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ORIGINAL PAPER

Rheological behaviour of a novel microbial polysaccharide

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MONICA MIRONESCU[1]*, ION DAN MIRONESCU[2]
[1]Department of Food Biotechnology, Faculty of Agricultural Sciences, Food Industry and Environmental Protection, "Lucian Blaga" University of Sibiu, I.Ratiu str. 7-9.; Romania.
[2]Department of Chemistry and Food Engineering, Faculty of Agricultural Sciences, Food Industry and Environmental Protection, "Lucian Blaga" University of Sibiu, I.Ratiu str. 7-9. Romania.
**Corresponding author: monica.mironescu@ulbsibiu.ro Phone 004 0269 211338*

Abstract

This paper analyzed the rheological behavior of a new microbial extracellular polysaccharide produced by the extreme halophilic archaeon Haloferax mediterranei (EPSH). Because EPSH is little known, its rheological properties were compared with xanthan, a well characterized microbial polysaccharide. 5% EPSH in aqueous solution with 200 g/l NaCl (the polysaccharide and salt concentrations in the natural environment), 100 g/l NaCl, 40 g/l, 20 g/l and 0 g/l NaCl was analyzed and compared with a 5% aqueous xanthan solution. Flow behavior and viscoelastic behavior were investigated. A Power-law model was obtained for the flow behavior. The flow and viscosity functions obtained in this work evidenced the pseudoplastic character of EPSH, which decreases with the decrease of salt content. The pseudoplastic character of EPSH is much less pronounced as those of xanthan, as the curves obtained practically and the coefficient of the Power-law model indicated. The viscoelastic measurements of the elastic and viscous modules showed that EPSH at 5 g/l and at 200 g/l NaCl (similar to its native state) behave as a weak gel with the viscous state predominant, being weaker as xanthan; destruction of the EPSH gel structure occurred at frequencies near $5s^{-1}$ whereas xanthan weak gels were destabilized at oscillation frequency around 50 s^{-1}. At lower salt content (100 g/l and less) EPSH acted differently, forming structures with the liquid state predominant. These results indicated that EPSH is a polyamphion with high ionic strength and the reduction of the salt content induces structural modifications of this biopolymer. The analysis of the loss tangent indicated that EPSH in saline environments has a higher molecular mass as xanthan. The analysis of complex modulus for aqueous and saline EPSH and aqueous xanthan solutions in oscillatory test combined with heating to and maintaining at 100°C showed that the polysaccharide maintains its initial structure, being more resistant as xanthan, which is destabilized at 100°C.

Keywords: polysaccharide, EPSH, *Haloferax mediterranei*, flow function, Power-law, viscoelastic

Introduction

Microorganisms growth is often associated with the production of extracellular polymeric substances composed especially from polysaccharides [1]. The extracellular polysaccharides (EPS) have specific biological functions as the adhesion to surfaces, as protective barrier or as structural elements of the biofilms [2, 3]. EPS as xanthan gum, scleroglucan, gellan gum, curdlan, bacterial alginate, dextran, pullulan, bacterial cellulose, etc. are already successful used in the food industry, in medicine, pharmacy, cosmetics or oil industry [4-6].

Current researches are oriented on finding new sources of EPS, as the marine extremophilic microorganisms [7-9]. *Haloferax mediterranei* is an extreme halophilic archaeon (haloarchaeon) which needs as special condition for growth very high NaCl concentrations (between 18% and 25%) [10]. The microorganism produces a biofilm having as major component a extracellular polysaccharide (abbreviated EPSH in this work), which gives the colonies a typical mucous character [11]. In terms of sugar composition, the EPSH

contains for neutral sugars, with mannose and glucose as predominant and smaller amounts of galactose and ribose; it has also two amino sugars glucosamine and galactosamine in small quantity [12]. The presence of sulphate groups in higher amount as the amino groups was also evidenced [10, 13].

Investigations with static light scattering showed that the polysaccharide forms aggregates with a molecular weight of around 10^8 Da [14]. The presence pf different ions influence the particles form and shape; in the presence of divalent ions, EPSH has a random coil conformation and in media containing monovalent ions elongated chain are formed. Its binding capacity of cations decreases in the order: $Ca^{2+}>K^{+}>Na^{+}>Mg^{2+}$ [15].

