-grown and glucose-grown cells were almost identical, although the strain produced considerably more EPS when grown on glucose. The activities of dTDP-rhamnose synthetic enzymes may have an effect on the composition of the EPS. In fructose-grown cells, no activity of these enzymes was detected. Since a low amount of rhamnose monomer was found in the EPS produced on fructose (Chapter 6), the enzymes involved in the production of dTDP-rhamnose must be active, but these activities may be below the detection level with the assays performed in Chapter 5. In glucose-grown cultures, the activities of dTDP-rhamnose synthesizing enzymes were detectable, and the EPS contained more rhamnose residues. Unfortunately, we were not able to measure levels of dTDP-glucose and dTDP-rhamnose in cell extracts. The ratio of UDP-glucose and UDP-galactose in the cells is caused by the relatively high activity of UDP-galactose 4-epimerase, having an equilibrium of UDP-glucose and UDP-galactose of 3:1. This ratio is apparently not important for the composition of the EPS, since more UDP-galactose is incorporated in the EPS repeating unit.

Sugar nucleotides are not only important for the production of EPS, but they also play a role in the synthesis of cell walls. Therefore, it may be interesting to investigate the influence of the carbohydrate source on the sugar composition of the cell walls of *Lb. delbrueckii* subsp. *bulgaricus* in relation to the composition of the EPS.

3. Structure of the EPS repeating unit

Depending on the carbohydrate source, two types of EPS were produced concurrently with different molecular weights and structure (Chapter 6). A fraction with a high molecular weight was produced on glucose but almost not on fructose, whereas a low molecular weight fraction was produced almost independently of the carbon source. A *Lb. delbrueckii* subsp. *bulgaricus* strain having the same ability has never been described before. *Lb. delbrueckii subsp. bulgaricus* strain rr was shown to produce an exopolysacccharide with most probably an identical composition of the repeating unit as the high molecular weight fraction of the EPS produced by strain NCFB 2772, but this strain apparently produces only one type of polymer [54].

It seems that *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 produces EPS with a lower amount of rhamnose when the optical density of the cultures is low. In Chapter 2, it was found that in the

early logarithmic growth phase, the EPS contained a higher amount of glucose, compared to rhamnose residues. When manganese or phosphate ions were present in growth limiting amounts, the same phenomenon was observed (Chapter 3). It may be possible that at low cell densities, the production of the low molecular weight fraction is favored compared to the production of the high molecular weight fraction.

Up to now, it is unclear how the production of these two different exopolysaccharide fractions is genetically and enzymatically regulated. Since the genetics of EPS production by *Lb. delbrueckii* subsp. *bulgaricus* strains is unexplored, it is hard to predict whether one or more *eps* gene clusters are involved in the production of the EPS fractions. The genes coding for enzymes responsible for the synthesis of the EPS repeating unit are of great importance. Although the total amount of sugar nucleotides in the cells grown on fructose and glucose is comparable, the production of the EPS fractions is considerably different. Therefore, the biosynthesis of the repeating units of both EPS types is probably regulated by different gene clusters and enzymes. This is a very interesting item for further research on the genetic aspects of EPS production by this strain.

4. Application of EPS-producing lactic acid bacteria

Due to the inefficient energy generation from carbohydrates by fermentative organisms, lactic acid bacteria producing extracellular heteropolysaccharides can synthesize only relatively low amounts of EPS, compared to organisms with a respiratory metabolism like *Xanthomonas campestris*. Therefore, the application of EPS produced by lactic acid bacteria as food additives will be economically unfavorable and the advantage of the utilization of food-grade organisms will be overcome by the high amounts and low costs of EPS produced by other micro-organisms. The in situ production of EPS by lactic acid bacteria as starter cultures in the manufacture of fermented food products is therefore an important aspect for applications, since the organisms can bring about ropiness of the product itself.

Not only the amount, but also the structure of the EPS and interactions with other medium components like proteins play an important role in the improvement of the texture, so the functionality of EPS in foods is hard to predict. Therefore, more studies on the structure and rheology of the EPS in relation to the structure of fermented food products should be carried out. Before results obtained by the work described in this thesis can be applied in the dairy industry, more investigations on the role of EPS in yogurt must be performed. Relevant aspects would be the influence of the *S. thermophilus* strain on the growth and EPS production by *Lb. delbrueckii* subsp. *bulgaricus*, or interactions of EPS with milk proteins. Furthermore, the role of vitamins must be clarified, since it will be a difficult and expensive process to remove vitamins or other components from milk, in order to get a higher polymer production.

