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Structural and quantitative analysis of exopolisaccharides and oligosaccharides produced by lactobacillus. I. Basic information, isolation, quantitative determination, molecular mass and monosaccharide composition

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Abstract. There are a number of lactic acid bacteria capable to produce exopolysaccharides (EPS), mucoid bacteria cultures, which, when utilized in yogurt production, can improve the structure of the product, leading to more favourable rheological properties and increased viscosity. The current focus of interest in the field of oligosaccharides are those compounds, which contain 2-10 molecules of glucose and/or fructose and galactose; these are called galacto-oligosaccharides (GalOS) and are considered to have a positive effect on the intestinal function, promote beneficial bifidobacteria proliferation, reduce the pH and the amount of unwanted products generated during the putrefaction process. In order to use the EPS and the GalOS in the food industry and to examine their physiological effects, there is a need to develop analytical methods, which allow the determination of the quantity and the composition of these carbohydrates.

In order to examine the EPS, they need to be extracted from their medium. For this process, in addition to the traditional methods, preparative chromatographic purification or enzymatic breakdown of unwanted materials has also been used. The amount of EPS was usually determined directly from the matrix or from the partially purified fermented liquid and rarely from the aqueous solution of the prepared extract. The quality characteristics examination of these products include the determination of the molecular weight, the determination of the monosaccharide composition and the determination of the interconnection region, the isomerism, the chain structure and the phosphor content of the monosaccharides with various methods.

For the quantitative measurement of EPS, the following methods were used: mass measurements, measurement of absorbance after derivatization, preparative ion chromatography, and most of the authors determined the molecular weight by using gel chromatography, also known as size-exclusion chromatography. To determine the monosaccharide composition of the EPS, gas chromatography or high-performance liquid chromatography was used with or without derivatization.

1 Introduction

Several bacteria produce such kind of polysaccharides, which they do not incorporate into their cell structure but excrete them outside the cell. These kinds of substances form mucus in the intercellular space or become fixed to the cell wall, surrounding it as a shell (*Sutherland*, 1972). These kinds of polysaccharides are called exopolysaccharides (hereinafter referred to as: EPS) because the polysaccharides can be found outside the cell wall.

A number of lactic acid bacteria are also capable of producing EPS (*Sutherland*, 1972; *Cerning*, 1990; *Cerning et al.*, 1988; *Doco et al.*, 1990; *Ariga et al.*, 1992). The use of these kinds of mucoid cultures in the yogurt production can improve the structure of the product (*Cerning*, 1990; *Andres*, 1982; *Schellhaass & Morris*, 1985; *Teggatz & Morris*, 1990) because of the effect of EPS results in a more favourable rheological property and an increased viscosity (*Macura & Townsley*, 1984; *Manca De Nadra* et al., 1985), while the breakup of the gel structure and the syneresis will be avoidable (*Cerning et al.*, 1988). By using some kind of EPS-producing lactic acid bacteria species, the utilization of stabilizers of vegetal origin is avoidable, producing this way a "natural," "without additives" yogurt (*Marshall & Rawson*, 1999; *De Vuyst et al.*, 1998; *Uemura et al.*, 1993) or ice cream (*Christiansen et al.*, 1999). The presence of EPS is not desirable in any kind of food product; the consistence of wines will be mucous and oily from it (*Dueñas-Chasco et al.*, 1998).

Some EPS could have positive effects on human health because they are non-digestible food ingredients (*Gibson & Roberfroid*, 1995), on the one hand, and they have been attributed to have anti-carcinogenic (*Oda et al.*, 1983; *Kitazawa et al.*, 1991) and cholesterol-lowering (*Nakajima et al.*, 1992) properties, on the other hand. In recent years, extensive studies have been carried out on the mapping of the structure of EPS (*Dueñas-Chasco et al.*, 1998; *Casteren et al.*, 1998; *Urashima et al.*, 1999; *De Vuyst et al.*, 1998; *Uemura et al.*, 1998) and the determination of the amount of EPS (*Dueñas-Chasco et al.*, 1998; *Casteren et al.*, 1998; *Sebastiani* and *Zelger*, 1998; *Urashima et al.*, 1999; *Marshall* and *Rawson*, 1999; *De Vuyst et al.*, 1998; *Uemura et al.*, 1998; *Casteren et al.*, 1998) produced by the various types of lactic acid bacteria species. According to some authors, depending on the fermentation conditions, the resulting structure of the EPS may differ even in the case of the same bacteria (*Petit et al.*, 1991; *Kojic et al.*, 1992; *Cerning et al.*, 1994; *Grobet et al.*, 1996). The amount of EPS produced, varies widely depending on the fermentation conditions and the species used. To exploit the benefits of the EPS-producing species in food industry, we have to know the quantity, structure as well as the production conditions of the EPS produced by any particular bacteria species used, on the one hand; on the other hand, we have to know if a particular amount of EPS with a particular structure significantly improves the physical properties of the product. In the case of undesirable EPS (e.g. glutinous wine), the goal is to design an enzymatic treatment which can reduce the viscosity, and for this the knowledge of the structure of the particular EPS is required as well (*Dueñas-Chasco et al.*, 1998).