EPSH separated together with salts presented in the nutritive broth (with NaCl as major component), can form a biofilm when dried in air; the biofilm has interesting properties: antimicrobial action, preserving activity and biodegradability [16].

They are relatively limited information on the rheological properties of EPSH. The rheological behavior of polysaccharides is important because it offers information on their properties [17-19], on the relations between microstructure and physical characteristics of the system where they are used [20] or on the textural characteristics [21]. Viscosity studies showed that the aqueous diluted EPSH solutions exhibit pseudoplastic behavior with high viscosity, the viscosity remaining relatively constant in a large temperature and pH interval [22].

The aim of this research is to improve the knowledge on the rheology of this biopolymer in semi-concentrated solutions containing NaCl, respective to analyze the flow and the viscoelastic behavior at room temperature and to analyze the influence of high temperatures on the dynamic rheological properties of EPSH. Taking into account the particular adaptation of the haloarchaeon to very high salinity, the influence of NaCl on the rheological behavior of EPSH in high saline, diluted and aqueous solutions is considered. Because xanthan is one of the most characterized microbial polysaccharide [23-25], it is used in this research as comparative biomaterial with EPSH.

Material and methods

Materials

EPSH was produced through the cultivation of *Haloferax mediterranei* ATCC 33500 strain (DSMZ Germany) in a glass stirrer tank bioreactor. For the cultivation, a complex medium with (in g/l): glucose 5; NaCl 200; $MgCl_2$ 50; K_2SO_4 5; $CaCl_2$ 0.133; yeast extract 5; peptone 5; was used. The cultivation parameters were: 38^{o}C, pH 7.2, air supply 1 l/min during the entire cultivation. The cultivation parameters were regulated trough a process control system based on LabVIEW [26].

For the rheological analysis of EPSH, salts were removed from the original substrate through centrifugation and dialysis, as described elsewhere [12, 13]. The cultivation medium containing EPSH was separated from cells by centrifugation at 11000 rpm, 4^{o}C for 30 min and salts were removed through dialysis against water (1 x 10^5 MW cut off) until the conductivity was smaller as 0.5 μs/cm. EPSH was precipitated with ethanol 96% in the proportion dialysate : ethanol = 1 : 2. The product obtained was lyophilized and stored in sealed recipients at room temperature. The fibrous material obtained was used for the rheological measurements.

Because *H. mediterranei* is very adapted to saline environments, the possibility that NaCl influences the EPSH rheological behavior was considered. Five sodium chloride concentrations were tested: 200 g/l, 100 g/l, 40 g/l, 20 g/l and 0 g/l (aqueous solution). The

EPSH concentration was 5 g/l in all samples, this concentration in polysaccharide being also found in its natural environment [11].

In order to compare the rheological behavior of the polysaccharide produced by *H. mediterranei* with a well characterized polysaccharide, aqueous solutions with 5 g/l of xanthan (Merck, Darmstadt, Germany) were used.

Rheological measurements

Rheological measurements were carried out on a Physica MCR 300 Rheometer (Anton Paar Austria) having cone-plate geometry CP 50-2 and tempering system TEK 150P-C. In all analyses, the space between the superior plate and the cone cap was 0.05 cm. The choice of this small distance was motivated by the small polysaccharide dimensions [27]. Before each analysis, the rheometer was calibrated with pure water. Before the rheological measurements, samples were maintained at room temperature for several hours, so that any stresses in the biomaterials were able to relax [22]. Then, each solution was left to stand inside the device for 10 minutes prior to eliminate the tensions, to stabilize the polysaccharide structure, to eliminate the gas bubbles and to tempering the solution. The duration of 10 minutes is considered as the time needed to recover the nonlinear properties of xanthan [28].

The flow properties (flow curves and viscosity curves) were measured at constant temperature (25°C) with linear variation of shear stress from 10^{-2} to 10^{1} Pa. The coefficients of the Power-law model, which is describing the pseudoplastic behavior [29], were also determined.

Small amplitude sinusoidal oscillatory tests at variables frequencies are often used to measure simultaneously the viscous and elastic properties of a polymer. Oscillatory tests at 1% constant amplitude and constant temperature (25°C) were performed; the elastic and viscous modules *G'* and *G"* (Pa) were determined; from these the complex modulus $|G^*|$ (Pa), the complex viscosity $|\eta^*|$ (Pa·s) and the loss tangent tanδ were calculated using the rheometer built-in functions.