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Summary

Extracellular polysaccharides (EPS) produced by lactic acid bacteria have gained great popularity in the food industry because of their thickening and stabilizing properties. Since lactic acid bacteria have the GRAS (Generally Recognized As Safe) status, these organisms and their products can be applied as food additives. In particular in the dairy industry, EPS producing lactic acid bacteria are used to improve the texture and viscosity of fermented milk products, like yogurt, viili and långfil and the prevention of syneresis (wheying-off).

Many lactic acid bacteria producing EPS have already been studied, but since they have numerous medium requirements, including carbohydrates, amino acids, vitamins, purines, pyrimidines and mineral salts, most investigations on the production of EPS were performed using very complex growth media, like milk, whey ultrafiltrate or complex synthetic media. However, these media are often not suitable when the influence of individual medium components on growth and EPS production is studied. Therefore, a chemically defined growth medium is required, containing all components necessary for growth and EPS production.

In this thesis, the influence of physiological factors and the medium composition on the growth, EPS production and the sugar composition of the EPS produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 is studied in detail. This lactic acid bacterium is, together with *Streptococcus thermophilus*, used in the manufacture of yogurt. In Chapter 2, a chemically defined medium is composed, in which the strain was able to grow and produce EPS. The exopolysaccharides were composed of glucose, galactose and rhamnose in the ratio 1 : 6.8 : 0.7. The EPS production was growth-related, and an excess of carbohydrate did not result in an increased polymer production. It was found that production of EPS was higher with glucose or lactose as the carbohydrate source, than when fructose was used. The amount and composition of the EPS produced on glucose and lactose were identical, the galactose monomer from lactose was not used for growth and polymer production. Limiting amounts of manganese and phosphate in the growth medium resulted in a lower EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 (Chapter 3). The amount of rhamnose monomer in the EPS was slightly lower than when manganese and phosphate were present in non-limiting concentrations.

In Chapter 4, the medium requirements of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 were studied using the technique of single and multiple omissions of medium components. Surprisingly, a multiple omission of vitamins from the growth medium caused a significant enhancement of the EPS production. A simplified defined medium was composed, from which many components were omitted which were individually not required for growth. Although the strain grew less well in the simplified medium, the amount of EPS produced was twofold higher than in the complete medium. Furthermore, the EPS production beyond the stationary growth phase was stronger when grown in the simplified medium.

The influence of the carbohydrate source on the production of EPS and the activities of enzymes involved in the production of sugar nucleotides as precursors for EPS biosynthesis is described in Chapter 5. *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 produced four times more EPS when grown on glucose than when fructose was used and it was found that the EPS produced on fructose contained no rhamnose monomer. Activities of enzymes involved in the synthesis of UDP-glucose, UDP-galactose and dTDP-rhamnose were observed when the strain was grown on glucose. But when grown on fructose, no activities were detected of enzymes leading to the production of dTDP-rhamnose and this may lead to the absence of rhamnose in the EPS produced on fructose. On the other hand, the amount of UDP-glucose and UDP-galactose in extracts of cells grown on fructose and glucose were comparable. Apparently the differences in the amount of EPS

produced on glucose and fructose are not caused by the activities of the enzymes leading to the production of these sugar nucleotides.

Lb. delbrueckii subsp. *bulgaricus* NCFB 2772 grown on glucose produces two types of EPS concurrently and in almost equal amounts, with molecular weights of 1.7×10^6 and 4×10^4 (Chapter 6). The exopolysaccharides produced with fructose as the carbohydrate source were composed of mainly a fraction with a low molecular weight of 4×10^4. The high- molecular-weight fractions were composed of branched repeating units with glucose, galactose and rhamnose in the ratio 1 : 5 : 1, whereas the low-molecular-weight fractions were more linear and contained glucose, galactose and rhamnose in the ratio 1 : 11 : 0.4. The production of the high-molecular-weight fraction was dependent on the carbohydrate source, whereas the low-molecular-weight fraction was produced more continuously. Since a low amount of rhamnose monomer was found in the EPS produced on fructose, the enzymes involved in the production of dTDP-rhamnose are active, in contrast to the results obtained in Chapter 5, but these activities may be below the detection level of the enzyme assays performed.

Lb. delbrueckii subsp. *bulgaricus* NCFB 2772 produces EPS when grown in a defined medium and the amount of EPS is dependent on the carbohydrate source and the presence or absence of several vitamins. For future research, it would be interesting to study the genetics and enzymology of the assembly of the different EPS repeating units in fructose-grown and glucose-grown cells and in cells grown at low optical densities. Furthermore, the mechanisms of the enhanced EPS production as a result of multiple vitamin omissions may be an interesting topic for future research.

