Influence of temperature on the rheological behavior of a new fucose-containing bacterial exopolysaccharide

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\textbf{A B S T R A C T}

The effect of temperature on the rheology of a new fucose-containing extracellular polysaccharide (EPS) was evaluated. The steady state data revealed a shear-thinning behavior, with the viscosity being immediately recovered when the shear rate was decreased. The mechanical spectra indicated viscous solutions with entangled polymer molecules in the range of temperatures studied (from 15°C to 65°C). In addition, the Time-Temperature Superposition principle was successfully applied and the Cox–Merz rule was valid, reinforcing the idea of a thermoregistically simple behavior for the EPS in aqueous solution. Furthermore, the viscous and viscoelastic properties at 25°C were maintained after consecutive heating and cooling cycles, indicating a good thermal stability under temperature fluctuations.

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1. Introduction

Polysaccharides have a wide range of applications, including food, pharmaceutical, cosmetics, paints, explosives, paper and oil industries, due to their diverse structures and physical properties. They are extensively used as thickening, gelling, stabilizing, binding, emulsifying and flocculating agents. Natural polysaccharide sources include plants (e.g. starch and pectins), algae (e.g. carrageenan, alginate and agar), animals (e.g. chitosan) and microorganisms (e.g. xanthan and gellan) [1]. However, production of polysaccharides by microbial fermentation has advantages in comparison to their extraction from other sources, since microorganisms usually exhibit higher growth rates and the manipulation of production conditions is much easier [2]. Unlike other sources, microbial fermentation is not influenced by climate changes or seasonality. Up to now, the main factor limiting commercial production of microbial polysaccharides is related to the high cost of the most commonly used substrates (e.g. glucose, starch and sucrose) [1].

Previous works on microbial polysaccharides production showed the potential of glycerol byproduct from the biodiesel production as sole carbon source. This byproduct is a low cost substrate, and its availability is increasing with the increase of biodiesel production. Using a \textit{Pseudomonas oleovorans} strain, a galactose-rich polysaccharide was produced [3]. It was mainly composed by galactose, with variable amounts of mannose, glucose and rhamnose, depending on the cultivation conditions. It also contained acyl groups substituents (acetate, pyruvate and succinate). Its characterization, regarding solution properties, rheological behavior, emulsifying and flocculating capacities, as well as film-forming ability, have been reported [3–7].

Afterwards, a distinct novel exopolysaccharide (EPS), containing fucose, has been produced by a newly isolated bacterial strain grown on glycerol byproduct. This strain has been identified and named \textit{Enterobacter A47} DSM 23139. Previous work was focused on the rheological and morphological characterization of the culture broth during EPS production in the bioreactor [8]. That study was determinant for the evaluation of the factors affecting the broth viscosity over cultivation time, which is closely related with heat and mass transfer rates that eventually have an impact upon bioreactor’s productivity and yield. A preliminary polymer characterization in terms of its chemical composition, molecular weight and intrinsic viscosity has also been presented [9]. The functional properties of this fucose-containing EPS, including its rheological behavior in aqueous medium, emulsion forming and stabilizing capacity and flocculating activity, were compared to other commercially available polymers, namely, fucogel, xanthan gum, guar gum, alginate, pectin and carboxymethylcellulose (CMC), to evaluate its potential industrial applications.

The characterization of the new fucose containing EPS continued and, in the present work, the attention was driven to study the influence of temperature on the rheological properties of the fucose-rich EPS. A similar insight was already performed in a previous work for the galactose-rich EPS produced by \textit{P. oleovorans}...
strain [6]. Bae et al. [10] and Durand [11] have also reported the temperature influence on the rheological properties of levan and chemically modified dextran, respectively. Polymers may establish interactions as the temperature is changed, forming aggregates or gel structures, as reported for gellan [12] and pectin [13]. The study of the effect of temperature on the rheological properties of the fucose-rich EPS is essential to evaluate the potential of this novel biopolymer, namely, in applications involving processes with thermal variations, such as in drilling fluids [14] for the oil industry, and food processing (e.g. pasteurization, evaporation).

2. Materials and methods

2.1. EPS production, extraction and purification

The EPS was produced in a bioreactor using an Enterobacter A47 (DSM 23139) in a mineral medium supplemented with glycerol byproduct from the biodiesel industry (SGC Energia, SGPS, SA, Portugal), as described by Alves et al. [8].

