Biotechnology Letters Vol 8 No 9 625-628 (1986)
Received as revised July 29

Isolation and characterization of exocellular polysaccharide produced by Lactobacillus bulgaricus.

Jutta **CERNING**,[*] C. **BOUILLANNE**,[**] M.J. **DESMAZEAUD**,[**] and Michèle **LANDON**[**]

[*] Laboratoire de Biochimie et Technologie Laitières,
[**] Laboratoire de Microbiologie Laitière,

I.N.R.A. 78350 Jouy-en-Josas, France.

SUMMARY **Lactobacillus bulgaricus** strains grown on skim milk produce a viscosifying exocellular watersoluble heteropolysaccharide composed of galactose, glucose and rhamnose in an approximately molar ratio of 4:1:1. The molecular weight is approximately 500.000.

INTRODUCTION

The production of exocellular polysaccharides is found in many species of both Gram-positive and Gram-negative bacteria and has been the subject of numerous investigations. In the dairy industry the use of polysaccharides-producing bacteria is of interest with respect to the improvement of body and texture of yoghurt, in particular in France and the Netherlands where addition of plant or animal stabilizers is prohibited. Dairy starter cultures that contain **Streptococcus thermophilus** and **Lactobacillus bulgaricus** capable of producing slime are commercially available, but the chemical composition and structure of the exopolymer have not been reported. Information on the amount of polysaccharides that are produced is also lacking and little information exists regarding the culture conditions, which affect the ability of the organism to produce polysaccharides and the resultant rheological properties of fermented milk systems. There appears little agreement among recent publications (Oda et al , 1983 ; Schellhaas, 1983 ; Manca de Nadra et al , 1985) as to the chemical composition of the polysaccharides. The findings of Oda et al (1983) and Schellhaas (1983) agree in as much as they indicate galactose and glucose to be released after hydrolysis of the polymer, but the molar ratios reported are very different. Already in 1973 Groux investigated the composition of slime secreted by a strain of **L. bulgaricus** and reported that galactose was the most frequently encountered monomer within the polysaccharide. Arabinose, mannose and glucose were also present. The polysaccharide recovered by Tamime (1978) from a yoghurt starter culture was reported as a glucan as glucose was the only monomer detected by gas chromatography. In

most of these investigations neither a precise description of the culture conditions nor the analytical details of sugar determination and identification are reported and results should therefore only be extrapolated with care. Here we describe the identification and quantitative estimation of an exocellular polysaccharide produced by 3 ropy strains of **L. bulgaricus** compared to one non ropy strain of the same species.

MATERIALS and METHODS

Microorganisms Ropy (CNRZ 416, CNRZ 737) and non ropy (CNRZ 398) strains of **Lactobacillus bulgaricus** were obtained from the collection of the Centre National de Recherches Zootechniques (CNRZ). The ropy strain CRL 420 was obtained from the Centro de Referencia para Lactobacilos Argentina.

Culture conditions A preculture was inoculated (0.1%) into sterilized (110°C, 10 min) reconstituted non fat dry milk (100g/l) and incubated at 43°C for 16 hours. The cultures were then cooled in ice water and cell number was counted using a microscope. The cell number for **Lactobacillus bulgaricus** is generally close to 3.10^8 cells/ml.

Viscosity Rotation viscosity measurements were made on a Haake Rotovisco RV2 coaxial cylinder viscosimeter using a MK 50 rotor assembly and an NV sensor system operating at 43°C. Viscosity is expressed in mPa.s.

EXOCELLULAR POLYSACCHARIDE ISOLATION

The culture broth was diluted twice and the casein hydrolyzed by Pronase (enzyme/substrate ratio 1/100, pH 7.5, 40°C, 16 h). Merthiolate (1/1000) was added during incubation to inhibit cell growth. After removal of the cells by centrifugation (16 000g,20 min. 4°C) the clarified supernatant (appr. 2 liters) was concentrated by ultrafiltration (membrane =10.000 M.W cut. off) under nitrogen to about 400 ml. The crude polysaccharide was precipitated by addition of 3 volumes of ethanol. The resultant precipitate was collected after centrifugation (6370 g, 4°C) and redissolved in water. The above procedure was repeated 3 times. The crude polysaccharide was then dissolved in distilled water, dialyzed and lyophilized.

The crude preparation was dissolved in 0.05 M phosphate buffer and applied to a DEAE TRIS ACRYL (IBF, France) column equilibrated with the same buffer. The non adsorbed fraction eluted with the same buffer and detected with the phenol reaction, was lyophilized after dialysis against water at 4°C for 3 days.

ANALYTICAL METHODS

The total carbohydrate content of the non hydrolyzed polysaccharide was determined by the phenol-sulfuric acid method of Dubois et al (1956), modified by Drapron and Guilbot (1962) and arbitrarily expressed as glucose. Protein content of the crude polymer preparation was determined according to the method of Koops et al (1975). The polymer was hydrolyzed for 5 h at 100°C in 2N trifluoracetic acid. Component sugars were identified by high

performance thin layer chromatography (HPTLC) and visualized with diphenylamine-aniline. Individual monosaccharides were converted to their alditol acetates and analyzed by gas liquid chromatography (Sawardeker et al , 1965). The molecular weight was determined by applying a solution of 4 mg polysaccharides in 2.5 ml 0.05M phosphate buffer on a CL Sepharose 4B (Pharmacia, France) column which had been calibrated with dextrans (SIGMA, U.S.A.) of known molecular weight (488 000, 74 300 and 35 600 daltons). Viscosity of the purified polysaccharide (0.2 % solution) was measured at 25°C at pH 4.6, 7.0 and in distilled water using a Contraves Low Shear 30 viscosimeter.

