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Chemical and Rheological Properties of the β-Glucan Produced by *Pediococcus parvulus* 2.6

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Some physicochemical and rheological properties of the exopolysaccharide (EPS) produced by *Pediococcus parvulus* 2.6 were examined. Structural characterization by NMR (^{1}H and 2D-COSY) showed that the same EPS, a 2-substituted (1,3)-β-D-glucan, was synthesized irrespective of sugar source used for growth (glucose, fructose, or maltose). The molecular masses of these β-glucans were always very high ($>10^6$ Da) and influenced by the culture medium or sugar source. The steady shear rheological experiments showed that all concentrations of the β-glucan aqueous solutions exhibited a pseudoplastic behavior at high shear rates. Viscoelastic behavior of β-glucan solutions was determined by dynamic oscillatory analysis. A critical concentration of 0.35% associated with the appearance of entanglements was calculated. The β-glucan adopts an ordered hydrogen bond dependent helical conformation in neutral and slightly alkaline aqueous solutions, which was partly denatured under more alkaline conditions.

KEYWORDS: Exopolysaccharides; rheological properties; lactic acid bacteria; fermentation; molecular mass

INTRODUCTION

Exopolysaccharides (EPSs) are extracellularly secreted microbial polymers associated or not with the cell surface, produced by a wide variety of bacteria. EPSs can be employed as thickeners, stabilizers, emulsifiers, gelling agents, or fat replacers (*1*). They exhibit a large variety of complex chemical structures, physiological functions, and a wide range of potential applications in various industrial areas.

The food industry is particularly interested in natural viscosifiers and texture enhancers, so-called biothickeners, and an increasing interest is observed in EPSs produced by lactic acid bacteria (LAB). The GRAS (Generally Recognized As Safe) status of these bacteria facilitates the application of their polysaccharides either as additives or as in situ produced thickeners. These exopolysaccharides play an important role in the rheology, texture, and mouthfeel of fermented milks (viili, långfil) and other fermented products (*2*). In addition, EPSs produced by LAB in food products may have health benefits for the consumer (*3*).

Of the polysaccharides with industrial uses, particular attention has been focused on (1,3)-β-D-glucans during the past decade. These polyglucans are produced by several bacteria as EPSs. They are also found as major components of cell walls in fungi and endosperm cells of cereals (*4*). Most of these polysaccharides, such as curdlan from *Agrobacterium*, exhibit interesting physicochemical properties, especially gelling capability, leading to their extensive use in food applications. In addition, this heterogeneous group of β-linked polyglucoses is attracting increasing attention from the pharmaceutical and functional food industries, because of their positive effects on human and animal health. Examples include bioactive and medicinal properties, such as immune stimulation, anti-inflammatory, antimicrobial, hepatoprotective, and cholesterol-lowering as well as antifibrotic, antidiabetic, and hypoglycemic activities (*4, 5*).

The rheological properties and the biological effects of (1,3)-β-D-glucans depend on their chemical structure, concentration, degree of branching, molecular mass, and conformation (*6*). In general, they are polymers of high molecular weight with highly ordered helical structures, which can exist as triple helix or in random coil depending on solvent conditions. Conformation in single helix has also been reported. Currently, it is well-documented that (1,3)-β-D-glucans exist in this spatial structure only under well-defined solvent conditions or in aqueous, neutral solution when the molecular mass is below a minimum limit required for the stability of triplexes (*7*). The formation of more complex structures has also been proposed by triple-helix aggregations (*8*).

The EPS-producing strain used in this study, *Pediococcus parvulus* 2.6, is a lactic acid bacterium that produces a neutral

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branched 2-substituted (1,3)-β-D-glucan when cultivated with glucose as sugar source (*9*). Some of the environmental conditions that influenced EPS synthesis by this strain have been examined (*2, 10*), and improved EPS formation was obtained in a medium containing high concentrations of glucose, Mn (5 mg L^{-1}), and ethanol (4.9% w/v). Thermodegradative behavior of the β-glucan was studied, showing a good thermal stability up to 220−230 °C and degrading significantly above 250 °C. In addition, the potential as possible starter culture of this strain has been shown in oat-based media. This strain increased the viscosity and ropiness of the fermented oat product. It has been also reported that a ropy, oat-based product fermented by *Pediococcus damnosus* 2.6 reduces cholesterol levels and stimulates the bifidobacteria flora in humans (*11*).

This paper focuses on the influence of culture media on molecular masses of the β-glucans produced by *P. parvulus* 2.6. In addition, our analysis of the rheology and three-dimensional conformation of these β-glucans complements previous basic findings on the viscoelastic properties of EPS from *P. damnosus* 2.6 (*12*). Industrially relevant shear thinning flow behavior, observed in other β-glucans, such as schizophyllan and scleroglucan, is also noted and explained.

MATERIALS AND METHODS

Microorganism and Fermentation Conditions for EPS Production. *P. parvulus* 2.6 was obtained from the UPV/EHU (University of the Basque Country) culture collection, and it was isolated from a ropy cider (*9*). It was first named *P. damnosus* 2.6, but lately was renamed *P. parvulus* 2.6 by sequencing information from a fragment of the 16S rRNA gene, after PCR amplification using conserved primers (*13*). Stock cultures were mantained at −80 °C in MRS broth containing 20% (v/v) of glycerol. Before experimental use, the bacterium was propagated twice in MRS broth containing ethanol (5%, v/v) at 28 °C (pH 4.8).

