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Señora Jefe de la
DIVISION DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

Trámite : 002
Evento : 001
Actuación : 444

Referencia : Expediente No. 03-112173
Asunto : Solicitud de patente para: BIOPOLIMERO CON BASE EN
LACTOCOCCUS LACTIS NRRL B-30656, EL PROCESO PARA EL
CULTIVO DE LACTOCOCCUS LACTIS NRRL Y EL PROCESO PARA
LA PRODUCCION DEL BIOPOLIMERO
Solicitante : INSTITUTO DE BIOTECNOLOGIA DE LA UNIVERSIDAD
NACIONAL
Clase : C08B 037/000, C12P 019/000, C12P 019/004
Fecha solicitud: Diciembre 23 de 2003

1. Yo, Emilio FERRERO, obrando como apoderado del solicitante en el asunto antes identificado, me refiero al Oficio No. 7678 notificado por fijación en lista del 12 de junio de 2008, y atentamente presento junto con este escrito el siguiente documento:

Manca de Nadra M. C., Strasser De Saad A. M., Pesce de Ruiz Holgado A.A. y Olivier G. (1985). *Extracellular polysaccharide production by lactobacillus bulgaricus* cri 420. *Milchwissenschaft* 40: 409-411.

2. Puesto que se han atendido a cabalidad las peticiones hechas por su Despacho sobre la presente solicitud de patente, atentamente solicito que se continúe su tramitación.

Señora Jefe,

EMILIO FERRERO

T.P.A. No. 31.929

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No. M-2008-133279\JMS

Extracellular polysaccharide production by *Lactobacillus bulgaricus* CRL 420

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1. Introduction

Numerous microorganisms produce exopolysaccharides, i.e., polysaccharides found outside the cell wall, either attached to it in the form of capsules or secreted into the extracellular environment in the form of slime. Such polymers vary considerably in their chemical structures (9).

Probably the largest, and certainly the most heterogeneous group of exopolysaccharides is composed of those heteropolysaccharides formed from repeating unit structures. These are widely distributed among eukaryotic and prokaryotic genera, but as yet, relatively few have been thoroughly studied to elucidate their chemical structures. Interest has primarily been concentrated on heteropolymers formed by microorganisms of medical importance, together with a small number of other polymers. Most are formed from relatively simple structural units ranging in size from disaccharides to tetrasaccharides, but one or more types of acyl groups may also be present (8).

Most of the exopolysaccharide producing microorganisms which have been studied utilize carbohydrates as their carbon and energy source and either an ammonium salt or amino acids as their source of nitrogen. Yet, as different ecological environments have been studied, it has become clear that a great variety of substances can be converted into polysaccharides.

We report the production and characterization of an extracellular polysaccharide by a strain of *Lactobacillus bulgaricus* isolated from a market yogurt.

2. Materials and methods

Microorganism: *Lactobacillus bulgaricus* CRL₄₂₀ was isolated from yogurt.

Cultural conditions: The strain was maintained by transference of the organism in sterile reconstituted non-fat dry milk.

The cells were grown in LAPT medium (4) supplemented with 0.5% (w/vol) sugar sources (glucose, fructose, sucrose or lactose). The medium was adjusted to pH 6.8 with 0.2 N NaOH and sterilized by autoclaving for 20 min at 118 °C.

Chemical composition of the polysaccharide: Cell free supernatant solution was obtained by centrifugation (10,000 g, 20 min, 4 °C) in a MSE refrigerated centrifuge, the soluble polysaccharide was precipitated from the supernatant fluid with three volumes of absolute ethanol, followed by storage overnight at 4 °C. The precipitate was recovered by centrifugation (20,000 x g, 15 min, 4 °C) evaporated at 37 °C and redissolved in distilled water to a final concentration of 3.5 mg/ml.

Acid hydrolysis: The polysaccharide was hydrolyzed with 4 N HCl in a sealed tube at 100 °C during 8 h and then neutralized with 4 N NaOH.

Enzymatic hydrolysis: Dextranase (1,6- α -D-glucan-6-glucanhydrolase, E.C. 3.2.1.11) from *Penicillium ilicium* was dissolved in 0.1 M phosphate buffer pH 6.0 to give a solution containing 300 units/ml.

α -Glucosidase (maltase, α -D-glucoside glucosylhydrolase, E.C. 3.2.1.20) from brewers yeast was dissolved in 0.1 M phosphate buffer pH 6.0 to give a solution containing 300 units/ml.

β -Glucosidase (emulsin, β -D-glucoside glucosylhydrolase E.C. 2.2.1.21) from almonds was dissolved in 0.1 M acetate buffer pH 5.0 to give a solution containing 300 units/ml.

The polysaccharide (3.5 mg/ml), purified by gel filtration on Sepharosa 4B (see below), was used as substrate. Duplicate 2 ml samples of polysaccharide were mixed with 1 ml of each enzyme solution and incubated in a shaking water bath for 2 h at 25 °C and 37 °C for α -glucosidase and β -glucosidase, respectively.

