Expolysaccharides from Lactic Acid Bacteria: Technological Bottlenecks and Practical Solutions

Luc De Vuyst*, Bart Degeest

Division of Industrial Microbiology, Fermentation Technology and Downstream Processing (IMDO), Department of Biotechnology, Faculty of Sciences, Vrije Universiteit Brussel (VUB), Pleinlaan 2, B-1050 Brussels, Belgium
tel.: +32-2-6293245, fax: +32-2-6292720, e-mail: ldvuyst@vub.ac.be

SUMMARY: Microbial exopolysaccharides are added to or occur in a wide variety of food products, where they serve as viscosifying or gelling agents. Exopolysaccharides with different composition, size and structure are synthesized by several strains of lactic acid bacteria. Structural analyses combined with rheological studies reveal that there is considerable variation among the different exopolysaccharides; some of them exhibit remarkable thickening and shear-thinning properties and display high intrinsic viscosities. Hence, several slime-producing lactic acid bacterium strains and their biopolymers have interesting functional and technological properties, which may be exploited towards different products. However, the production of exopolysaccharides from lactic acid bacteria is low and often unstable, and their downstream processing is difficult. Strain improvement, enhanced productivities and advanced modification and production processes (both enzyme and fermentation technology) may contribute to their economic soundness.

Introduction

Most of the biothickeners in current use by the food industry are polysaccharides from plants (e.g. starch, pectin, locust bean gum, guar gum) or seaweeds (carrageenan, alginate). The

 CCC 1022-1360/99/$ 17.50+.50/0

animal hydrocolloids gelatin and casein (proteins) are used too. Their functional properties in foods are determined by quite subtle structural characteristics[1,2]. However, these polysaccharides are not always readily available in the quality needed or their rheological properties may not exactly match those required. Most of the plant polysaccharides used are chemically modified to improve their structure and rheological properties. Their use is hence strongly restricted and, in the European Union, for food applications the food products need to be labelled with an E-number. An alternative class of biothickeners are the microbial exopolysaccharides (EPS). Examples are dextrans, xanthan, gellan, yeast glucans, pullulan, bacterial alginates, etc. However, microbial EPS represent only a small fraction of the current biopolymer market. A disadvantage of using microbial EPS is the high costs of recovery. Xanthan is a microbial EPS approved in the European food industry, mainly because of its unique rheological properties in foods. It is produced by *Xanthomonas campestris*, a phytopathogenic bacterium that is not GRAS (Generally Recognized As Safe). EPS from both mesophilic and thermophilic lactic acid bacteria (LAB) (GRAS microorganisms) have potential for development as functional food ingredients with both health and economic benefits[3-5]. For instance, the creamy, smooth texture is one of the aspects of the quality of yoghurt which seems to be improved by the ability of the yoghurt bacteria to form EPS, even though only small amounts of EPS are being produced.

Exopolysaccharides from lactic acid bacteria

EPS from LAB are composed of branched repeating units consisting of mainly galactose, glucose and rhamnose, in different ratios[3-5]. They possess apparent molecular masses that range from 0.5×10^6 to 2.0×10^6 Da. Repeating unit structures of several EPS produced by LAB have been elucidated recently[6-18] (Fig. 1). Their size may range from di- to heptasaccharide. They furthermore show few common features, which raises the question about the relationship between these EPS structures and the texturising properties they confer. It would be of interest to understand this relationship and to have the means to modify the biopolymers to influence the properties of the native EPS. Enzymes having a specific action on EPS from LAB might be used for tailoring the chemical structure and hence the functional properties. These data in combination with additional biochemical, molecular biological and process engineering studies of EPS from LAB can form the basis for future 'polysaccharide engineering'.