To obtain the polysaccharide, a semidefined (SMD) broth was used as the basic EPS production medium without yeast extract, beef extract, or peptone, as these ingredients interfere with EPS quantification and purification. It contained (in grams per liter of distilled water) casamino acids (Difco), 5; sodium acetate, 5; Bacto yeast nitrogen base (BYNB) (Difco), 6.7; K_2HPO_4, 2; $MnSO_4 \cdot H_2O$, 0.05; diammonium citrate, 2; Tween 80, 1; adenine, uracil, xanthine, and guanine, 0.005; and L-malic acid, 4. Depending on the experiment, 20 g of glucose, fructose, or maltose was added. Sugars and BYNB were sterilized by filtering them through a 0.22 μm pore size Millex-GS filter unit (Millipore, Bedford, MA) and added after autoclaving. The pH of the SMD medium was adjusted to 4.8 prior to sterilization. To compare the molecular masses of the EPSs obtained in different culture media, a MSTv broth was also used (*2*). This medium was optimized for EPS production by *P. parvulus* 2.6 and contained 50 g/L of glucose, 5 ppm of Mn, and 5% (v/v) of ethanol. Fermentations were carried out in a 10 L fermenter for 96 h at 28 °C, without pH control. Bacterial counts were measured by serial dilution plating on MRS agar (Difco).

Isolation, Purification, and Quantification of EPS. Bacterial cells were removed from fermented culture medium by centrifugation (13000*g*, 4 °C, 40 min). The exopolysaccharides were precipitated from the supernatant with 3 volumes of cold ethanol (96% v/v) and mantained during 12−14 h at 4 °C. The precipitate was recovered by centrifugation at 13000*g* for 20 min at 4 °C, washed three times by resuspension in ethanol at 80% (v/v), and centrifuged. Finally, the precipitated EPS was resuspended in deionized water and exhaustively dialyzed against distilled water, using a dialysis membrane (Medicell International, Ltd., London, U.K.) having a cutoff of 12−14 kDa. After dialysis, the precipitate was lyophilized. The total carbohydrate content of the EPS was measured by the phenol−sulfuric acid method as described by Ibarburu et al. (*14*), using glucose as standard. Each given value is the average of three independent determinations.

Analysis of Fermentation Products. To perform chemical analysis, fermentation supernatants from centrifuged samples (16000*g*, 30 min) were filtered through a 0.22 μm membrane filter (Millipore). The concentration of sugars and organic acids in the fermentation broth was quantified by HPLC (Agilent 1100, Hewlett-Packard, Germany) using an ion exclusion, cation exchange column (Aminex HPX-87H, Bio-Rad, Hercules, CA), coupled with an ultraviolet−visible (UV−vis) and refractive index (RI) detector. The column was eluted with diluted sulfuric acid (pH 2.2) at 65 °C, at a flow rate of 0.6 mL/min.

Characterization of the Polysaccharides. *NMR Spectroscopy.* NMR analyses of the EPS synthesized by *P. parvulus* 2.6 in different sugar sources were performed using a spectrometer Avance 500 (Bruker Instruments Inc.). One milligram of each EPS was resuspended in 1 mL of deuterated water (deuterium oxide, 99 atom % D, Aldrich), and the solutions were stirred to complete homogenization. ^{1}H monodimensional and 2D-COSY analyses at 60 °C spectra were carried out according to the conditions described by Dueñas et al. (*9*). To confirm the stability of the β-glucan in alkaline solutions at room temperature, the polysaccharide (1 mg) was dissolved in 1 mL of NaOD (0.4 M) and analyzed by NMR as above indicated. To prevent alkaline hydrolysis of the β-glucan from taking place, quantification of reducing sugars was also done, according to the method described by Anthon and Barret (*15*) and using glucose as standard for calibration purposes.

Size Exclusion Chromatography. The molecular masses of the exopolysaccharides were determined by high-performance size exclusion chromatography (HP-SEC, GPCV 2000, Waters), which included a differential RI detector and a multicapillary viscometry detector. The apparatus was equipped with a guard column and Pl-aquagel-OH 40, 50, and 60 (Polymer Laboratories) columns in series. The use of such a capillary viscometer allows determination of the intrinsic viscosity, which is defined as

$$[\eta] = \lim_{c \to 0} \frac{\eta - \eta_s}{c\eta}$$

where η is the solution viscosity and η_s is the solvent viscosity. The relationship between η and η_s (relative viscosity) is measured by the viscometer detector.

The samples of the dialyzed polysaccharide (0.05%) were dissolved and eluted with a solution (pH 7) containing 0.2 M $NaNO_3$ and 0.01 M NaH_2PO_4 at a flow rate of 0.7 mL/min. As standards for the calibration curve, five dextrans (Fluka), 1.5×10^5, 2.7×10^5, 4.1×10^5, 6.7×10^5, and 1.4×10^6 Da, and four dextrans (Pharmacia), T10, T40, T70, and T2000, were used. The standard T2000 was interpreted as proposed by Wittgren and Wahlund (*16*), taking for the high molecular mass (M_ω) component the value of 5×10^6 Da.