Samenvatting

In de voedingsmiddelenindustrie is het gebruik van extracellulaire polysacchariden (EPS) geproduceerd door melkzuurbacteriën zeer populair, vanwege hun verdikkende en stabiliserende eigenschappen. Omdat melkzuurbacteriën de zogenaamde GRAS (Generally Recognized As Safe)-status hebben, kunnen deze micro-organismen en hun producten worden toegepast als voedseladditieven. EPS producerende melkzuurbacteriën worden met name toegepast in de zuivelindustrie bij de bereiding van gefermenteerde melkproducten, zoals yoghurt, viili en långfil om de textuur en viscositeit van deze producten te verhogen en synerese (afscheiding van wei) tegen te gaan.

Verscheidene onderzoeken aan EPS-producerende melkzuurbacteriën zijn al verricht, maar melkzuurbacteriën hebben complexe groeimedia nodig, met daarin koolhydraten, aminozuren, vitaminen, purines, pyrimidines en minerale zouten. Daarom zijn de meeste onderzoeken uitgevoerd in melk, wei-ultrafiltraat of complexe synthetische media, maar deze kweekmedia zijn in het algemeen niet geschikt als de invloed van individuele mediumcomponenten op de groei en EPS-productie wordt bestudeerd. Derhalve is een chemisch gedefinieerd medium noodzakelijk, met daarin alle componenten noodzakelijk voor de groei en EPS productie.

In dit proefschrift is nader onderzoek verricht naar de invloed van fysiologische factoren en de mediumsamenstelling op de groei, EPS-productie en de suikersamenstelling van EPS geproduceerd door *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772. Samen met *Streptococcus thermophilus* wordt deze gebruikt in de bereiding van yoghurt. In Hoofdstuk 2 is een chemisch gedefinieerd medium beschreven, waarin *Lb. delbrueckii* subsp. *bulgaricus* in staat was te zich te vermeerderen en EPS te produceren. De exopolysacchariden waren samengesteld uit glucose, galactose and rhamnose in de verhouding 1 : 6.8 : 0.7. De synthese van EPS was gebonden aan de groei van de micro-organismen en een overmaat aan koolhydraten in het medium leidde niet tot een hogere EPS-productie. De productie van EPS was hoger met glucose of lactose als koolstofbron, dan met fructose. De hoeveelheid en suikersamenstelling van EPS geproduceerd op glucose en lactose waren identiek, bij groei op lactose werd alleen het glucosemonomeer gebruikt. Een limiterende hoeveelheid mangaan of fosfaat in het kweekmedium zorgde voor een verlaging van de EPS-productie. Het gehalte aan rhamnose monomeren in EPS was iets lager dan wanneer mangaan en fosfaat in voldoende mate aanwezig waren (Hoofdstuk 3).

In Hoofdstuk 4 zijn de mediumbehoeften voor groei van *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 nader bekeken door een of meer mediumcomponenten weg te laten. Het tegelijkertijd weglaten van verschillende vitaminen leidde tot een aanmerkelijke verhoging van de EPS-productie. Een vereenvoudigd gedefinieerd medium werd samengesteld, waaruit diverse componenten waren weggelaten die individueel niet nodig bleken te zijn voor de groei. Hoewel de groei minder was in dit medium, was de hoeveelheid geproduceerd EPS twee keer hoger dan in het complete medium. Verder ging de EPS-productie in het vereenvoudigde medium na het bereiken van de stationaire groeifase veel sterker door.

De invloed van de koolstofbron op de productie van EPS en de activiteit van enzymen betrokken bij de synthese van suikernucleotiden als precursor voor EPS-synthese is beschreven in Hoofdstuk 5. *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 produceerde vier keer meer EPS bij groei op glucose dan wanneer fructose werd gebruikt, terwijl in EPS geproduceerd op fructose geen rhamnose monomeren aanwezig waren. Enzymen in de synthese van UDP-glucose, UDP-galactose en dTDP-rhamnose waren aktief in glucosegekweekte cellen, maar in fructosegekweekte cellen werd geen enzymactiviteit gevonden in de synthese van dTDP-rhamnose. Dit zou kunnen leiden tot de afwezigheid van rhamnose in het exopolysaccharide geproduceerd met fructose. Aan de andere kant was het gehalte aan UDP-glucose en UDP-galactose in celextracten van *Lb.*

delbrueckii subsp. *bulgaricus* NCFB 2772 gekweekt op fructose en glucose even hoog. Blijkbaar wordt het verschil in de hoeveelheid geproduceerd EPS tussen glucosegekweekte en fructosegekweekte cellen niet veroorzaakt door de activiteit van de enzymen betrokken bij de synthese van deze suikernucleotiden.