Temperature tests with heating from 30°C to 100°C and maintenance at 100°C (heating rate 7°C/min) combined with oscillatory tests at 1% constant amplitude and constant frequency ($50s^{-1}$) were also performed, in order to investigate the polysaccharide resistance to high temperatures.

Results and Discussion

Figure 1 shows the flow and the viscosity functions resulted for EPSH and xanthan. The curves evidence the pseudoplastic behavior of EPSH and show that the pseudoplastic character of EPSH in saline environment is less pronounced as those of xanthan. At different salt concentration, the flow and viscosity curves change, showing the importance of salt on the rheological properties of EPSH.

To quantify the pseudoplastic behavior of this specific EPS, the data were fitted to a Power law model. The Power-law model is an easy-to-use model that is ideal for pseudoplastic, relatively mobile fluids such as weak gels and low-viscosity dispersions. The coefficients of this model for all solutions analyzed are presented in Table 1. The consistency coefficient *K* describes the overall range of viscosities across the part of the flow curve that is being modelled. The high value of K for xanthan, compared with those of EPSH, indicates that xanthan is much more viscous as the polysaccharide excreted by *H. mediterranei*. The analysis of *K* for EPSH indicates a decrease of viscosity with the increase of dilution of the highly saline EPSH solution. The result suggests a destabilization of EPSH in the absence of salt at very high concentrations.

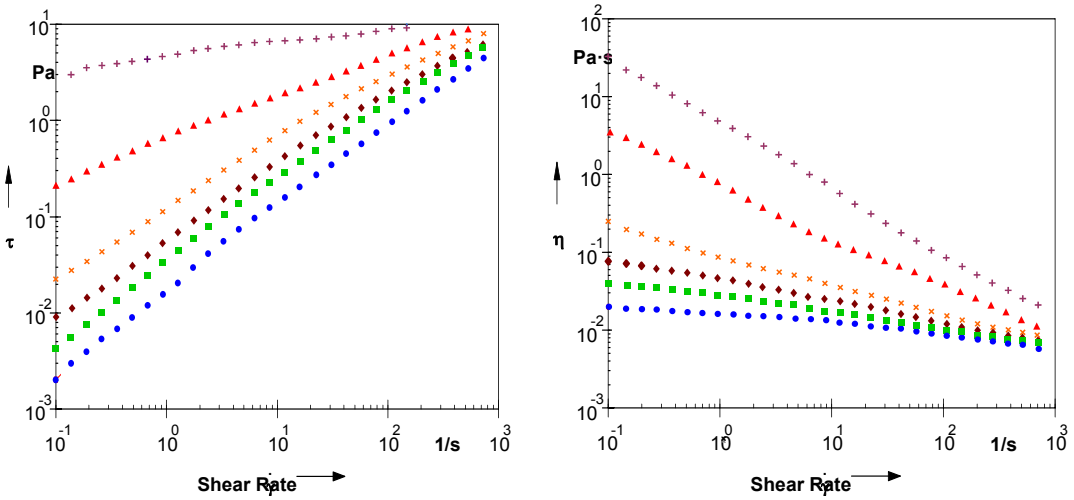

Figure 1. Flow curves (a), respectively viscosity curves (b) for 5% EPSH in in saline medium with 200 g/l NaCl (triangle), 100 g/l NaCl (rosette), 40 g/l NaCl (diamond), 20 g/l NaCl (quadrate), water (circle) compared with aqueous xanthan solution (cross)

Table 1. Coefficients of the Power-law model for EPSH at different salt concentrations and for xanthan

Sample	*K*	*n*
5% EPSH in saline medium with 200 g/l NaCl	0,6212	0,4275
5% EPSH in saline medium with 100 g/l NaCl	0,1199	0,6703
5% EPSH in saline medium with 40 g/l NaCl	0,0585	0,7503
5% EPSH in saline medium with 20 g/l NaCl	0,0332	0,8246
5% EPSH in aqueous medium	0,0171	0,8534
Aqueous solution of 5% xanthan	4,6943	0,1432

The exponent *n* (known as the Power-law index) has values between 0 and 1 for a shear thinning fluid; the more pseudoplastic the product, the closer *n* is to zero [29]. Compared with xanthan (which shows a high pseudoplastic behavior, indicated by a very small value for *n*), EPSH has a less pronounced pseudoplastic character, which decrease with the decrease of salt content in the environment.