Rheological Analysis. The lyophilized EPSs were dissolved in deionized water by magnetic stirring at room temperature to avoid denaturation effects that could occur at high temperatures. The obtained 0.25, 0.35, 0.5, 1, and 1.5% β-glucan solutions were stable, with no symptoms of phase separation. Rheological measurements were made in a Termo-Haake Rheostress I viscoelastometer, equipped with a cone-plate (60 mm diameter, 2° cone angle) geometry, which allows continuous flow and oscillatory flow measurements. The viscoelastometer has a solvent trap unit that was eventually used for comparison purposes, but, in any case, time influence was not observed during testing, confirming the absence of dehydration of samples. Each experiment was repeated at least three times, detecting differences below 5%. Experiments carried out after different storage times (up to 1 month) give perfectly reproducible results, confirming the stability of the solutions.

Continuous steady-state flow behavior was studied through a shear-rate range of 0.001−200 1/s (shear stress between 1 and 15 Pa) at 10 °C. The consistency index (K) and the pseudoplasticity index (n) were calculated using software based on the power law equation $\sigma = K\dot{\gamma}^n$, where σ is the shear stress and $\dot{\gamma}$ is the shear rate. The influence of temperature on the shear thinning nature of a 0.5% aqueous β-glucan solution was analyzed in a 0−20 °C interval.

The viscoelastic behavior was studied by dynamic small-amplitude oscillatory flow measurements within the linear viscoelastic regimen. These experiments consisted in applying to the solution a shear stress as a sinusoidal time function, analyzing the strain response. Dynamic viscoelastic functions such as the storage modulus G', loss modulus

G'', and complex viscosity η^* of aqueous EPS solutions at 0.25, 0.35, 1, and 1.5% were determined over a frequency range of 0.01−10 Hz at 0 °C. The linear viscoelastic range was determined by a strain amplitude sweep test from which a strain γ value of 1.4% was deduced as the most suitable for all experiments. The Carreau−Yasuda model (*17*) was used to determine the Newtonian viscosity of the different polysaccharide concentrations

$$\eta = \frac{\eta_0}{1 + (\lambda \dot{\gamma}^n)} \quad (1)$$

where η_0 is the Newtonian or linear viscosity (given in Pa s), λ (in s) is the relaxation time, and n is the nondimensional power law exponent. The model can be adapted to complex viscosity data, substituting η by η^* and $\dot{\gamma}$ by the frequency ω.

Preliminary Conformational Studies. The change in the absorption maximum of the dye Congo Red in the presence or absence of polysaccharide preparations was measured. EPS solutions were prepared at 0.1% in different NaOH concentrations (from 0 to 0.4 M). Congo Red (91 μM) was added to all of the solutions. The absorption spectra were recorded from 400 to 600 nm at 25 °C with a UV α-Helλos (Thermospectronic) spectrophotometer. As negative control, solutions of pure dye were used at the same NaOH concentrations.

RESULTS AND DISCUSSION

Structural Analysis and Molecular Mass Determination of β-Glucans Produced by *P. parvulus* 2.6 in Different Culture Media. As described by several authors, the composition of culture medium can significantly influence the monomer composition and molecular mass of microbial exopolysaccharides (*3*, *18*). In *P. parvulus* 2.6, both bacterial growth and EPS production were clearly dependent on sugar source. This strain grew better on glucose than on maltose or fructose in SMD medium, and the maximal biomass yields obtained were 2.4×10^9, 1.4×10^9, and 6.7×10^8 CFU/mL, respectively. *P. parvulus* 2.6 fermented the three sugars by the homolactic pathway, therefore producing lactic acid as sole fermentation product (*19*). EPS amounts were also significantly higher in glucose (140 mg/L) than in maltose (80 mg/L) or fructose (25 mg/L) after 4 days of incubation. A similar influence of sugar source on EPS production has been described for the strains *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 and *Lactococcus lactis* NIZO B40, which synthesized more EPS in glucose than in fructose (*20*, *21*).

Structural characterization of the three polysaccharides was also performed by ^{1}H and 2D-COSY NMR analyses (**Figure 1**). The chemical shifts for the ^{1}H resonances of the polysaccharides were compared, being identical between them and to those previously described by Dueñas et al. (*9*) for this strain. From these results, we concluded that *P. parvulus* 2.6 produces always a β-glucan with the same primary structure: a trisaccharide repeating unit, with two (1→3)-β linked residues in the main chain, one of which is substituted in position 2 by a terminal glucose residue. This polymer shows a close structural similarity to type 37 polysaccharide from *Streptococcus pneumoniae*. The latter has also a linear backbone of →3-β-D-Glc-(1→, but the C_2 linked glucoses are attached to each glucose residue of the main chain (*22*). On the other hand, the structure of EPSs from *P. parvulus* 2.6 is identical to that of the β-glucans produced by *Lactobacillus* sp. G-77 and *Oenococcus oeni* (*14*), which were also isolated from ropy ciders. This fact has been explained by horizontal transfer of the *gtf* gene mediated by plasmids involved in the β-glucan synthesis (*13*).