Lb. delbrueckii subsp. *bulgaricus* NCFB 2772 produceert bij groei op glucose twee typen exopolysacchariden tegelijkertijd en in ongeveer gelijke hoeveelheden, met respectievelijke molecuulgewichten van 1.7×10^6 and 4×10^4 (Hoofdstuk 6). De exopolysacchariden geproduceerd met fructose als koolstofbron bestonden voornamelijk uit een fractie met een laag molecuulgewicht van 4×10^4. De fracties met een hoog molecuulgewicht waren samengesteld uit vertakte repeterende eenheden met glucose, galactose and rhamnose in de verhouding 1 : 5 : 1, terwijl de fracties met het lage molecuulgewicht meer lineair waren en glucose, galactose and rhamnose bevatten in de verhouding 1 : 11 : 0.4. De productie van de fracties met het hoge molecuulgewicht was afhankelijk van de gebruikte koolstofbron, terwijl de productie van de fracties met een laag molecuulgewicht meer onafhankelijk van de koolstofbron was. Er werd echter wel een kleine hoeveelheid rhamnose monomeren gevonden in het EPS geproduceerd op fructose. Dit duidt erop dat de enzymen, die verantwoordelijk zijn voor de productie van dTDP-rhamnose, wel actief zijn bij groei op fructose. Dit is in tegenstelling tot de resultaten beschreven in Hoofdstuk 5, maar de enzymactiviteiten kunnen bij de gebruikte assays beneden het detectieniveau hebben gelegen.

Lb. delbrueckii subsp. *bulgaricus* NCFB 2772 produceert EPS in een gedefinieerd medium en de hoeveelheid EPS is afhankelijk van de koolstofbron en de aan- of afwezigheid van diverse vitaminen. Voor toekomstig onderzoek is het wellicht interessant om onderzoek te verrichten aan de enzymologie en genetica van de vorming van de repeterende eenheden in glucose- en fructosegekweekte cellen en in cellen gekweekt bij lage optische dichtheden. Daarnaast zijn de mechanismen van de verhoogde EPS-productie veroorzaakt door het weglaten van verschillende vitaminen nog onduidelijk en wellicht een interessant onderwerp voor verder onderzoek.

Nawoord

Het proefschrift dat voor u ligt is het resultaat geworden van een stuk werk dat mij de afgelopen jaren niet alleen veel bloed, zweet en tranen heeft gekost, maar bovenal veel plezier heeft gegeven. Het is het resultaat geworden van ruim vijf jaar experimenten bedenken en uitvoeren, medium maken, prutsen met fermentoren, frustraties door infecties of mislukte experimenten, voldoening door goede resultaten, het draven van verdieping naar verdieping in het Biotechnion, het schrijven van verslagen en artikelen, het begeleiden van studenten tijdens afstudeervakken en practica, excursies, congressen en vooral het dagelijkse leven bij IM. Ik heb veel directe en indirecte steun van diverse personen gehad die ik graag zou willen bedanken. Een aantal van hen wil ik met name in dit nawoord noemen.

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Curriculum vitae

Gerrit Jan Grobben werd geboren op 21 december 1963 te Hengelo (Ov.). Hij behaalde het VWO (Gymnasium-β) diploma aan de Openbare Scholengemeenschap Bataafse Kamp te Hengelo (Ov.) en begon in 1983 met de studie Biologie aan de Rijksuniversiteit Groningen. In februari 1989 werd de studie afgerond met als hoofdrichtingen Microbiële Fysiologie en Biochemie. Van februari 1989 tot oktober 1989 was hij als vrijwilliger werkzaam bij de werkgroep Microbiële Fysiologie, Rijksuniversiteit Groningen. Tussen november 1989 en december 1990 vervulde hij zijn militaire dienstplicht als chauffeur/radiotelefonist bij de Koninklijke Landmacht. Van mei 1991 tot februari 1992 was hij werkzaam als onderzoeksmedewerker bij de werkgroep Microbiële Oecologie, Rijksuniversiteit Groningen. Vervolgens was hij van 1992 tot 1996 als Assistent in Opleiding verbonden aan de sectie Industriële Microbiologie van de Landbouwuniversiteit Wageningen, en verrichte het onderzoek dat is beschreven in dit proefschrift. Sinds 1 februari 1998 is hij werkzaam als wetenschappelijk onderzoeker aan het Instituut voor Agrotechnologisch Onderzoek (ATO-DLO) in Wageningen.

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