As the flow curves show and because *H. mediterranei* grows and forms polysaccharide in extreme salty environments, the possibility that the EPSH changes its conformation in the absence of salts has to be considered. These changes can be evidenced through rheological measurements, too [20]. The analysis of the viscoelastic behavior of EPSH in saline (200 g/l NaCl) and aqueous solution, realized in comparison with xanthan is presented in Figure 2. Both polysaccharides have similar behavior in solutions 5% with different solvents: water for xanthan and salty solution (with 200 g/l NaCl) for EPSH. As observed in Figure 2, G' > G" in a first stage for both polysaccharide solutions, indicating the predominance of the solid character of biopolymers at low frequencies. This behavior is specific to the polymers gel networks, showing that xanthan in aqueous media and EPSH in salty media form a hydrogel, phenomena frequently for polysaccharides; G' is not much higher as G" because the gel formed is not very strong [30]. In these conditions xanthan forms a so-called "weak" gel through chains association and intermolecular networks formation in aqueous media [31]. Both values of elastic and viscous modules are smaller for EPSH, indicating that this polysaccharide forms a gel weaker as the xanthan gel.

There is a critical frequency at which the two responses for the elastic and viscous modules are equal. As observed in Figure 2, the initial gel structure is destroyed for both EPS,

at frequencies near 5 s^{-1} for EPSH and 50 s^{-1} for xanthan. After the structure of both EPS destabilizes, G" values rest near the initial ones and G' decreases very much, results which evidence the pass in a form having the liquid form as predominant. The final structure can be considered stable because the two modules evolutes in parallel.

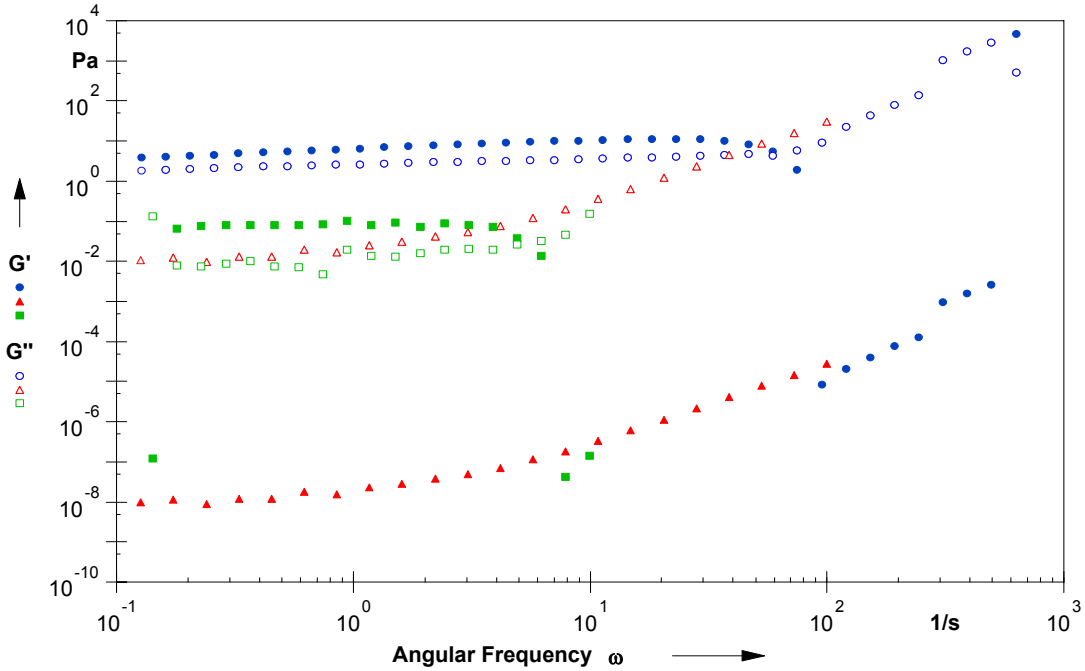

Figure 2. Variation of the elastic modulus G' and viscous modulus G" as function of frequency for 5% aqueous xanthan solution (circle), aqueous EPSH solution (triangle) and salty EPSH solution (200 g/l NaCl) (quadrate)

Compared with the high saline medium (200 g/l salt), structural modifications occur in an medium with 100 g/l NaCl (Figure 3).