Several authors have reported that the composition of the culture medium can also influence the size of exopolysaccharides produced by lactic acid bacteria. Molecular mass characterization

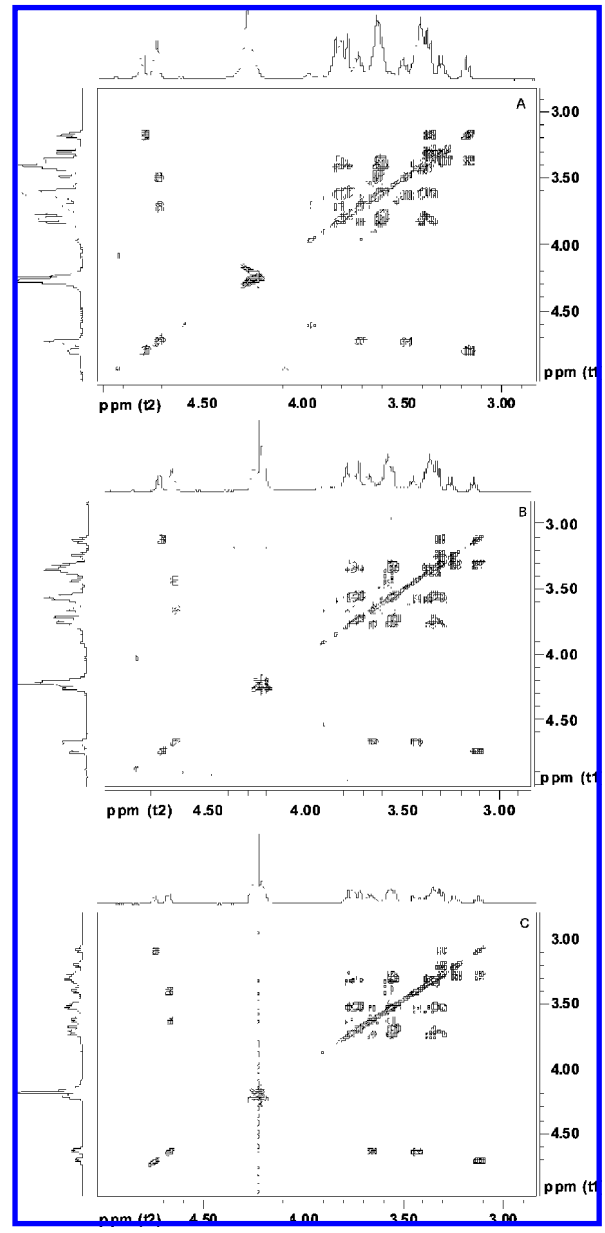

Figure 1. ^{1}H and 2D-COSY NMR spectra of β-glucan synthesized by *P. parvulus* 2.6 on different sugar sources: glucose (**A**), fructose (**B**); maltose (**C**).

of β-glucans was carried out by size exclusion chromatography (SEC), using different dextrans as molecular mass standards. All chromatograms showed the presence of several fractions. When glucose was used as sugar source, a majority fraction, representing >85% of the population, was detected both in MSTv (**Figure 2**) and in SMD media. It exhibited the highest molecular mass, reaching values of 9.6×10^6 and 6.8×10^6 Da in these media, respectively. However, this high molecular mass fraction was quantitatively of minor importance in fructose (**Figure 2**) and maltose, showing lower molecular masses, around 2.5×10^6 Da. The same trend was described for *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772, which produced different molecular mass heteropolysaccharides depending on the sugar source (*20*). In addition, all chromatograms exhibited low molecular mass fractions, which were lower compared with the lowest molecular mass of dextran standards used (10 kDa) and, so, the sizes were difficult to determine. These low fractions

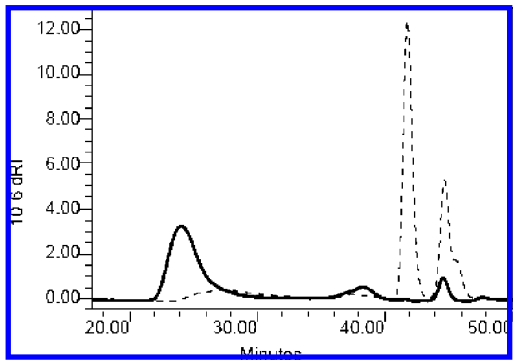

Figure 2. Size exclusion chromatograms of the β-glucans produced using as culture media MSTv with glucose (—) and SMD with fructose (- - -).

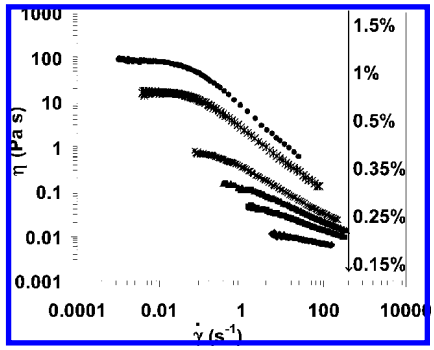

Figure 3. Viscosity (η) as a function of shear rate ($\dot{\gamma}$) for β-glucan solutions in water. Constant temperature: $T = 10$ °C.

could represent oligomers of the β-glucans and were quantitatively more important in maltose or fructose, precisely the sugar sources in which the β-glucan production was diminished.