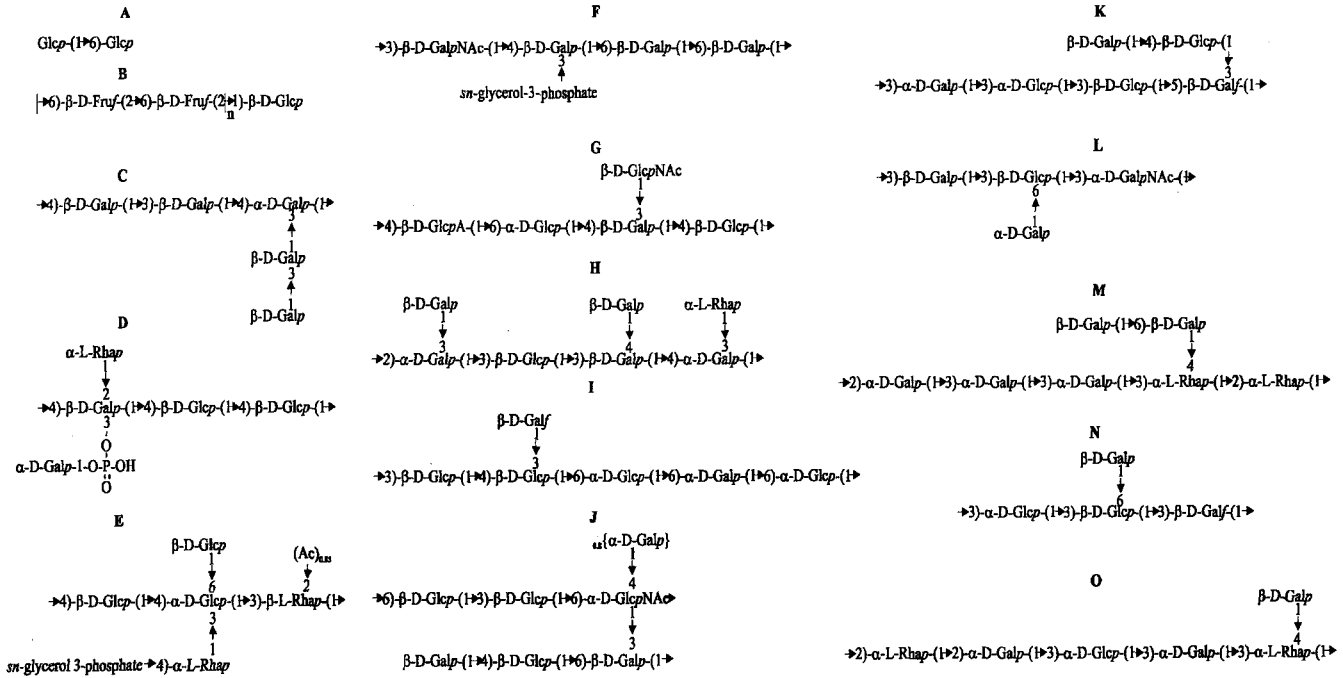

Fig. 1. Primary structure of EPS produced by LAB: *(i)* homopolysaccharides: (A) dextran, (B) levan, (C) polygalactan, *L. lactis* subsp. *cremoris* H414[8]; *(ii)* heteropolysaccharides from mesophilic LAB: (D) *L. lactis* subsp. *cremoris* SBT 0495[7], (E) *Lb. sake* 0-1[12], (F) *Lb. paracasei* 34-1[14]; *(iii)* heteropolysaccharides from thermophilic LAB: (G) *Lb. acidophilus* LMG 9433[15], (H) *Lb. delbrueckii* subsp. *bulgaricus* rr[9,24], (I) *Lb. helveticus* NCDO 766[13], (J) *Lb. helveticus* TY1-2[10] and (K) its mutant TN-4[11], (L) *S. thermophilus* Sfi6[6,16], (M) *S. thermophilus* OR 901[17], (N) *S. thermophilus* SFi32[18], and (O) *S. thermophilus* SFi12[18]. Glc: glucose; Gal: galactose; Rha: rhamnose; GlcNAc: N-acetyl-glucosamine; GalNAc: N-acetyl-galactosamine; Ac: acetyl. The D- (D) and L- (L) configuration, and pyranose (*p*) and furanose (*f*) structure are indicated.