On the basis of their chemical structure, EPS produced by lactic acid bacteria can be divided into three groups (*Cerning*, 1995):

- α-glucans, which are mainly formed from glucose units linked by $\alpha-1,6$ and $\alpha-1,3$ bonds, for example, dextrans;
- fructans, which are mainly formed from fructose molecules linked by $\beta-2,6$ bonds, for example, levans;
- heteropolysaccharides, which are composed of several monosaccharides.

The oligosaccharide structures containing 2-10 molecules of glucose and/or fructose and galactose, which are called galacto-oligosaccharides (hereinafter referred to as GalOS) (*Hyun-Jae Shin et al.*, 1998), have been currently in the focus of interest (*Hyun-Jae Shin et al.*, 1998; *Rustom et al.*, 1998; *Yanahira et al.*, 1998). GalOS have a positive effect on the intestinal function: they promote the growth of beneficial bifidobacteria, reduce the pH and the amount of products formed during the putrefaction process (*Yanahira et al.*, 1998). Since they are non-digestible, they have a physiological effect similar to the fibres, i.e. they lower the blood pressure and the level of cholesterol in the blood serum (*Tomomatsu*, 1994), and serve as a low-energy-containing sweetening agent applicable as a food or cosmetic additive (*Hyun-Jae Shin et al.*, 1998). Due to the above reasons, the industrial production of GalOS is also studied, which – according to our current knowledge – is possible in three different ways (*Hyun-Jae Shin et al.*, 1998):

- chemical or enzymatic hydrolysis of galactan and lactose (*Prenosil et al.*, 1987; *Iwasaki et al.*, 1996; *Bonnin* and *Thibault*, 1996);
- chemical or enzymatic synthesis (*Cote* & *Tao*, 1990; *Thien*, 1995);
- fermentation with eukaryotic cell cultures (*Gorin et al.*, 1964; *Ohtsuka et al.*, 1990; *Shin et al.*, 1995).

Due to its high lactose content, whey is a suitable raw material for the enzymatic production of GalOS (*Rustom et al.*, 1998). The productivity of enzymatic methods has been studied (*Hyun-Jae Shin et al.*, 1998), and in the case of a particular method the fermentation parameters have been optimized (*Rustom et al.*, 1998). These studies also require the quantitative (*Hyun-Jae Shin et al.*, 1998; *Rustom et al.*, 1998) and structural (*Yanahira et al.*, 1998) determination of GalOS.

As the lactose digestion of individuals with lactose intolerance is not as effective as those of healthy individuals, there is a need to reduce the amount of

lactose intake, which can only be done if the lactose content of dairy products is known. *Richmond et al.* (1987) investigated the changes of lactose, glucose and galactose during the production of yogurt. It was found that the lactose content is reduced during the initial heat treatment and the subsequent fermentation.

In summary, it can be stated that in order to use EPS and GalOS in food production and to examine its possible physiological effects there is a need for the existence of analytical methods which enable the quantification and the determination of the composition of these kinds of carbohydrates.

2 Determination of the structure and the quantity of the exopolisaccharides (EPS)

Before the examination, EPS have to be extracted from the medium, which is mostly a fermented liquid, a complex biological system containing living and dead cells, macromolecules "released" into the solution and small molecules as well. This complex task involves the use of traditional methods (separation based on different sedimentation or solubility rates), on the one hand, while in the final step, purification with preparative chromatographic methods or by the application of enzymatic methods for the breakdown of the unwanted materials. The amount of EPS is usually determined directly from the matrix or from the partially purified ferment, and rarely from the aqueous solution of the obtained mixture. The quality characteristics examination of these products includes the determination of the molecular weight, of the monosaccharide composition and that of the interconnection region, the isomerism, the chain structure and the phosphor content of the monosaccharides with various methods.