Rheological Properties. *Continuous Flow Steady State Measurements.* The viscosity function of the aqueous solutions of the β-glucan produced in glucose by *P. parvulus* 2.6 was determined, repeating each experiment at least two times. The results at $T = 10$ °C of the different aqueous solutions of the β-glucan, obtained in SMD medium with glucose as sugar source, are presented in **Figure 3**. A viscosity enhancement with polysaccharide concentration was found. All concentrations of the β-glucan exhibited a non-Newtonian pseudoplastic behavior with a shear thinning zone at high shear rates. However, a Newtonian or linear plateau was noted at low shear rates, defining a shear rate independent viscosity. As observed in general in polymer solutions (*23*), the onset of the pseudoplastic behavior shifts to lower shear rates as the polysaccharide concentration increases.

The existence of a structure or physical network in the solution, which is due to entanglements between β-glucan chains, can explain the viscosity decrease observed in **Figure 3**. Above a certain shear rate, entanglements are progressively disrupted by shearing forces, giving rise to a viscosity reduction (*24*). Similar observations have been reported on hydrolyzed oat gum rich in β-glucans (*25*). It is then possible to attribute the origin of the solution structure or network to associations between β-glucan chains. These associations should be labile and stress sensitive and will be, therefore, disrupted by mechanical shearing. The specifity of β-glucan chain entanglements is treated in the last part of the paper.

Pseudoplastic or shear thinning behavior has also been reported for other biopolymers with industrial applications, such as xanthan and gelan or other β-glucans such as scleroglucan (*26*) and schizophyllan (*24*). Shear thinning behavior of a polysaccharide has several potential advantages in food applications. Viscosity reduction as shear rate increases becomes favorable in industrial operations such as mixing and pumping. In addition, pseudoplasticity is important in helping to provide good sensory qualities, such as mouthfeel and flavor release and suspension properties of food products (*27*).

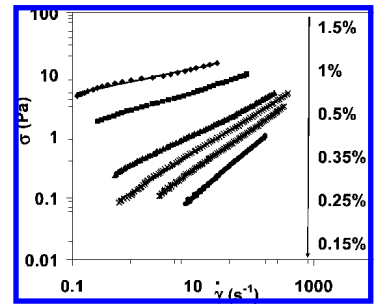

Figure 4. Flow curves and corresponding power law fits (see text and **Table 1**) for β-glucan solutions in water ($T = 10$ °C).

Table 1. Values of the Parameters K and n of the Power Law $\sigma = K\dot{\gamma}^n$ for Different β-Glucan Concentrations at $T = 10$ °C

concentration %	K (Pa s)	n
0.15	0.02	0.83
0.25	0.06	0.69
0.35	0.13	0.62
0.5	0.35	0.48
1.0	2.58	0.30
1.5	7.77	0.20

Table 2. Values of the Parameters K and n of the Power Law $\sigma = K\dot{\gamma}^n$ for a 0.5% β-Glucan Concentration at Different Temperatures

T (°C)	K (Pa s)	n
0	0.44	0.47
4	0.39	0.48
7	0.36	0.49
10	0.34	0.49
15	0.29	0.51
20	0.25	0.52

The pseudoplastic degree was evaluated by fitting the shear thinning region of the flow curves to the power law equation (described under Materials and Methods), as depicted in **Figure 4**. Results of the pseudoplasticity index (n) and the consistency index K are summarized in **Tables 1** and **2**. As seen in **Table 1**, for concentrations higher than 0.25%, the aqueous solutions of β-glucan exhibited a notable shear thinning behavior, as the values of the pseudoplasticity index (n) were considerably lower than $n = 1$ (which indicates Newtonian or linear behavior). A decrease of n parameter values with regard to the increase of the polysaccharide concentration was observed, showing the most concentrated solution (1.5%) having the lower pseudoplasticity index ($n = 0.2$). Thus, the lower the concentration was, the closer to Newtonian was the behavior of β-glucan solutions. Scleroglucan, a β-glucan synthesized by *Sclerotium rolsfii*, showed similar values of the pseudoplasticity index (*26*). With respect to the consistency index K, a remarkable increase with β-glucan concentration was observed (**Table 1**). This result is compatible with a stronger and consolidated molecular structure, as has also been reported by Moreno et al. (*28*) for extracellular polysaccharide produced by the cyanobacterium *Anabaena* sp. ATCC 33047.

Table 2 shows the influence of temperature on the pseudoplastic properties of the aqueous solutions of β-glucan. The value of the pseudoplasticity index (n) increased with temperature.