Colorimetric determinations: The polysaccharide produced in the culture medium was detected by the iodine reaction (1).

Polysaccharide concentration was determined by the phenol sulphuric method (2).

Glucose was determined by the glucose oxidase method (Glucostat; Worthington Diagnostic).

Fructose was determined by the ROE method (5, 6, 7).

Paper chromatography: For descending paper chromatography (Whatman No. 1) the solvent used was butanol — pyridine — water (6:4:3). The chromatogram was developed following the TREVELYAN method (10).

Determination of molecular weight: A portion (0.5 ml) of the polysaccharide was put on a Sepharosa 4B (Pharmacia) column (2 x 50 cm), equilibrated and eluted with 0.05 M phosphate buffer pH 7.0. Fractions (3.5 ml) were collected at a flow rate of 12.6 ml/h at 4 °C. The polymer was eluted in fractions number 43, 44 and 45 (Fig. 1). The column was

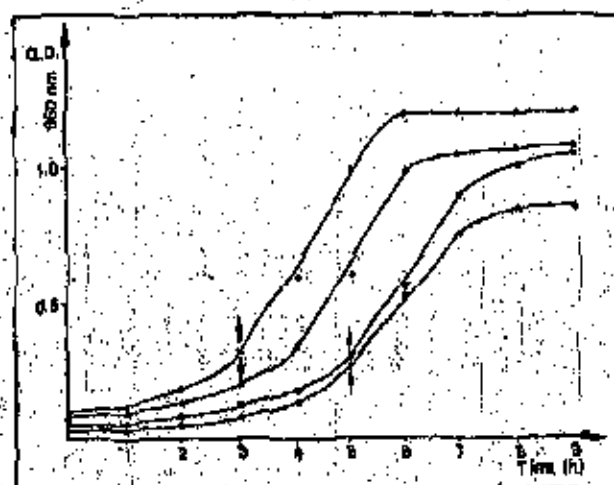


Fig. 1: Growth of *L. bulgaricus* CRL₄₂₀ in LAPT medium with 0.5% (w/vol) glucose, Δ fructose, $\square$ lactose, $\bullet$ sucrose. 1 indicates the initiation of polysaccharide production. For experimental details see the text.

¹ CRL: Centro de Referencia para Lactobacilos.

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calibrated by using a mixture consisting of dextrans with the following molecular weights: 42,500; 71,200 and 494,000 (Sigma) at a concentration of 0.25 mg/ml each.

The molecular weight of the polysaccharide was determined by using a graphic plot of the molecular weight log of the dextrans against elution volume.

3. Results and discussion

Influence of carbon source on the polysaccharide production: *L. bulgaricus* produces an extracellular polysaccharide from the following carbon substrate: glucose, fructose, sucrose and lactose. Polysaccharide production was apparently a feature of the early logarithmic phase of batch-culture growth.

The maximum rate of synthesis occurred during exponential growth and a small amount of polymer was formed when growth ceased. MIAN *et al.* (3) found the same results for *Pseudomonas aeruginosa*.

When the carbon source was glucose or fructose the polysaccharide production started after 3 h of incubation. If the carbon source was lactose or sucrose the polysaccharide synthesis started after 5 h of incubation. It was assumed that when the carbon source is a disaccharide the bacteria must hydrolyse it to grow and produce the polysaccharide (Fig. 2).

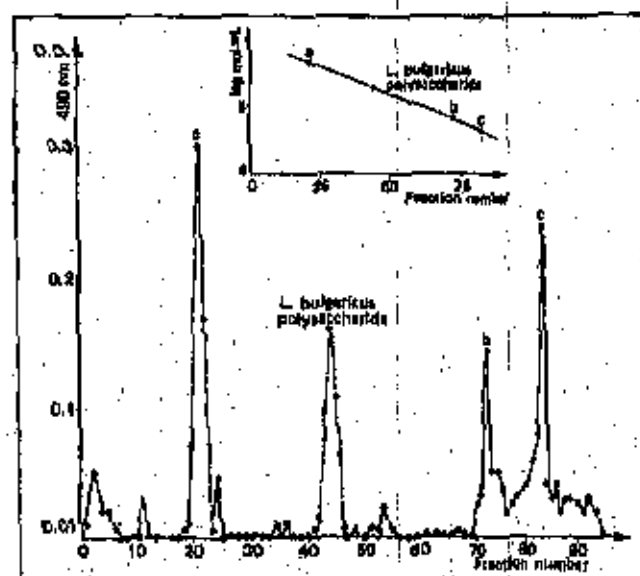


Fig. 2: Elution profiles of polysaccharides in Sepharose 4B column. The polysaccharides eluted were measured as total sugars. The results are expressed as O.D. at 490 nm. The following dextrans of the known molecular weights were used: a, 494,000; b, 71,200, and c, 42,500. Details of the experiment are given in the text. Inset figure: Graphic plot of the log of the molecular weight as a function of the elution volume.