2.1 Isolation of the exopolysaccharides

The optional steps of the extraction process of exopolysaccharides are the following:

- removal of microorganisms by centrifugation,
- precipitation of proteins from the supernatant solution,
- precipitation of the EPS using an organic solvent,
- cleaning of the EPS with enzymes,

- purification and breakdown into fractions of the EPS by ion-exchange chromatography.

The medium from which the EPS has to be extracted is usually a dairy-based ferment. *Dueñas-Chasco et al.* (1998) first removed the cells with direct centrifugation (20,000 g, 30 min.) and then with centrifugation (11,000 g, 20 min.) applied after cooling (6 °C, 1 hour) (*Urashima et al.*, 1999). In both cases, the following step was the alcoholic precipitation of the EPS from the supernatant. The step of removing the proteins was omitted in the first case (*Dueñas-Chasco et al.*, 1998), while in the second case the authors attempted to remove the proteins by ion-exchange chromatography (*Urashima et al.*, 1999). To precipitate the proteins, *Casteren et al.* (1998) stirred the "raw EPS," which was obtained after centrifugation, ultrafiltration and then lyophilization of the ferment in a solution containing 4% trichloroacetic acid for 2 hours at a temperature of 4 °C followed by centrifugation (28,100 g, 30 min.).

Other authors (*Sebastiani* and *Zelger*, 1998; *Marshall* and *Rawson*, 1999; *De Vuyst et al.*, 1998; *Uemura et al.*, 1998) omitted the first step and began immediately with the precipitation of the proteins followed by the removal of the precipitate. Most of the authors used the trichloroacetic acid method (*Sebastiani* and *Zelger*, 1998; *Marshall* and *Rawson*, 1999; *De Vuyst et al.*, 1998); *Uemura et al.* (1998) reached their goal by acidifying the solution and then boiling it. *Sebastiani* & *Zelger* (1998) added 70 cm^3 of 80% trichloroacetic acid to 400 cm^3 of the solution, leaving it to stand for one night and then centrifuged at a temperature of 4 °C (13,000 g, 30 min.). *Marshall* & *Rawson* (1998) added 85% trichloroacetic acid to 1 cm^3 of the yogurt during the precipitation of the protein. *De Vuyst et al.* (1998) added an equal volume of 20% trichloroacetic acid to the ferment and removed the proteins and the cells by centrifugation (25,000 g) for 20 minutes. After diluting the ferment to the two-fold of its initial volume, *Uemura et al.* (1998) precipitated first the casein fraction by adjusting the pH of the ferment to the value of 4.6. The casein and the microbes were removed from the whey applying centrifugation (10,000 g, 4 °C) for 20 minutes. After the neutralization process, the whey was boiled in a water bath for 30 minutes and the insoluble proteins were sedimented by centrifugation (10,000 g, 20 min).

In the third step, most of the authors (*Dueñas-Chasco et al.*, 1998; *Sebastiani & Zelger*, 1998; *Urashima et al.*, 1999; *Marshall & Rawson*, 1999; *Uemura et al.*, 1998; *Casteren et al.*, 1998) precipitated the EPSs with a concentrated alcoholic solution. *De Vuyst et al.* (1998) examined the functionality

of methanol, isopropanol and acetone for this purpose, and decided to use acetone. They added an equal volume of acetone to the deproteinized solution, left it to stand overnight and then centrifuged it (25,000 g, 30 min, 4 °C). After the aqueous dissolution of the pellet, another trichloroacetic acid clarification and a centrifugation followed, closing the procedure with a second precipitation with acetone.