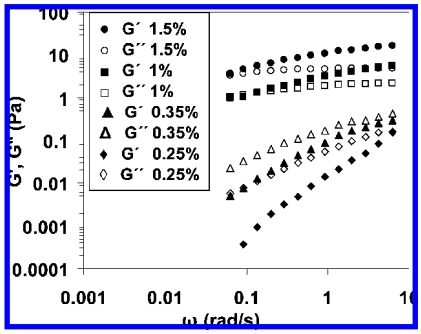

Figure 5. Storage (G') and loss moduli (G'') as a function frequency, evaluated at $T = 0$ °C and $\gamma = 0.014$ (linear viscoelastic region), for different β-glucan concentrations.

Therefore, we can assert that the shear thinning character of the solution is reduced as temperature is raised. This tendency, which is relevant in relation to the potential use of these polymers in food industry, was also observed for the rest of the β-glucan solutions analyzed.

Dynamic Viscoelastic Measurements of β-Glucan Solutions. Linear viscoelastic behavior of β-glucan from *P. parvulus* 2.6 was studied by dynamic oscillatory analysis, the most commonly used method for examining the viscoelastic properties of food components. For each concentration of β-glucan in water the interval of linear viscoelasticity was determined. The storage shear modulus G' and the loss shear modulus G'' were analyzed as a function of frequency in the range from 0.06 to 10 rad/s at 0 °C. The results are depicted in **Figure 5**.

For 0.25 and 0.35% β-glucan concentrations, the viscous modulus (G'') predominated over elastic modulus (G') in the whole range of frequencies, showing that these solutions behaved as viscous fluids, although a cross-over ($G' = G''$) at approximately 6 rad/s could be envisaged. However, the most concentrated solutions (1 and 1.5%) displayed a different behavior. Thus, the viscous modulus (G'') was lower than the elastic modulus (G') at practically all tested frequencies. The cross-over of the two curves was observed at low frequencies, around 0.06−0.1 rad/s (**Figure 5**). Moreover, a weak frequency dependence of both dynamic moduli was noted, because $G' \alpha\ \omega^{0.32}$ and $G'' \alpha\ \omega^{0.07}$ functions were obtained for 1.5% and $G' \alpha\ \omega^{0.39}$ and $G'' \alpha\ \omega^{0.16}$ for 1% solutions. This frequency dependence was much slighter than that reported by Lambo-Fodje et al. (*12*) for 2−4% concentrations of β-glucans produced from *P. damnosus* 2.6. In comparison, the lowest values of the power law indices found by these authors for their β-glucans are $n' = 0.9$ (for $3G' \propto \omega^{n'}$) and $n'' = 0.7$ (for $G'' \alpha\ \omega^{n''}$), at a 4% concentration.

Considering the basis established by Guenet (*29*), our viscoelastic results ($G' > G''$ and very slight dependence on frequency) indicate the formation of a weak gel for concentrations ≥1%. This weak gel behavior, which has also been described in other β-glucans, such as the scleroglucan (*30*), favors the potential use of these polysaccharides as thickening agents. The constitution of a gel network in β-glucan solutions is not surprising, considering the capacity of this polysaccharide chain to adopt an ordered hydrogen bond dependent helical conformation, as shown in the last part of this paper. Actually, gels based in such structures have been reported in other polysaccharides such as agarose or carrageenans (*29*).

To verify if our β-glucan solutions fit the Cox−Merz rule, which gives an idea of the homogeneity of a solution, the results obtained under continuous steady flow and oscillatory flow were compared. Thus, a double-logarithmic representation of the

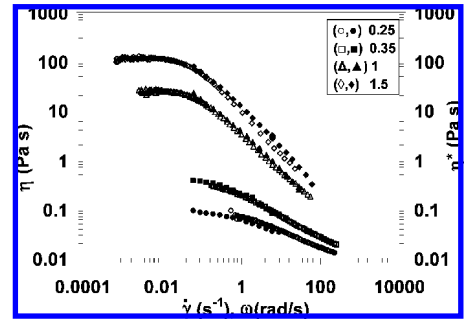

Figure 6. Double-logarithmic plot of the complex viscosity (η^*) (solid symbols) and the steady state viscosity (η) (open symbols) versus the oscillation frequency and the shear rate, respectively.

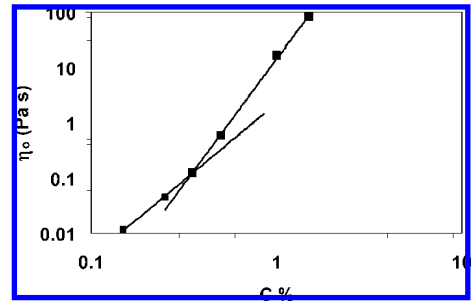

Figure 7. Double-logarithmic plot of the Newtonian viscosity versus polymer concentration in β-glucan aqueous solutions at $T = 0$ °C.

complex viscosity (η^*) and the steady-state viscosity (η) versus the oscillation frequency and the shear rate, respectively, was performed (**Figure 6**).

The good superposition of the results obtained with both types of flow indicated the existence of only one phase in β-glucan solutions. On the contrary, Grassi et al. (*30*) pointed out that scleroglucan aqueous solutions of 2% do not fulfill the Cox−Merz rule, because the complex viscosity was significantly higher than the steady-state viscosity. This observation suggested the existence of certain heterogeneity in their samples.