At the end of the run, the cultivation broth was collected and diluted with deionized water for viscosity reduction, followed by its centrifugation (13000 rpm, during 30 min) for cell separation. The EPS was precipitated from the cell-free supernatant with cold acetone (3:1), dissolved in deionized water and freeze dried. The dried EPS was dissolved again in deionized water and subjected to a second precipitation. After this procedure an EPS solution was subjected to dialysis with 3500 MWCO membrane (SnakeSkin TM Pleated Dialysis Tubing 68035 – Thermo Scientific), against deionized water for 48 h. The dialysis solution contained 5 ppm sodium azide to prevent biological degradation of the EPS. Finally, the EPS was freeze dried.

2.2. EPS chemical characterization and molecular weight evaluation

The EPS was characterized in terms of its sugar composition, acyl groups, inorganic and protein content, as described by Freitas et al. [5]. Number and weight average molecular weights (Mn and Mw, respectively), as well as the polydispersity index (PD = Mw/Mn) were obtained by size exclusion chromatography (SEC) in a low temperature Waters Co. apparatus, equipped with a Waters Ultra hydrogel Linear column and a differential refractive index detector (Waters 2410). A 0.1 M NaCl solution at 30 °C was used as eluent, and the polymer concentration was less than 0.1 wt.%, thus ensuring the pumping of essentially non aggregated polysaccharides in coil conformation by a Waters 510 Solvent Delivery System. The values of Mw and Mn were calculated using a calibration curve generated with monodisperse pullulan standards (Shodex, Showa Denko, Japan).

2.3. Rheology measurements

The purified polymer obtained as described in Section 2.1 was mixed with deionized water in order to obtain a solution with an effective polymer concentration of 0.81 wt.%. Preliminary results show that this concentration is well above the critical concentration value (C* ≈ 0.09 wt.%). The rheology of this solution was studied using a controlled stress rheometer (AR2, TA Instruments Inc., New Castle, DE, USA), equipped with a cone and plate geometry (diameter 4 cm, angle 2°). During the experiments, the shearing geometry was covered with paraffin oil to prevent water evaporation. Flow curves were determined using an steady state flow ramp in the range of shear rate from 1 s⁻¹ to 700 s⁻¹. Frequency sweeps were carried out using a strain of 0.1, within the linear viscoelastic region. Both oscillatory and steady-state tests were carried out at different temperatures ranging from 15 °C to 65 °C. The purified EPS solution was also submitted to temperatures cycles of consecutive heating and cooling steps. After recording the mechanical spectrum and the steady-state data at 25 °C, the sample was heated up to 40 °C at a rate of 3 °C min⁻¹, followed by an oscillatory time sweep (strain = 0.2 and f = 1 Hz) at that temperature for 10 min. Afterwards, the sample was cooled down at a rate of –3 °C min⁻¹ to 25 °C, and new oscillatory and steady-state tests were performed at the same conditions. The cycle was repeated by heating the sample up to 55, 70 and 80 °C.

3. Results and discussion

3.1. EPS production and characterization

The cultivation run of Enterobacter strain A47 (DSM 23139) on glycerol byproduct took 7 days, during which 13.3 g L⁻¹ of EPS, quantified by acetone precipitation, were produced. The glycosyl composition analysis of the purified EPS obtained at the end of the cultivation run revealed that it was mainly a heteropolysaccharide composed of neutral sugars: fucose (25%), galactose (32%) and glucose (38%). Both mannose and rhamnose were detected in trace amounts (<1%). The acyl groups analysis showed that the EPS contained pyruvyl (3.8%), acetyl (6.8%) and succinyl (1.0%) as substituents, accounting for 11.7% of the polymer’s dry weight.

After dialysis with the 3500 MWCO membrane, the extracted polymer contained non-sugar components, namely, proteins and ash that accounted for less than 8.0% and 4.0%, respectively, of the EPS dry weight. These components were probably remnants from the culture broth, as their content could be further reduced by dialysis with a higher MWCO membrane (data not shown). Moreover, the dried polymer also had a residual water content of 1.9%.

The average molecular weight (Mw) and the polydispersity index of the fucose-containing EPS, determined by SEC analysis, were 5.8 × 10⁵ and 1.3, respectively, showing a homogeneous polymer.