Metabolic engineering

Several enzymes and/or proteins are involved in the biosynthesis and secretion of EPS: glycosyltransferases, phosphoglucomutase, isoprenoid glycosyl lipid carrier, etc. Specific glycosyltransferases are exclusively involved in the production of the repeating units and hence determine the EPS monomeric composition. However, other steps are encoded by the household genes of the host, including glucose-P interconversions *via* phosphoglucomutase. Phosphoglucomutase could be a key enzyme linking the lactose degradation pathway to EPS biosynthesis (Fig. 2). Assuming that this linkage occurs at this branching point of the pathway, it is tempting to speculate that it is possible to engineer overproduction of EPS since the galactose moiety could be catabolized completely *via* the glycolysis, whereas the glucose moiety could be used for EPS production. Therefore, the flux *via* phosphoglucomutase should be made sufficiently high, and the question is whether this can be realized[19]. The lipid carrier is also involved in the biosynthesis of cell wall polymers so that there is competition for this facilitating membrane component during different phases of growth[20]. Thus, the nature and composition of EPS are influenced by both medium composition, biosynthetic pathways, growth phase, and rate of microbial growth.

Genetic engineering

The problems associated with most ropy strains used for milk fermentation are that the ability to produce EPS is often an unstable characteristic at the genetic level and the ropy texture itself[3-5] is unstable. Consequently, not all ropy strains are suitable for large-scale industrial fermentations, and ropy strains in use have to be periodically reselected from the culture to conserve the EPS production characteristics in industrial strains. Thus manufacturers still mainly rely on prefermentation processing such as increasing milk solids and/or additives for product stability. Consequently, *in situ* production of EPS and exploitation of the EPS production characteristic has economic benefits.

A complex genetic organisation is responsible for EPS production and secretion[16,21]. More knowledge of the molecular organisation and of the factors regulating expression of EPS from LAB will make it possible to enhance EPS production under defined growth or fermentation conditions, to increase the number of possibilities for modifying the structure and function of EPS, and to realize simultaneous production of different EPS.