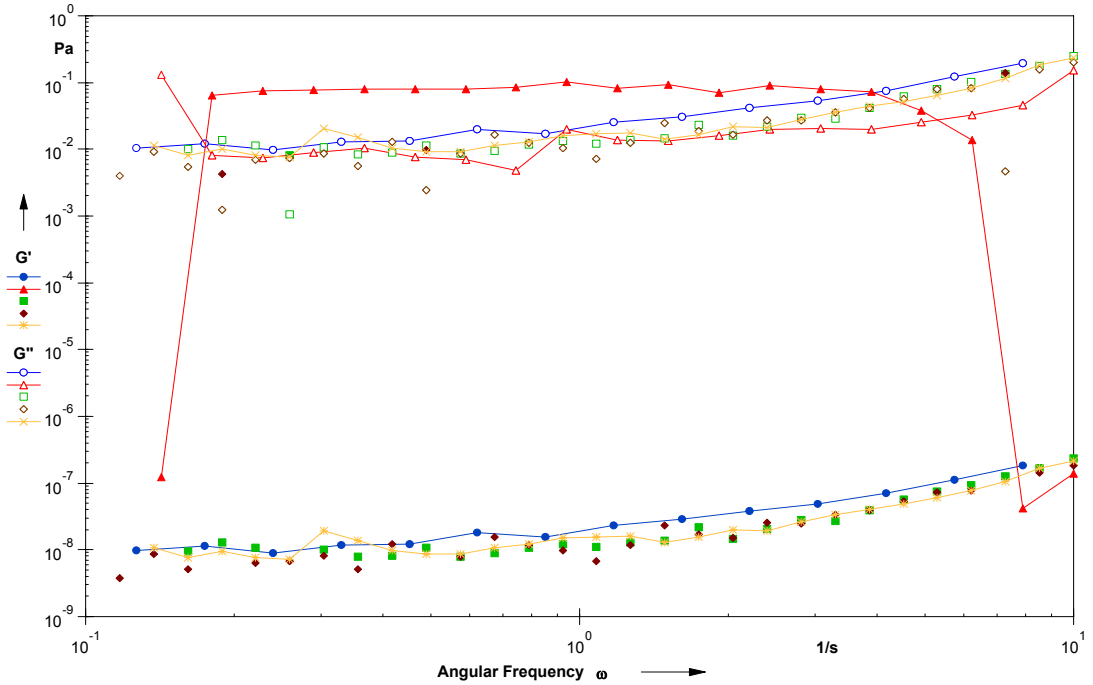

Figure 3. The elastic modulus G' and the viscous modulus G" as function of frequency in solutions 5% EPSH in saline medium with 200 g/l NaCl (triangle), 100 g/l NaCl (rosette), 40 g/l NaCl (diamond), 20 g/l NaCl (quadrate), in water (circle)

In the frequency domain 10^{-1} – 10^{1} s^{-1} the EPSH behaves as a structured compound with the viscous and elastic modules evolving in parallel and the liquid state predominant (G”> G’). When the sodium chloride in the EPSH solution decreases more (to 40 g/l or 20 g/l) and in the absence of NaCl, the viscoelastic behavior of this polysaccharide remains the same as in media with 100 g/l (Figure 3), indicating the formation of a structure with predominant liquid state (G”> G’). G” is less frequency dependent, phenomenon observed for xanthan too [23].

The influence of sodium chloride on the EPSH behavior can be directly correlated with the ionic charge. In the extreme saline environments (with around 200 g/l salts), the ionic strength is very high (I ≅ 425) and the positive and negative charges of EPSH are neutralized; the gel has a stable conformation. The dilution with water at 1:2 and more decreases the ionic strength; in these conditions the EPSH charges are not total neutralized. The result is the destruction of the initial structure with formation of a new structure. Such behavior is observed for polyampholytes (called also polyamphions or dipolar polyions) with high ionic strength [33]. So, it can be affirmed that EPSH behaves as a polyamphion with high ionic strength. Taking into account the polysaccharide composition, the polyanionic character of EPSH is more pronounced, given by the carboxyl and sulfate groups; the cationic character is given by the amino groups from aminosugars, in reduced number in the biopolymer composition.