With the exception of *Casteren et al.* (1998), the authors cited that they repeated the clarification with alcohol several times: they dissolved the precipitate formed as a result of the addition of ethanol in water and removed the insoluble parts, then added ethanol to the supernatant again. After the removal of the cells, *Dueñas-Chasco et al.* (1998) added three units of cold ethanol to one unit of supernatant, left the solution stand overnight at 4 °C and then centrifuged it at 4,500 g for 20 min. The resulting precipitate was solved in distilled water and then repeated the treatment with ethanol three times, after which they dissolved the final precipitate in distilled water and dialysed it. *Sebastian & Zelger* (1998) added also three units of alcohol to one unit of the deproteinized solution, kept the mixture at 20 °C for 30 minutes, and centrifuged it at 4 °C for 30 minutes at 13,000 g. The precipitate was dried up under vacuum and then, depending on the amount and solubility of pellets, dissolved it in 5-25 cm^3 of sterile distilled water. The insoluble fraction was separated from the soluble fraction by centrifugation. The insoluble fraction was suspended in distilled water again and three times as much ethanol was added again to the soluble fraction. After centrifugation, the pellet was drained again and dissolved in water. *Urashima et al.* (1999) added three units per volume of cold ethanol to the one unit per volume supernatant. They left it to stand overnight at 4 °C and then centrifuged it at 5,000 g for 10 minutes. The pellet was dissolved in hot water and centrifuged again in the solution for 10 minutes. This operation was repeated three times, after which the resulting polysaccharide was dialysed and lyophilized. *Marshall & Rawson* (1999) added an equal unit per volume of ethanol to the supernatant resulted after the treatment with tricloroacetic acid, suspended the precipitate and carried out a second alcohol precipitation treatment. *Uemura et al.* (1998) also used the same unit per volume of 99.5% ethanol as the volume of the aliquot part of the deproteinized solution to precipitate the EPS. The solution was stirred overnight at 4 °C; then the precipitate was obtained by centrifugation, after which it was dissolved in water and the ethanol precipitation was repeated twice more. After neutralizing the tricloroacetic acid supernatant with 2 M NaOH, *Casteren et al.* (1998) realized the extraction of EPS by adding two units per volume of 96% ethanol at 4 °C. After the centrifugation (28,100 g,

30 min, 4 °C), the precipitate was dissolved in distilled water, was dialysed and lyophilized, and then the sugar content of the fractions was subsequently determined. The "raw" EPS contained 51% by weight of sugar, the EPS precipitated with tricloroacetic acid, consisting mainly of protein substances, contained 17% by weight of sugar, the substances precipitated with ethanol contained 63% by weight of sugar and the supernatant of the alcohol precipitation contained 9% by weight of sugar. As expected, the precipitate of the ethanol solution contained the decisive part of the sugar. The sugar content increased from 51% to 63% after the removal of proteins. The difference in the polysaccharide-monosaccharide composition of the "raw" EPS and the alcohol precipitation was very low.

After purification with tricloroacetic acid and alcohol, *Uemura et al.* (1998) removed the remaining proteins and DNAs with enzymes. The polysaccharides were dissolved in a 0.05 M Tris-HCl buffer (pH=8.0), which also contained 1 mM $MgCl_2$, and were treated at 37 °C for 6 hours with 2 $\mu g/cm^3$ Dnase (type IV, EC.3.1.21.1, Sigma, St. Louis, USA) and RNase (type I-AS, EC.3.1.27.5, Sigma). The protein contamination in the sample was degraded by adding 0.2 mg/cm^3 proteinase K (EC.3.4.21.14, Sigma) at 37 °C for overnight. The reaction was stopped with a heat treatment at 90 °C for 10 minutes. The EPS fraction was precipitated with ethanol, dialysed with distilled water, and then lyophilized.

Urashima et al. (1998), *Uemura et al.* (1998) and Casteren et al. (1998) realized the removal of the remaining proteins as well as the fractionation of EPS by preparative ion-exchange chromatography (IE-HPLC). *Dueñas-Chasco et al.* (1998); *Sebastiani & Zelger* (1998); *Marshall & Rawson* (1999) and *De Vuyst et al.* (1998) omitted this purification and at the same time the purity control step, too. *Sebastiani & Zelger* (1998) did not perform chromatographic purity test, and thus, according to their statement, they "could not detect either peptides or proteins in their EPS mixture".

Urashima et al. (1999) introduced the aliquot part of the polysaccharide solution through a DEAE- Sephadex A-50 (Pharmacia Fine Chemicals) 1.5×20 cm ion-exchange column. The column was equilibrated with a 50 mM Tris- aminomethane – HCl buffer (pH 8.7) and the same buffer was also used for eluting with 250 cm^3 of eluent; after this, a linear NaCl gradient was supplied with further 250 cm^3 of liquid until reaching a concentration of 0-1 M. The presence of proteins was determined using a spectrophotometric detector by measuring the absorbance at 280 nm, while the presence of sugars was determined using the phenol – sulphuric acid method (*Hodge & Hofreiter*, 1962), measuring the wavelength at 490 nm. Using this method, it was possible

to separate well the neutral polysaccharides (which are found at the beginning of the chromatogram) from a fraction containing mainly proteins. However, in addition to the proteins, this fraction contained carbohydrates too, which are probably acidic carbohydrates with acidic properties on the pH of the eluent, whereas they were bound to the column better and could be eluted from the column only due to the "ion exclusion" effect of the NaCl gradient from the column.