RESULTS and DISCUSSION

Isolation and purification of the exocellular polysaccharide from coagulated milk systems is difficult and tedious. The crude preparations of polysaccharides contain approximately 10 % carbohydrates and between 60 and 80 % protein. Purification by DEAE TRIS ACRYL yields a preparation containing up to 90 % carbohydrates. However experiments carried out to produce polysaccharides by the aboved mentioned strains on media other than milk failed. Furthermore, since the composition of exopolysaccharides is dependent on the carbon and energy source provided (Sutherland, 1982), the main interest of this study was to investigate the possible formation of exopolysaccharides in the presence of milk i.e. under conditions close to those existing in the dairy industry.

Table I Polysaccharide production and viscosity in skim milk by ropy and non ropy strains of **Lactobacillus bulgaricus.**

	Titrable acidity	Viscosity mPa.s	mg polysaccharide/ liter of culture
Non ropy strain			
CNRZ 398	68	7	14
Ropy strains			
CRL 420 [a]	116	61	55
CNRZ 416 [b]	71	3	57
CNRZ 416 [b]	85	164	285
CNRZ 737 [b]	84	218	424

a : skim milk
b : skim milk + 1 % casein

The non ropy strain CNRZ 398 produced only very small amounts of polysaccharide (table 1). The ropy strain 416 formed more of the polymer, however as seen from the extremely low viscosity (2.6 mPa.s) this strain lost its ropy character. The instability of the thickness producing trait in **Lactobacillus** has been reported by numerous investigators and seems to be normal. However even with a very low viscosity strain 416 produces more polysaccharide (57 mg/l) than the non ropy strain 398 (14 mg/l). Strain 416 by several transfers recovered the ropy property in a skim milk culture to which 1 % casein has been added. Viscosity (164 mPa.s) and the amount of produced polysaccharide (285 mg/l) increased considerably. Another ropy strain 737 gave even more viscosity (215 mPa.s) and higher quantities of polysaccharide (424 mg/l). It should be born in mind that our culture conditions (43°C, 16 h) are close to those used in yoghurt

manufacture. It is possible that the strains might have produced more polysaccharides at lower temperatures. Growth of lactic acid bacteria at lower temperatures (35°C) can induce an increase in polysaccharide production (Surtherland, 1972 ; Schellhaas, 1983). The polymers produced by the ropy and non ropy strains have the same sugar components. In all cases galactose is the major monosaccharide, glucose and rhamnose are present in smaller amounts. Traces of arabinose were identified also. Gas liquid chromatography confirmed the HPTLC identification and revealed that the polymer is composed of galactose, glucose and rhamnose in a molar ratio of approximately 4:1:1. HPTLC and GC suggest that uronic acid groups and aminosugars are absent, in agreement with results reported elsewhere (Schellhaas, 1983). It is important to underline that the qualitative characterization of the sugar components by HPTLC was carried out on the purified and non purified polysaccharide to make sure that all the monomers originally present were identified and none of them lost during the purification procedure.

The elution volume of the polysaccharide (strain 416a, table 1) from the CL Sepharose 4B column was almost the same as that of dextran of molecular weight of 488 000 daltons and a single sharp peak was observed indicating that in this case the exopolysaccharide consisted of a single polymer. Compared to the high viscosities measured in the culture broth (table 1) the purified polysaccharide at a 0.2 % concentration produced a semi solid Newtonian solution of low relative viscosity : 2.8 mPa.s in water, 5.4 mPa.s at pH 4.6 and 6.9 mPa.s at pH 7.0. This seems to indicate that interactions might take place in the culture medium between the polysaccharide and the other milk constituents, in particular the casein.

Achnowledgements The authors are grateful to Marie-Thérèse TOLLIER (Laboratoire de Chimie Biologique et Photophysiologie, I.N.A. Paris-Grignon, France) for help with gas chromatography analysis and B. LAUNAY and G. CUVELIER (Département Science de l'Aliment, ENSIA, Massy, France) for help and discussion concerning viscosity measurements.

REFERENCES

DRAPRON, R., and GUILBOT, A. (1962) Ann. Technol. agric. 11, 175-218.
DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A., and SMITH, F. Anal. Chem. (1956) 28, 350-356.
GROUX, M. (1973) Schweiz. Milchz. 4, 2-8.
KOOPS, J., KLOMP, H., and ELGRSMA, R.H.C. (1975) Neth. Milk Dairy J. 29, 169-180.
MANCA DE NADRA, M.C., STRASSER DE SAAD, A.M., PESCE DE RUIZ HOLGADO, A.A., and OLIVER, G. (1985) Milchwissenschaft 40, 409-411.
ODA, M., HASEGAWA, H., KOMATSU, S., KAMBE, M., and TSUCHIYA, F. (1983) Agric. Biol. Chem. 47, 1623-1625.
SAWARDEKER, J.S.F., SLONEKER, J.H.A., and JEANES, A. (1965) Anal. Chem. 37, 1602-1604.
SCHELLHAAS, S.M. (1983) Ph. D. Thesis, University of Minnesota, U.S.A.
SUTHERLAND, I.W. (1972) Bacterial exopolysaccharides. Adv. Microb. Physiol. 8, 143-213.
SUTHERLAND, I.W. (1982) Biosynthesis of microbial exopolysaccharides. Adv. Microb. Physiol. 23, 79-150, Academic Press, London.
TAMIME, A.Y. (1978) Milchwissenschaft 33, 209-212.