The analysis of the variation of loss tangent tanδ with the angular frequency (Figure 4) gives information on the molecular mass. This is obviously a well defined point and conveniently this “cross-over” has been shown to depend on the molecular weight and molecular weight distribution of some polymers [32]. The moment of passing from a smaller value to a higher value is directly correlated with the molecular mass, this change occurring faster in systems with higher molecular mass [24].

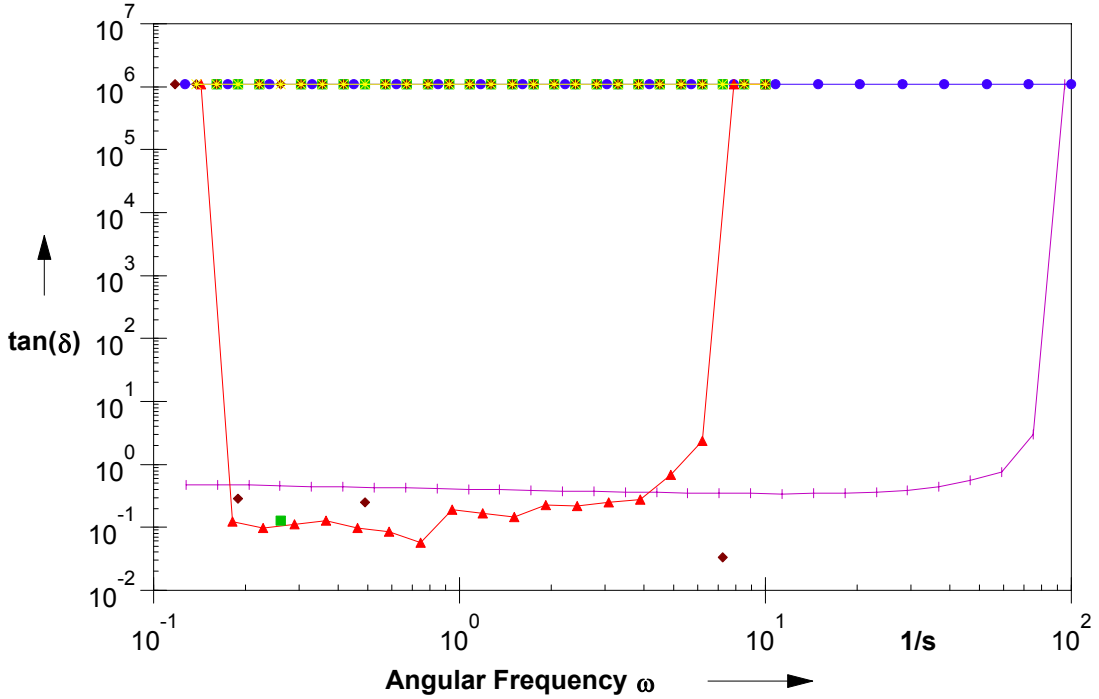

Figure 4. Loss tangent tanδ as function of frequency in solutions 5% EPSH in saline medium with 200 g/l NaCl (triangle), 100 g/l NaCl (rosette), 40 g/l NaCl (diamond), 20 g/l NaCl (quadrate), water (circle) compared with aqueous xanthan solution (cross line)

As shown in Figure 4, tanδ changes faster for EPSH as for xanthan, indicating its higher molecular mass in saline environments as xanthan in water. The value of loss tangent is a quantitative measure of solid-like elastic body or liquid-like viscous fluid of a system.

The constant values of loss tangent for the samples having EPSH in a smaller amount of NaCl as in the natural environment are the result of the predominance of the liquid state in the polysaccharide gel.

Complex viscosity $|\eta^*|$ decreases for all analyzed solutions during the treatment at low frequencies, as observed in Figure 5. $|\eta^*|$ increases when frequency increases, this change being distinct for EPSH in saline concentrated solutions with 200 g/l NaCl and xanthan; they have a gap of the complex viscosity when ω attires values around 10 s^{-1} for EPSH and 100 s^{-1} for xanthan, indicating some structure degradation, which could be correlated with a decrease of the molecular weight and chain length. The samples with less salt don't have this gap, most probably because their structure remains relatively stable, as the analysis of G' and G" revealed, too. However, the rheological tests have to be correlated with other methods to certify this assumption.