Casteren et al. (1998) dissolved the previously purified EPS in 5 mM NaOAc (pH=6) and equilibrated the analytical column with the same buffer (DEAE Sepharose fast flow (52×5 cm) (Pharmacia), Biopilot system). They increased the flow rate during the measurement from 10 cm^3/min to 50 cm^3/min, while the concentration of NaOAc increased from the initial 5 mM to 2 M. The substances which remained on the column were cleaned off the column by applying a flow rate of 20 cm^3/min and a concentration of 0.5 M NaOH. The absorbance of the eluent was measured at 280 nm, while the concentration of the proteins was measured based on the β-casein extinction factor (*Swaigsgood*, 1992). The sugar content of the fractions (approx. 100 mL) was measured with the orcinol method (*Tollier & Robin*, 1979), using a standard glucose curve. In this case, the chromatogram contained two large carbohydrate fraction peaks, of which the second peak, similar to the chromatogram provided by the *Urashima et al.* (1999), coincided with the protein fractions peak.

In such circumstances, *Urashima et al.* (1999) and *Casteren et al.* (1998) were able to purify only the neutral EPS from the proteins in the ion-exchange column. If there is a need to purify the acidic EPS fraction too, a new chromatographic method has to be developed, which allows the separation of the acidic EPS and the proteins, or the sample preparation methods have to be changed. During the pretreatment, *Casteren et al.* (1998) applied a trichloroacetic acid treatment, while *Urashima et al.* (1999) applied no trichloroacetic acid treatment. Despite this fact, *Casteren et al.* (1998) detected a significant amount of proteins; so, the trichloroacetic acid treatment (4% trichloroacetic acid solution, stirring at 4 °C for 2 hours, followed by centrifugation) alone was not a sufficient method for the deproteinization. Based on the reported information, the comparison of the carbohydrate/protein ration of the two methods in the co-eluting fraction (acidic EPS and protein) is not possible, as *Urashima et al.* (1999) communicate only the absorbance values and do not supply information about the concentration of the carbohydrate and protein fractions. The initial composition of the mixture was also different. Therefore, it is possible to suggest the application of tricholoroacetic acid treatment during the sample preparation, because of its property to re-

duce the amount of the protein fraction interfering with the acidic EPS, only on the basis of theoretical considerations.

Uemura et al. (1998) loaded the EPS dissolved in 0.05 M Tris - HCl buffer (pH 8.6) into a DEAE 650M Toyopearl 650 M anion-exchange column (Tosoh, Tokyo, Japan, 2.6×20 cm), equilibrating it previously with the same buffer. After loading, the column was washed with 350 cm^3 buffer having the same composition as the equilibrating solution, and then a NaCl linear gradient ranging from 0 M to 0.5 M was applied. For the detection of neutral sugars in the eluent, the method based on the phenol-sulphuric acid reaction, designed by *Dubois et al.* (1956), was applied and the absorbance of the proteins was measured at the wavelength of 280 nm. The carbohydrate containing fraction was lyophilized after dialysing it at 4 °C by treating it first with 2 M NaOH and then with distilled water. The EPS was separated into two main fractions, non-binding neutral polysaccharide (NPS) and binding acidic polysaccharides (APS). Based on the chromatogram reported, none of the resulting EPS fractions contained a significant amount of eluted proteins. The deproteinization processes associated with the acidification, boiling, and subsequent enzymatic breakdown processes probably removed the bulk amount of the proteins efficiently, or larger amounts of proteins eluted from the column only during its regeneration. However, during the studies based on gel chromatography applications, a protein fraction appeared on the chromatogram, which was separated from the APS.

In summary, if there is a need to have protein-free APS fractions after the isolation of EPS, the steps described by *Uemura et al.* (1998) have to be used, which are the following:

- precipitation of the protein by acidifying and then boiling the solution,
- precipitation of the EPS with ethanol,
- purification of the EPS with enzymes,
- purification and breakdown into fractions of the EPS by ion-exchange or gel chromatography.

If the goal is to extract only NPS fraction, it is sufficient to apply the pretreatment methods described by *Urashima et al.* (1999), which are the following:

- removal of the microorganisms by centrifugation,
- precipitation of the EPS with ethanol,

- purification and breakdown into fractions of the EPS by ion-exchange chromatography.