The presence of interactions between polymer chains, which are designated in general terms as entanglements, is delimited by defining a critical concentration (C^*). This critical concentration for entanglements can be calculated by performing a double-logarithmic plot of the linear or Newtonian viscosity (η_0) versus the β-glucan concentration. As explained under Materials and Methods, both viscosity functions, $\eta(\dot{\gamma})$ and $\eta^*(\omega)$, can be fitted to eq 1, allowing us to determine the Newtonian viscosity η_0 from continuous and oscillatory data. The results of **Figure 7** clearly suggest that the Newtonian viscosity increase with β-glucan concentration should be adjusted to two straight lines. A slope change from 3 to 4.4 (equivalent to the same change of b exponent in $\eta_0 = KC^b$ equation) at a concentration of 0.35% was observed. Within experimental limits, 0.35% can be taken as an approximate value of the critical concentration for entanglements for the β-glucan from *P. parvulus* 2.6. Entanglements between polymer chains are envisaged indistinctly for random coil and rod-like polymers (*31*). Random coil (flexible) polymers show b exponent values in the range of 5−6 (*32*), but lower b values are observed for stiff polymers (*33*). Indeed $b = 4.4$ (determined from the upper slope of **Figure 7**) can be considered as low enough to be compatible with a helical conformation, associated with a rigid rod-like behavior.

Helix−Coil Transition Analysis. (1→3)-β-D-Glucans with a helical conformation have been reported to form complexes with Congo Red in dilute alkaline solutions. The formation of these

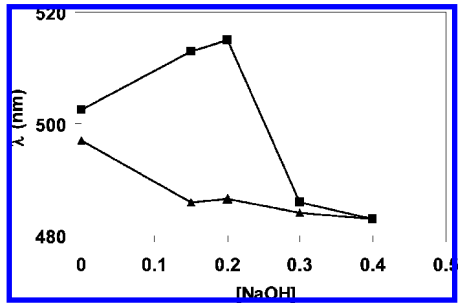

Figure 8. Change in λ_{max} of Congo Red (▲) and Congo Red−β-glucan complex (■) at different NaOH concentrations. The solutions contain 91 μM Congo Red and 0.5 mg/mL β-glucan. The λ_{max} of Congo Red was determined at the same NaOH concentrations as for Congo Red complexes.

complexes and the resulting shift of the maximum absorption wavelength (λ_{max} value) of Congo Red constitute a rapid method for detecting helical structures. Polymers having exclusively a random coil conformation are not able to form these complexes (*34*).

Figure 8 shows the variation of λ_{max} of Congo Red for different concentrations of NaOH, in the presence or absence of β-glucan. At low concentrations of NaOH ($\leq$0.2 M), the λ_{max} of the Congo Red−β-glucan complex shifted to a longer wavelength (512−515 nm), reaching the longest wavelength at 0.2 M alkaline concentration. At higher concentrations, λ_{max} dropped sharply, reaching the same value as that of the control solution of Congo Red (483 nm). This λ_{max} displacement is consistent with an order−disorder transition, which may be attributed to the breakage of hydrogen bonds (*35*).

These results suggested that the β-glucan synthesized by *P. parvulus* 2.6 adopts an ordered hydrogen bond dependent helical conformation in neutral and slightly alkaline aqueous solutions. This helical conformation is in agreement with the three-dimensional model of a representative fragment of this 2-substituted β-(1→3)-glucan proposed by Lambo-Fodje et al. (*12*). This tertiary structure was denatured under strong alkaline conditions (c_{NaOH} > 0.2 M), probably transformed into a random coil, as found for other (1,3)-β-D-glucans. For instance, the triple helix of the β-glucan curdlan is denatured in alkaline solutions, provoking the loss of the thickening capacity of this polysaccharide (*6*). Other β-glucans, such as the schizophyllan and the scleroglucan with branched structures, also adopt a configuration in triple helix in water and in random coil when they are denatured in dimethyl sulfoxide or in NaOH (*22*).

Viscometric Characterization of the β-Glucan in Alkaline Solution. The relative viscosities η_r of the β-glucan throughout 96 h in alkaline solution (0.4 N NaOH) were studied by SEC−viscometry measurements. As shown in **Figure 9** a significant reduction of relative viscosity was observed in alkaline solution (0.4 N NaOH) over time, reflecting a continuous denaturation process of the ordered conformation of the β-glucan and suggesting that hydrogen-bonding structure broke down gradually in alkaline solution. To reject the possibility of the results being due to β-glucan hydrolysis, all samples were analyzed by ^{1}H NMR together with reducing sugars analysis. The results showed no hydrolysis of the polysaccharide in the alkaline medium at room temperature.