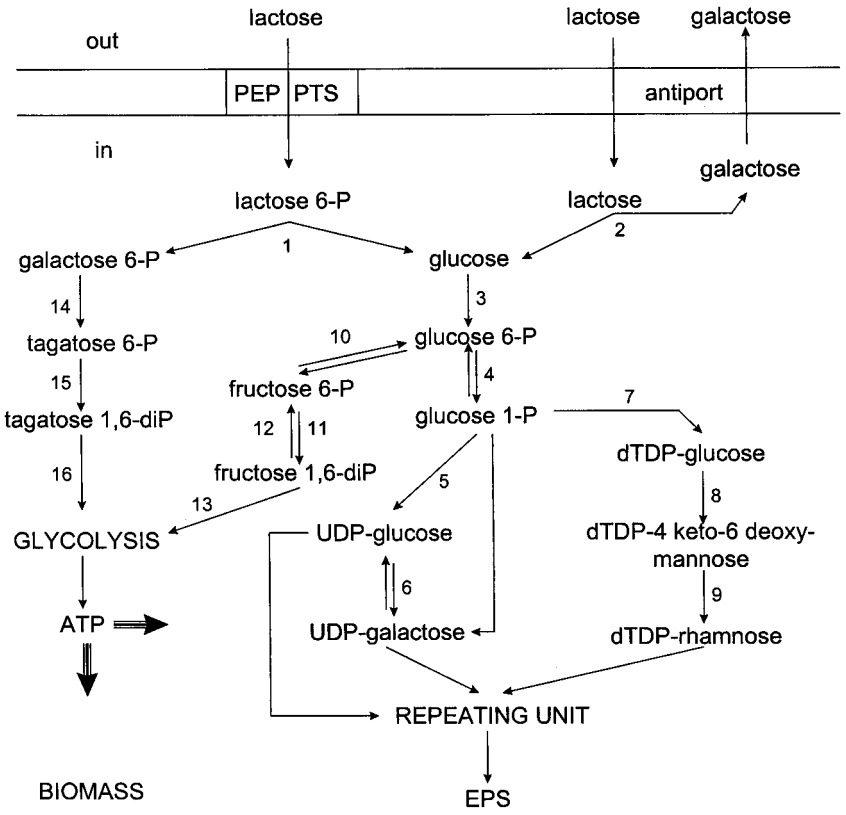

Fig. 2. Schematic representation of pathways involved in lactose catabolism (left) and exopolysaccharide (EPS) biosynthesis (right) in lactose-fermenting *Lactococcus lactis*, (lactose transport via a lactose-specific phosphoenolpyruvate dependent phosphotransferase (PEP PTS) primary transport system), and galactose-negative *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (lactose transport via a lactose/galactose antiport secondary transport system) strains. The numbers refer to the enzymes involved: (1) phospho-β-galactosidase, (2) β-galactosidase, (3) glucokinase, (4) phosphoglucomutase, (5) UDP-glucose pyrophosphorylase, (6) UDP-galactose-4-epimerase, (7) dTDP-glucose pyrophosphorylase, (8) dehydratase, (9) epimerase reductase, (10) phosphoglucose isomerase, (11) 6-phosphofructokinase, (12) fructose-1,6-bisphosphatase, (13) fructose 1,6-diphosphate aldolase, (14) galactose 6-phosphate isomerase, (15) tagatose 6-phosphate kinase, (16) tagatose-1,6-diphosphate aldolase.

Changing production by cloning the *eps* genes in suitable heterologous hosts was realized with a *S. thermophilus eps* gene cluster that could be expressed in a non-producing *L. lactis* strain[16]. However, only low amounts of EPS were produced in this heterologous host. Another approach is to engineer the EPS production in the production host itself or in related strains. This was realized with plasmid-encoded lactococcal *eps* genes that could be conjugally transferred to the genetically well-characterized and well-transformable *L. lactis* strain MG 1363[21]. In these and other production hosts, inactivation by site-directed or random mutagenesis of *eps* genes or relevant household genes coding for specific sugar activations, may affect the incorporation of activated nucleotide sugars or other steps in the polymerisation process. Alternatively, existing or new *eps* genes may be overexpressed. This could result in mutant EPS that have altered structures and properties. Such EPS engineering offers not only the possibility to study structure-function relationships of EPS but also creation of EPS with novel properties that can be used as industrial thickeners. However, genetically modified microorganisms and their products will require legal approval and need to be accepted by both the food processer and the consumer.

Process engineering

The total yield of EPS produced by LAB depends on the composition of the medium (carbon and nitrogen sources) and the conditions in which the strains grow, *i.e.* temperature, pH, oxygen tension and incubation time. Whereas for mesophilic strains low temperatures often markedly induce slime production, EPS from thermophilic strains display maximal production levels under optimal growth conditions[22-28]. Optimal pH conditions for production of EPS are generally close to pH 6.0[25-29]. Aeration would become a problem under conditions that EPS are produced using engineered strains capable of catabolizing galactose and producing additional ATP through the production of acetate *via* acetate kinase[19]. The production of intracellularly synthesized EPS by different LAB strains varies roughly from 0.045 to 0.350 g/L when the bacteria are grown under non-optimized culture conditions. Optimal culture conditions result in EPS yields from 0.150 to 0.800 g/L[3-5]. Not only the nature of the carbon source and sometimes the combination of monosaccharides, but also their concentration can have a stimulating effect on EPS biosynthesis[30]. An optimal carbon/nitrogen ratio in both milk and MRS media resulted in 1.1 g/L with *S. thermophilus* LY03[27]. With *Lb. sake* 0-1, EPS yields of approximately 1.4 g/L are achieved[25]. A marked