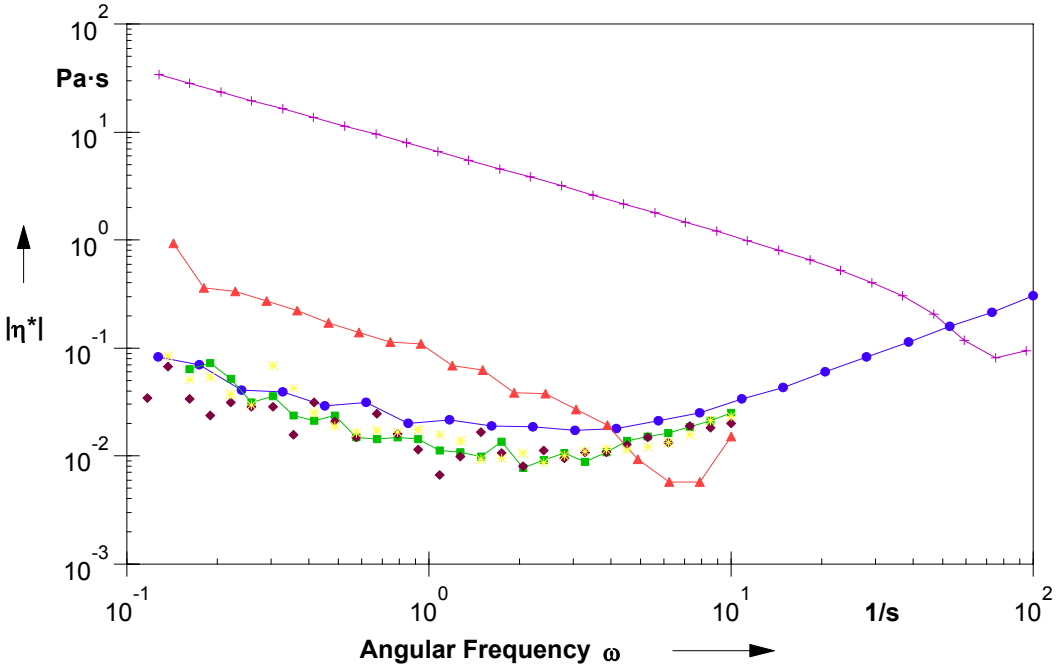

Figure 5. Complex viscosity $|\eta^*|$ as function of frequency in solutions 5% EPSH in saline medium with 200 g/l NaCl (triangle), 100 g/l NaCl (rosette), 40 g/l NaCl (diamond), 20 g/l NaCl (quadrate), in water (circle) compared with aqueous xanthan solution (cross line)

The evolution of complex modulus for aqueous EPSH and xanthan solutions at the oscillatory test combined with heating to 100°C and maintenance at 100°C is presented in Figure 6. $|G^*|$ has different values for the three compounds analyzed, in order $|G^*|_{xanthan} < |G^*|_{EPSH_saline} < |G^*|_{EPSH_aqueous}$. $|G^*|_{EPSH_aqueous}$ remains at a constant value (around 415 Pa) during heating from 30°C to 100°C; small modifications of $|G^*|$ appears after the temperature of 100°C is reached, but EPSH restores its structure after two minutes and the complex modulus equilibrate at the initial value. $|G^*|_{EPSH_saline}$ has a similar behavior as $|G^*|_{EPSH_aqueous}$, its value resting quasi-constant around 360 Pa with small changes around this value after 100°C is reached. $|G^*|_{xanthan}$ has initially 325 Pa and no significant changes of occurs till 45°C; then, between 50°C and 100°C the complex viscosity increase to 340 Pa and

oscillates slightly around this value. The rapid decrease of $|G^*|_{xanthan}$ at heating at 100°C indicates a destabilization of this EPS, without stabilization at a constant level after 5 minutes.

It can be concluded that EPSH is more resistant at the temperature of 100°C, the viscosity remaining constant for longer time in the presence or in the absence of NaCl. Based on this property, some applications for EPSH could be found, as viscosity agent at boiling temperatures.