2.2 Determination of the quantity of exopolysaccharides

The quantitative measurement of the EPS was carried out using the following procedures. *De Vuyst et al.* (1998) and *Uemura et al.* (1998) measured the weight of the EPS extracted from a mixture with a particular volume. *Marshall & Rawson* (1999) and *Sebastiani & Zelger* (1998) generated a colour reaction by adding the adequate reagents to the aqueous solution of the purified EPS, then determined the extinction and from this the concentration. *Urashima et al.* (1999), *Uemura et al.* (1998) and *Casteren et al.* (1998) reacted the fractions, which were separated by preparative ion-exchange chromatography, with reagents leading to colour reaction in the presence of carbohydrates, then determined the concentration after measuring the extinction.

According to *De Vuyst et al.* (1998), "PS determinations are based mainly on indirect methods. The colorimetric techniques result in that the amount of EPS is given in glucose or dextran equivalent values, and there is not necessarily a relationship between the results of the viscosity measurements and the amount of EPS." The amount of EPS was determined by applying the already described EPS isolation procedure on 0.5 L sample, and then washed the polysaccharides, which have been considered to be pure with acetone, dried it for 48 hours at 42 °C, and then measured its weight. The quantity of EPS was expressed in polymer solids/L (mg PDM/L).

Marshall & Rawson (1999) dissolved the purified EPS in 1 cm^3 distilled water, and added 1 cm^3 5% phenol solution and 5 cm^3 concentrated sulphuric acid. The extinction values of the solution were measured at 490 nm, and then determined the concentration of the EPS using standard glucose curves (*Dubois et al.* 1956).

Sebastiani & Zelger (1998) worked with two methods. In the case of the phenol-sulphuric acid method, they dissolved 0.2 cm^3 of EPS preparation in 1.8 cm^3 of bi-distilled water, and then added 50 µl distilled phenol and 5 cm^3 97% sulphuric acid. The mixture was held at room temperature for 10 minutes, stirred with rotation, and then incubated for another 15 minutes at 27 °C. The samples were put into quartz cuvettes and their absorbance was measured at 490 nm against water as a blank. The concentration of glucose was determined using standard glucose calibration curve.

During the other study, 4 cm^3 of ferment was stirred with 0.7 cm^3 80% tricloroacidic acid, and then centrifuged at 2,000 g for 10 minutes. 900 µl

has been examined from the supernatant applying the dextran test method designed by *Keniry et al.* (1969), which was modified according to *Garcia-Garibay & Marshall* (1991). The amount of EPS was determined using calibration curves made of diluted dextran solutions.

During the study of the ferment containing multiple different lactic acid bacteria species or bacteria variety, the two methods gave contradictory results: the dextran test showed detectable levels of EPS only in two cases of five, while the sulphuric acid method produced measurable results in all five cases. The explanation cannot be the greater sensitivity of the latter method since, according to the sulphuric acid test, the difference between the content of the five EPS fractions may be at most a three-fold one. Moreover, the sulphuric acid method resulted in a lower EPS content in the case of a ferment which was detected by the dextran method compared to the ferments where the dextran method did not show the presence of any EPS.

To find the cause of the discrepancy, the EPS mixture was examined for its content of glucose, galactose or lactose by the *Boehringer-Mannheim* enzyme test. The result was negative, after which no proteins or peptides could be detected from the mixture. Performing a quantitative determination of the amount of monosaccharides by HPLC, after the hydrolysis of monosaccharides, the concentration of monosaccharides showed a good match with the EPS results of phenol sulphuric acid method.

From those performing the ion-exchange examination, *Urashima et al.* (1999) and *Uemura et al.* (1998) determined the EPS content of the eluent with the phenol-sulphuric acid method already mentioned (*Dubois et al.*, 1956). *Uemura et al.* (1999) measured the EPS content of 1 L ferment to be 58.4 mg. *Casteren et al.* (1998) measured the sugar content of the fractions using the orcinol method calibrating with glucose solutions.

The applied indirect photometric methods (phenol-sulphuric acid test and dextran method) gave conflicting results. Based on a method comparison reported by *Sebastiano & Zelger* (1998), it seems that the phenol-sulphuric acid method resulted in a better match with the HPLC measurement results than the use of the dextran method; so, the former method is more preferred. Almost all the authors applied the phenol-sulphuric acid method (*Sebastiani & Zelger*, 1998; *Urashima et al.*, 1999; *Marshall & Rawson*, 1999; *Uemura et al.*, 1998), only *De Vuyst et al.* (1998) and *Casteren et al.* (1998) deviated from this method. The exclusive weight determination applied by De Vuyst et al. (1998) can only be used to determine the amount of EPS if the mixture does not contain significant quantities of contaminants (e.g. proteins) or if there is no significant EPS loss during the purification procedures.