reduction in the EPS yield upon prolonged fermentation seems to be dependent on the strain used and both chemical and physical conditions (temperature, pH, etc.) possibly due to glycohydrolase activity[23,26,28,29,31]. Changing the process conditions or harvesting at the appropriate time may overcome the latter problem. However, EPS production by LAB strains can hardly compete with bacteria such as *X. campestris*, as is reflected in the production levels of xanthan gum (30-50 g/L). From an economics point of view, a tenfold increase in EPS production by LAB, to obtain 10 to 15 g/L, is required to use these EPS as a food additive. Since EPS production seems to be coupled to growth and since LAB growth is presumably inhibited by the formation of lactate, there is potential for productivity improvement by reducing the concentration of lactate in the culture broth, either *via* fed-batch cultivation, extractive fermentation, etc. Additionally, a controlled feeding strategy may make it possible to produce 'tailor made' EPS without the need of manipulating the production strains genetically. For instance, it has been shown that polysaccharide production increased and that carbohydrate and uronic acid distribution in polysaccharides altered in favour of galactose with decreasing lactose feed rate in fed-batch cultivation with a galactose-fermenting *S. thermophilus* strain[32]. EPS produced in continuous culture was composed of glucose and galactose in a ratio of 1.0:2.4 when the *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 strain was grown on fructose, whereas glucose, galactose and rhamnose in a ratio of 1.0:7.0:0.8 were found when the strain was grown on either glucose or glucose and fructose[33].

Downstream processing

EPS often cause – even at low levels – a significant viscosity increase of the culture broth: this presents a diffusion barrier or mass transfer problem for nutrients, such that in fact EPS biosynthesis is early stopped by the producer strain. Also, cell separation, product recovery and purification are much more complex as compared to conventional fermentation products. The main problem in such preparations is the high viscosity of the slime solutions which hinders deposition of the cells and EPS separation from the carbohydrates and protein material of the (milk) medium itself. Addition of electrolytes (salts) may be useful in precipitation of EPS by neutralizing charges on the polysaccharide. If the polysaccharide is in capsule form it must be first detached from the cells. EPS is usually obtained after repeated trichloroacetic acid (to remove contaminating proteins), and alcohol or acetone (to

isolate EPS) precipitation steps[28]. Purification of EPS from LAB has been successfully achieved by anion exchange chromatography and/or gel permeation[25,27,34].

Applications of exopolysaccharides from lactic acid bacteria

EPS from LAB are not yet intentionally exploited by industrial manufacturers. A few exceptions exist[2]. Dextran derivatives and activated dextrans find several commercial uses. Industrial dextrans are used in the manufacture of gel filtration products and as blood volume extenders and blood flow improvers. Further possible uses of dextrans are in paper and metal-plating processes and as food syrup stabilizers. Also levan may find application in foods as a thickener. Alternan has potential commercial applications as low-viscosity bulking agent, extender, etc. in foods and cosmetics. Scandinavian fermented milk drinks display a firm, thick, slimy consistency. They rely upon the souring capacity of mesophilic ropy strains of *L. lactis* and the concomitant production of EPS for texture. Dairy starter cultures that contain slime-forming LAB strains are also commercially available in other parts of Europe and the United States. Ropy, thermophilic LAB starter cultures for yoghurt production are largely used in France and The Netherlands because the addition of stabilizers is prohibited in unfruited yoghurts. As mentioned above, a problem is that thermophilic LAB produce less EPS and the ropy character is unstable.

The intentional and controlled use of EPS from LAB as natural food additives or of functional starter cultures, *i.e.* strains producing interesting EPS, could result in a safe, natural end-product, and may have an important impact on the development of novel products (both fermented and non-fermented food products), especially food products with enhanced, rheological properties and improved texture and stability. For instance, low viscosity, gel fracture or high syneresis (whey separation) problems, which may occur during yoghurt manufacture, can be solved in both ways. Hence, the use of EPS from LAB may form a new generation of biothickeners to be used in fermented milks, dairy desserts, soups, sauces and salad dressings. For instance, the viscosity of the *Lb. sake* 0-1 EPS was higher throughout a range of increasing shear rates, while shear-thinning properties, *i.e.* a drop in viscosity with increasing shear rate, were as good as those of xanthan gum[25]. Good shear-thinning properties are important for food applications when considering processing costs (manufacturer) and mouthfeel (consumer) of food products. Viscosity is further highly dependent on the average molecular mass distribution. As the molecular mass of both