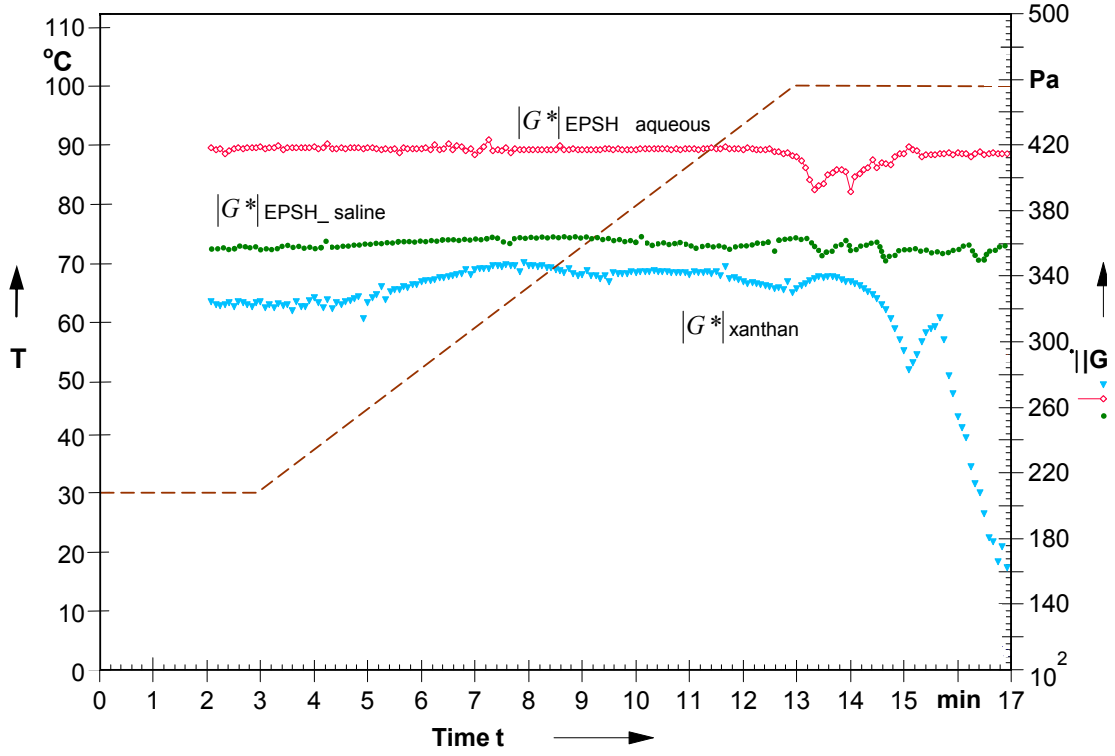

Figure 6. Influence of temperature (pointed line) on the complex modulus $|G^*|$ for solutions 5% of EPSH in water (diamond), EPSH in saline solution (200 g/l NaCl) (circle) and of xanthan in water (triangle) at the oscillatory test at constant amplitude 1 % and frequency (50 s^{-1}) combined with temperature test

Conclusions

The rheological measurements realized in this work offer valuable information on the behavior of 5% EPSH solutions with various salt concentrations and at high temperatures, which help to characterize the biopolymer in its natural environment, and after dilution with water.

The flow and viscosity curves obtained for EPSH in saline solution with concentrations from 0 to 200 g/l show the pseudoplastic character of this polysaccharide and indicate the strong influence of salt on its flow properties. The EPSH is less pseudoplastic as xanthan. This characteristic is evidenced by the flow behavior, but also by the coefficients of the Power-law models obtained in this research; both consistency coefficient *K* and Power-law index *n* indicate that the higher the salt content, the higher viscosity.

The dynamic analysis revealed some EPSH structural changes; in the frequency range analyzed (10^{-1} – 10^{1} s^{-1}) the polysaccharide in high concentrated saline solution (200 g/l NaCl, similar to its natural environment) acts as a weak gel with the solid phase dominant; this behavior is similar with xanthan, but the EPSH gel is weaker. When the NaCl concentration decreases to 100 g/l and less, EPSH behaves as a stable gel with the liquid phase predominant. This result indicates that EPSH has a strong ampholytic character.

The plotting of loss tangent as function of frequency helps to appreciate that the molecular mass of EPSH is higher then that of xanthan.

The biopolymer in saline or in aqueous solution maintains his structure during treatment at high temperature, being resistant at 100°C longer as xanthan.

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