Figure 10 shows the double-logarithmic plot of the intrinsic viscosity [η], defined under Materials and Methods, versus the molecular mass M_ω. The data were taken at different times after solution preparation, showing a molecular mass decrease as elapsed time augments. Instead of a Mark−Houwink type

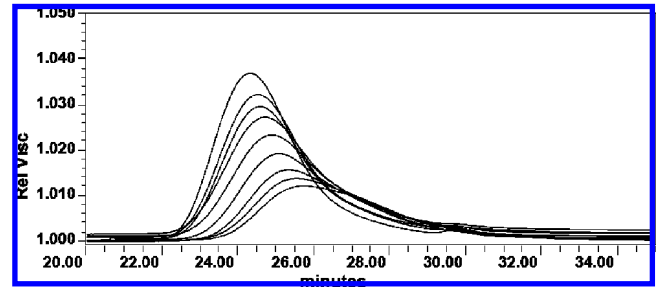

Figure 9. Change of the relative viscosity η_{rel} of β-glucan (0.5 mg/mL) with time in 0.4 M NaOH at T = 23 °C: (from top to bottom) 35 min; 1 h, 35 min; 2 h, 35 min; 3 h; 12 h; 24 h; 48 h; 72 h; 96 h.

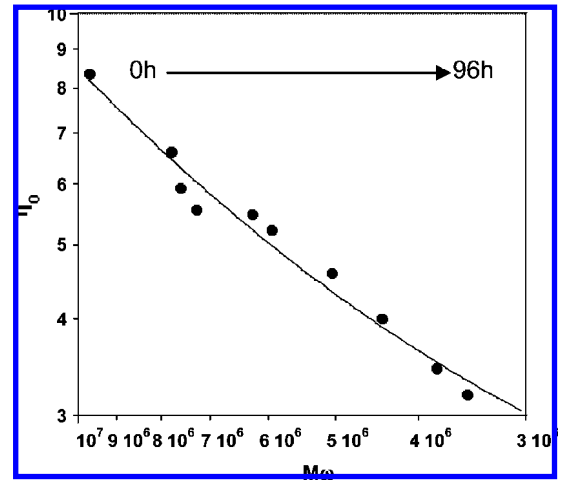

Figure 10. Double-logarithmic plot of the intrinsic viscosity versus the molecular mass for β-glucan at 0.4 N concentration of NaOH taken at different elapsed times as indicated by the arrow. Data are fitted to the equation log [η] = 0.29 (log M_ω)^2 − 3.02 log M_ω + 7.951.

equation ([η] = KM_ω^α), which would give a straight line in the double-logarithmic plot of **Figure 10**, the data follow a curve expressed by the equation log [η] = 0.29 (log M_ω)^2 − 3.02 log M_ω + 7.951. This signifies that a different α exponent value stands for each molecular mass. Therefore, the slope of the curve at each M_ω allows us to define a molecular mass dependent $\alpha(M_\omega)$ exponent of a Mark−Houwink type equation.The value of the exponent decreases from 1.08 for the aqueous solution to 0.62 for the alkaline solution after 96 h. From literature results we recall that the value of α ranges between 0.5 and 0.8, for random coil polymers. However, α > 1 values are typically observed for stiff polymers, such as poly-γ-benzyl-L-glutamate (*30*), so our results suggest that the polysaccharide would present initially a rigid rod behavior (α = 1.00), helicoidal ordered with flexible zones, evolving to a structure in random coil (α = 0.71) due to destruction of the helical zones. A similar conformation in random coil with partially helical areas was proposed by Kulicke et al. (*31*) for other β-glucans.

Time-dependent molecular mass decrease of β-glucans in alkaline solution occurs also for schizophyllan and scleroglucan (*22*). This has been explained by denaturation of the triple-helix structure as a consequence of the ionization of the hydroxyl groups and the subsequent electrostatic repulsion between chains (*5*). In both polysaccharides reported in the literature, the decrease of the molecular mass comes closer to a third of its initial value. A possible explanation for the larger decrease of our β-glucan molecular mass (from 9.6 × 10^6 to 1.7 × 10^6 Da, as observed in **Figure 10**) would be the separation of aggrega-

tions, constituted by bonding among helical segments, as indicated for the PGG−β-glucan isolated of *Saccharomyces cerevisiae* (*8*).

In conclusion, we have shown that the molecular mass of the (1,3)-β-D-glucan synthesized by *P. parvulus* 2.6 depends on culture conditions, indicating that synthesis of high molecular mass polysaccharides takes place when a more suitable culture medium for EPS production is used. A remarkable pseudoplastic or shear thinning flow behavior of β-glucan solutions is observed, especially for 1 and 1.5% glucan concentrations, which constitutes a helpful factor to provide good sensory qualities and favors industrial operations such as mixing and pumping. Therefore, the potential use of this polysaccharide as a biothickener is proved. Rheological results are compatible with hydrogen bonds, the presence of which has been proved by the absorption spectra analysis of complexes with Congo Red in dilute alkaline solutions. This analysis suggests that the β-glucan synthesized by *P. parvulus* 2.6 adopts an ordered hydrogen bond dependent helical conformation in neutral and slightly alkaline aqueous solutions and this tertiary structure agrees with the helical conformation proposed by Lambo-Fodje et al. (*12*). In addition, it is observed that the intrinsic viscosity decreases with time in alkaline solutions, leading to a decrease of the exponent of the Mark−Houwink equation. According to this result it is proposed that the polysaccharide would present initially a rigid rod behavior, helicoidal ordered with flexible zones, evolving to a structure in random coil due to destruction of the helical zones.

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