xanthan and the *Lb. sake* 0-1 EPS is of the same order of magnitude, the high viscosity of the 0-1 EPS is an intrinsic property and is not due to large differences in molecular mass. Also other EPS from LAB display interesting intrinsic viscosities, indicating that the polymers have remarkable thickening properties[4,6,27,30]. As mentioned above, an alternative way to improve yoghurt viscosity and decrease susceptibility to syneresis and graininess is by utilizing the slime-producing strains in the starter culture. Ropy strains may further contribute to the consistency of stirred-type yoghurt, produced on a large scale, because yoghurt containing viscosifying EPS is supposed to be less damaged mechanically from pumping, blending and filling machines. This ultimately leads to the manufacture of natural yoghurt without the addition of stabilizers. This type of production process gains increased popularity, because of the increased desire of the consumer for 100 % natural products. Finally, EPS may play an important role in the production of yoghurt drinks and low milk solids yoghurts, as well as in the production of creamier yoghurts with low or no fat content and an enhanced smoothness of mouthfeel. Because these products require increasing thickening properties, the use of slime-producing starter cultures in their manufacture is proposed[35].

Conclusion

Such a large number of possible EPS structures from LAB exists that more polymers of potential industrial value will almost certainly be obtained. If these are to be developed commercially, however, they must be cost effective. The use of whey could be a means of upgrading this dairy byproduct by lactose fermentation with concurrent production of a useful polymer for both food and non-food uses. Thus, major improvements must be sought in fermentation techniques to ensure that conversion of substrate to product is maximal. Control of fermentation conditions must be ensured otherwise product yield as well as quality cannot be guaranteed. Also, downstream processing to recover the product can probably be improved and costs lowered. While some of these developments will be achieved by the industrial producers, others may well result from basic studies in academic laboratories. Knowing the environmental and genetic factors regulating expression of the EPS, genetic approaches can be designed which enhance expression of desired EPS under defined growth or fermentation conditions. Finally, both genetic approaches and enzyme and fermentation technology will increase the number of possibilities for modifying the structure

and function of EPS. This polysaccharide engineering may lead to the development of 'designer polysaccharides' for applications that may or may not be food related. Although a popular high-tech concept, the technology for tailoring polymers to specific uses is still in its infancy.

Acknowledgements

The authors appreciate financial support from the University Research Council (VUB), the Flemish Institute for Science and Technology (IWT), the Fund for Scientific Research (FWO - Flanders), and the Institute Danone (Navorsingskrediet voor Fundamenteel Voedingsonderzoek).

References

[1] I.W. Sutherland, *Microbiol. Sci.* **3**, 5 (1986)
[2] S. Roller, I.C.M. Dea, *Crit. Rev. Biotechnol.* **12**, 261 (1992)
[3] J. Cerning, *FEMS Microbiol. Rev.* **87**, 113 (1990)
[4] J. Cerning, *Lait* **75**, 463 (1995)
[5] L. De Vuyst, *Proc. Lactic'97.* In press. (1997)
[6] T. Doco, J.-M. Wieruszeski, B. Fournet, D. Carcano, P. Ramos, A. Loones, *Carbohydr. Res.* **198**, 313 (1990)
[7] H. Nakajima, T. Hirota, T. Toba, T. Itoh, S. Adachi, *Carbohydr. Res.* **224**, 245 (1992)
[8] M. Gruter, B.R. Leeflang, J. Kuiper, J.P. Kamerling, J.F.G. Vliegenthart, *Carbohydr. Res.* **231**, 273 (1992)
[9] M. Gruter, B.R. Leeflang, J. Kuiper, J.P. Kamerling, J.F.G. Vliegenthart, *Carbohydr. Res.* **239**, 209 (1993)
[10] Y. Yamamoto, S. Murosaki, R. Yamauchi, K. Kato, Y. Sone, *Carbohydr. Res.* **261**, 67 (1994)
[11] Y. Yamamoto, T. Nunome, R. Yamauchi, K. Kato, Y. Sone, *Carbohydr. Res.* **275**, 319 (1995)
[12] G.W. Robijn, D.J.C. Van den Berg, H. Haas, J.P. Kamerling, J.F.G. Vliegenthart, *Carbohydr. Res.* **276**, 117 (1995a)
[13] G.W. Robijn, J.R. Thomas, H. Haas, D.J.C. Van den Berg, J.P. Kamerling, J.F.G. Vliegenthart, *Carbohydr. Res.* **276**, 137 (1995b)
[14] G.W. Robijn, H.L.J. Wienk, D.J.C. Van den Berg, H. Haas, J.P. Kamerling, J.F.G. Vliegenthart, *Carbohydr. Res.* **285**, 129 (1996a)
[15] G.W. Robijn, R. Gutiérrez Gallego, D.J.C. Van den Berg, H. Haas, J.P. Kamerling, J.F.G. Vliegenthart, *Carbohydr. Res.* **288**, 203 (1996b)
[16] F. Stingele, J.-R. Neeser, B. Mollet, *J. Bacteriol.* **178**, 1680 (1996)
[17] W.A. Bubb, T. Urashima, R. Fujiwara, T. Shinnai, H. Ariga, *Carbohydr. Res.* **301**, 41 (1997)
[18] J. Lemoine, F. Chirat, J.-M. Wieruszeski, G. Strecker, N. Favre, J.-R. Neeser, *Appl. Environ. Microbiol.* **63**, 3512 (1997)