2.3 Determination of the molecular weight of the exopolysaccharides

Most authors carry out the molecular weight determination by gel chromatography methods, also known as size exclusion chromatography methods. *Urashima et al.* (1999) determined the molecular weight of the EPS by HPLC, equipped with a Tohsoh SD 8022 pump, TSK gel G6000 PWXL (7.8 mm ID×30 cm) column and a refractometric detector (Tohsoh RI-8020), using distilled water as eluent during the measurement. The molecular weight was determined with the help of a pullulan standard kit (P-800, Shodex). According to their evaluation, the neutral EPS fraction appeared in a single peak on the chromatogram, its molecular weight was around 4.5×106 Da. The acidic fractions and the protein contamination have not been studied.

Uemura et al. (1998) worked also with gel chromatography, using an Asahipak GS-710 type column (7.6×500 mm, Asahi Chemical Industry Co., Kawasaki, Japan). The eluent was a 5 mM acetic acid-triethylamine buffer (pH=5.0). The calibration of the retention volumes was carried out with the elements of the pullulan kit, P-800, P-400, P-200, P-100, P-50, P-20, P-10 and P-5 (Showa Denko KK, Tokyo, Japan). The molecular weight of the NPS eluted as a single peak; it was estimated to be 1.2×10^6 Da. A lower molecular weight protein containing component also appeared in the chromatogram (5.8×10^3 Da), which was completely separated from the APS with a molecular weight of 1.1×10^6 Da.

Casteren et al. (1998) determined the molecular weight using a high-performance size-exclusion chromatography (HPSEC). The measurements were carried out by a SP8700 (Spectra-Physics) HPLC apparatus, equipped with a 300×7.8 mm Bio-Gel TSK column series (60XL, 40XL and 30XL), each of them including a TSK XL column (40×6 mm). The eluent was 0.4 M NaOAc (pH=3.0), the temperature of the column space was 30 °C, the flow rate was 0.8 cm^3/min. The eluent flowed through a Viscotek refractometer or online; for the calibrations, there were used dextrans, where the molecular weight varied from 4,000 to 500,000. Static "light-scattering" (SLS) detector was also used during the analyses. The molar mass of the purified EPS was measured to be 6.8×10^5 g/mol.

2.4 Monosaccharide composition of exopolysaccharides

The determination of the monosaccharide composition consists of the following possible steps:

- breakdown of the polysaccharide to monomer units by acid hydrolysis or by methanolysis,
- derivatization (pyridyl amine monomers, alditol acetates, trimethylsilylated derivatives),
- qualitative and quantitative determination by high-performance liquid chromatography (HPLC) or gas chromatography (GC).

Sebastiani & Zelger (1998) incubated 2 cm^3 of EPS with 1 M sulphuric acid solution in a 5 cm^3 hermetically sealed tube for 3 hours at 100 °C. The determination of the monosaccharide composition was carried out by HPLC, by separating 1 cm^3 mixture (REZEX organic acid column, 50 °C). Water was used as mobile phase at a flow rate of 0.6 mL/min. As EPS components glucose, galactose and rhamnose were detected.

De Vuyst et al. (1998) purified the already purified EPS once again by precipitating it with acetone, and then lyophilized the precipitate. 15 mg/L of the lyophilized solution was hydrolysed with 1-1 cm^3 at 100 °C for 3 hours with 6 M of trifluoroaceti acid or at 100 °C for 4 hours with 0.6 M of hydrochloric acid. The monosaccharide composition of the hydrolysates was calculated as the average value of the four HPLC measurements, where the relative ratio of the peak areas was used to estimate the monomer composition. For the measurements, a Waters HPLC was used (Waters Corp., Milford, MA, USA) equipped with the following modules: Waters 410 differential refractometer, a Waters column thermostat, "Waters 717 plus" sampler and Milennium software version 2.10. 30 µl of the sample was loaded at 35 °C to a Polispher OA KC column (Merck, Darmstadt, Germany). The mobile phase was 0.005 M sulphuric acid solution, the flow rate was 0.4 cm^3/min. In addition to the concentration of glucose and galactose, it was also possible to determine the concentration of lactose and lactic acid; thus, the HPLC assay was suitable to determine the fermentation profile in addition to the EPS composition.