Polysaccharide production in LAPT medium was maximum with glucose 0.5%, initial pH 6.8 and 37 °C.

Chemical composition of the polysaccharide: The molecular weight of the exopolysaccharide was estimated by gel filtration on Sepharose 4B (Fig. 1). It was found to be 197,440.

After acid hydrolysis the products obtained were examined by paper chromatography. Glucose and fructose were the constituent sugars in the polysaccharide.

Total sugars, glucose and fructose concentrations are shown in Table 1. The results demonstrate that the concentration of fructose is two-fold higher than that of glucose.

Table 1: Hydrolysis products of the polysaccharide produced by *L. bulgaricus*

Type of hydrolysis	Total sugars mg	Glucose mg	Fructose mg
4 N HCl	6.94	—	—
α -Glucosidase	4.86	2.22	4.54
Dextranase	—	0.09	2.20
α -Glucosidase + dextranase	—	1.04	2.90
		2.0	4.32

The results obtained by polymer hydrolysis with α -glucosidase and dextranase suggest that these sugars would be linked by α -1,4- and α -1,6-glucosidic linkages in a ratio 1:1.

The polymer was not degraded by β -glucosidase. The results obtained referring to molecular weight and sugars composition of the polysaccharide suggest that the polymer is formed by approximately 1,000 hexose molecules.

L. bulgaricus CRL-420 produces a soluble exopolysaccharide whose composition was independent of the nature of the carbon source in the substrate. SUTHERLAND (9) suggests that exopolysaccharides can be considered as belonging to five distinct groups. The polysaccharide produced by our strain could be included in group IV.

Exopolysaccharide formation may be useful for the stirred (as opposed to the gel) type of yogurt. The species which dispose of such property occur in the commercial starters for yogurt manufacture.

Acknowledgements

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We wish to thank Mónica Lucasele de Ruiz Pezo for her efficient technical assistance.

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5. Summary

MANCA DE NADRA, M.C., STRASSER DE SAAD, A.M., PESCE DE RUIZ HOLGADO, A.A., OLIVER, G.: Extracellular polysaccharide production by *Lactobacillus bulgaricus* CRL 420. *Milchwissenschaft* 40 (7) 409-411 (1985).

26 *Lactobacillus bulgaricus* (polysaccharide production)

Lactobacillus bulgaricus CRL 420 isolated from market yogurt produces a soluble exopolysaccharide. It is constituted by glucose and fructose in a ratio 1:2. The predominant linkages are α -1,4- and α -1,6-glucosidic in a ratio 1:1. The molecular size is 197 400 and the polymer would be formed by approximately 1 000 hexose molecules. Polysaccharide is produced from various carbon sources: glucose, fructose, sucrose and lactose, at the early logarithmic phase of growth.

MANCA DE NADRA, M.C., STRASSER DE SAAD, A.M., PESCE DE RUIZ HOLGADO, A.A., OLIVER, G.: Extraceluläre Polysaccharidbildung durch *Lactobacillus bulgaricus* CRL 420. *Milchwissenschaft* 40 (7) 409-411 (1985).

26 *Lactobacillus bulgaricus* (Polysaccharidbildung)

Aus handelsüblichem Joghurt isolierter *Lactobacillus bulgaricus* CRL 420 bildet ein lösliches Exopolysaccharid. Es setzt sich aus Glukose, Fruktose im Verhältnis 1:2 zusammen. Die vorherrschenden Bindungen sind α -1,4- und α -1,6-glukosidisch in einem Verhältnis von 1:1. Das Molekulargewicht beträgt 197 400, und das Polymer wird von ca. 1 000 Hexosemolekülen gebildet. Polysaccharid wird aus verschiedenen Kohlenstoffquellen gebildet, und zwar aus Glukose, Fruktose, Saccharose und Laktose in der frühen logarithmischen Wachstumsphase.

MANCA DE NADRA, M.C., STRASSER DE SAAD, A.M., PESCE DE RUIZ HOLGADO, A.A., OLIVER, G.: La producción de polisaccharido extracelular por *Lactobacillus bulgaricus* CRL 420. *Milchwissenschaft* 40 (7) 409-411 (1985).

26 *Lactobacillus bulgaricus* (producción de polisaccharido)

MANCA DE NADRA, M.C., STRASSER DE SAAD, A.M., PESCE DE RUIZ HOLGADO, A.A., OLIVER, G.: Producción de polisaccharido extracelular por *Lactobacillus bulgaricus* CRL 420. *Milchwissenschaft* 40 (7) 409-411 (1985).

26 *Lactobacillus bulgaricus* (producción de polisaccharido)

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