[19] W.M. De Vos, *A. van Leeuwenhoek* **70**, 223 (1996)
[20] I.W. Sutherland, *Adv. Microbiol. Physiol.* **8**, 143 (1972)
[21] R. Van Kranenburg, J.D. Marugg, N.J. Willem, I. Van Swam, W.M. De Vos, *Mol. Microbiol.* **24**, 387 (1997)
[22] F. Gancel, G. Novel, *J. Dairy Sci.* **77**, 685 (1994)
[23] F. Mozzi, G. Oliver, G. S. De Giori, G. F. De Valdez, *Milchwissenschaft* **50**, 80 (1995)
[24] G.J. Grobben, J. Sikkema, M.R. Smith, J.A.M. De Bont, *J. Appl. Bacteriol.* **79**, 103 (1995)
[25] D.J.C. Van den Berg, G.W. Robijn, A.C. Janssen, M.L.F. Giuseppin, R. Vreeker, J.P. Kamerling, J.F.G. Vliegenthart, A.M. Ledeboer, C.T. Verrips, *Appl. Environ. Microbiol.* **61**, 2840 (1995)
[26] M.A. Gassem, K.A. Schmidt, J.F. Frank, *J. Food Sc.* **62**, 171 (1997)
[27] B. Degeest, S. Van de Ven, L. De Vuyst, *Proc. 11th Forum for Applied Biotechnology. Med. Fac. Landbouww. Univ. Gent* **62/4a**, 1199 (1997)
[28] L. De Vuyst, F. Vanderveken, S. Van de Ven, B. Degeest, *J. Appl. Microbiol.* In press (1998)
[29] F. Mozzi, G. S. De Giori, G. Oliver, G. F. De Valdez, *Milchwissenschaft* **49**, 667 (1994)
[30] J. Cerning, C.M.G.C. Renard, J.F. Thibault, C. Bouillanne, M. Landon, D.M. Desmazaud, L. Topisirovic, *Appl. Environ. Microbiol.* **60**, 3914 (1994)
[31] F. Gancel, G. Novel, *J. Dairy Sci.* **77**, 689 (1994b)
[32] C. Petit, J.P. Grill, N. Maazouzi, R. Marczak, *Appl. Microbiol. Biotechnol.* **36**, 216 (1991)
[33] G.J. Grobben, M.R. Smith, J. Sikkema, J.A.M. De Bont, *Appl. Microbiol. Biotechnol.* **46**, 279 (1996)
[34] T. Doco, D. Carcano, P. Ramos, A. Loones, B. Fournet, *J. dairy Res.* **46**, 279 (1991)
[35] H.L. Rawson, V.M. Marshall. *Int. J. Food Sc. Technol.* **32**, 100 (1997)