Uemura et al. (1998) determined the sugar composition according to *Hase et al.* (1978) by HPLC method. The samples were hydrolysed in a 2 M trifluoroacetic acid solution at 100 °C for 2 hours, after which a pyridyl amination (PA) and a reduction occurred. The PA-monosaccharides were identified using a HPLC apparatus equipped with the following elements: Hitachi L-6200 "Intelligent Pump" (Hitachi Ltd., Tokyo, Japan), Palpak "A-type" column (4.6 mm × 150 mm, Takara, Kyoto, Japan) and an F-1080 fluorescence detector (Hitachi Ltd.). The elution took place under isocratic conditions using a ratio of 9:1 (v/v) of 0.7 M potassium borate buffer (pH=9.0)/acetonitrile, at a flow rate of 0.3 cm^3/min and at temperature of 65 °C. The detection of PA sugars

was carried out based on the values of their fluorescence (Ex: 310 nm, Em: 380 nm). The area under the peaks was determined using D-5500 Chromate software (Hitachi Ltd.). The assay showed that the EPS contained glucose and galactose.

Casteren et al. (1998) incubated the preparations treated with acid and ethanol, by first using a 12 M sulphuric acid solution at 30 °C for an hour, and then hydrolysing it according to the method of *Sebastiani & Zelger* (1998), with the use of an internal inositol standard by using a 1 M sulphuric acid solution at 100 °C for 3 hours. From the liberated sugars, they formed alditol acetate derivatives according to the method described by *Englyst & Cummings* (1984). These were separated from each other with a Carlo Erba 4200 gas chromatograph, on a 15 m × 0.53 mm size J W DB-225 column. The temperature programme was as follows: 1 min at a temperature of 180 C, heating from 180 °C to 220 °C at a speed of 2.5 °C/min, and then isothermal at 220 °C for 3 minutes. The temperature of the flame ionization detector (FID) was 275 °C and helium was used as a carrier gas. This method could be used to detect the following monomers: glucose, galactose, rhamnose, mannose and xylose.

Using the chemical modifications performed on the EPS, the authors concluded that if the carbohydrate chains contain galactose phosphate esters, there is a risk that the hydrolysis does not take place perfectly in the case of galactose phosphate bonds, thus obtaining a lower amount of alditol-acetate derivatives, and the detected amount of galactose will be lower than the real amount.

Dueñas-Chasco et al. (1998) have analysed the monosaccharides in the form of trimethylsilylated methyl glycosides based on the method of *Chaplin* (1982). The polysaccharides were treated at 80 °C with 0.625 M hydrochloric acid methanol solution for 16 hours, then silylated at 80 °C for 16 hours with the mixture of 1:1 ratio of pyridine-BSTFA. Isobutanol was added to the mixture and then dried in a nitrogen gas stream. The TMS derivatives were analysed by GLC-MS (gas-liquid chromatography–mass spectrometry), whereby a Kratos MS80RFA device was connected to a 25 m × 0.32 mm id. sized CP-Sil5-CB (WCOT ("Wall Coated Open Tubular") column. The temperature of the column space was isotherm for 2 minutes at 140 °C, and then increased to 250 °C at a speed of 8 °C/min.

Urashima et al. (1999) methanizated the polysaccharides in a 2% hydrochloric acid methanol solution at 80 °C for 20 hours, followed by the N-acetylation with acetic anhydride in methanol solution (1:3, v/v). The 2% hydrochloric acid methanol solution was prepared from a 5% HCl methanol solution so

that the solution was diluted with anhydrous alcohol. The methanol isolate was trimethyl silylated with a TMS-HT kit (Tokyo Kasei Co., Tokyo, Japan) and the derivatives were analysed using a Shimadzu 13B GC. The detector was a FID, the column was a 30-m-sized quartz capillary (123-1732 DB-17, Shimadzu, Kyoto, Japan). During the measurement, a temperature gradient from 150 °C to 250 °C was used at a speed of 3 °C/minute. D-galactose and L-rhamnose could be detected in the EPS.

The steps of composition determination were carried out in different ways. In the case of some HPLC methodologies, there is no need for derivatization, while in the case of GC analysis this step cannot be omitted since the reactive groups of alcohol must be sealed with the adding of a less active group. There is no report about the qualitative identification process of the monomers. Only one author used MS (*Dueñas-Chasco et al.*, 1998) to detect monosaccharide derivatives; in the case of the rest of the authors, the identification was perhaps based on the retention time of the standard materials. The principle of quantification is not detailed except by *De Vuyst et al.* (1998), who used the relative ratio of the peak area to determine the ratio of the monomers.

References

The references are located at the end of the second part of the article.