The intrinsic viscosity, obtained by extrapolation to zero concentration of Huggins and Kraemer equations, was 11.0 dL/g [8]. This value is within the range of that presented for commercial polysaccharides, such as xanthan and guar gum, 5–50 dL/g [15].

3.2. Rheological studies

3.2.1. Steady-shear behavior of purified EPS solution at different temperatures

The purified EPS solution at a concentration of 0.81 wt.% was submitted to a shear rate range from 1 s⁻¹ to 700 s⁻¹, at different temperatures. The flow curves obtained, increasing and decreasing the shear rate, were coincident, which may indicate that the sample has not a strong internal structure that could be disrupted by the strong stress imposed, and it is able to recover instantly. The viscosity measurements showed a shear-thinning behavior, since the apparent viscosity decreased as the shear rate increased, for all temperatures tested (Fig. 1).

On the other hand, the apparent viscosity decreased with the temperature increase, showing the influence of this parameter on the polymer’s rheological properties. The EPS molecules are high molecular weight structures able to establish interactions in solution (e.g. entanglements and hydrogen, electrostatic and hydrophobic bonds). The energy transferred to the sample influences the creation or disruption of such interactions. At low shear rates, the disruption of interactions is balanced by the formation of new ones, resulting in a constant apparent viscosity (Newtonian plateau) [16]. At high shear rates, the disruption of those interactions predominates and the molecules align in the direction of the flow, resulting in the observed decrease of the apparent vis-
cousity (shear-thinning). It is notorious in Fig. 1 that the shear rate corresponding to the transition from Newtonian to shear-thinning behavior moves to higher values as the temperature increases, which means that for higher temperatures the formation of new interactions is faster. This behavior is similar to that observed in a previous work, for the galactose-rich EPS produced by P. oleovorans from glycerol byproduct [6].

The Carreau model (Eq. (1)) [17] was used to describe the steady shear viscosity dependence with the shear rate, at different temperatures:

$$\eta_\text{a} = \frac{\eta_0 + \eta_\infty}{1 + (\lambda \dot{\gamma})^N}$$  \hspace{1cm} (1)

where $\dot{\gamma}$ is the shear rate (s$^{-1}$), $\eta_\text{a}$ is the apparent viscosity (Pa s), $\eta_\infty$ is the infinite shear rate viscosity (Pa s), $\eta_0$ is the zero-shear rate viscosity (Pa s), $\lambda$ is a time constant (s) and $N$ is a dimensionless constant. Since the second Newtonian plateau was never approached, Eq. (1) was simplified assuming $\eta_\infty$ much higher than $\eta_0$ and $\eta_\text{a}$. The model fitted quite well the experimental results, and the parameter values are presented in Table 1.

As expected, $\eta_\text{a}$ and $\lambda$ decreased with increasing temperature. The relaxation time constant ($\lambda$) decreased from 0.334 s$^{-1}$ to 0.037 s$^{-1}$ as the temperature increased from 15 to 65 °C, respectively. This fact indicates that less time is needed to form new interactions between polymer molecules at higher temperatures. Consequently, the transition from Newtonian plateau to shear-thinning regime is less notorious and moves to higher shear rate values.

### 3.2.2. Rheological properties under dynamic shear

The mechanical spectra for the EPS solution at 15 and 65 °C (Fig. 2A) indicated the presence of viscous samples of entangled polymer chains. At low frequencies, the loss modulus ($G''$) was higher than the storage modulus ($G'$), indicating a liquid-like behavior for the polymer solution. At high frequencies, a cross-over was detected at 15 °C, after which the elastic contribution predominates. However, this behavior was not observed at 65 °C. At lower temperatures, a higher viscosity was observed (Fig. 1). Hence, less energy is needed to transfer to those samples in order to store more energy than that dissipated, and perceive a $G'$ and $G''$ cross-over.

As expected, $G'_\text{a}$ and $\lambda$ were higher for the EPS solutions at 15 °C (reference temperature), as indicated by the marked temperature-dependent shift factor $\alpha_T$ to extend the frequency axis by several orders of magnitude [18]. According to this principle, all contributions to the dynamic moduli should be proportional to $T_0$ and all relaxation times should have the same temperature dependence. The change of temperature from $T$ to $T_0$ (reference temperature) will change $G'$ and $G''$, corresponding to the multiplication by $T_0/\rho_0 T$ (temperature density ratio) defined as $Br$ [19]. This shift factor was necessary to have a good superposition of the measurements done at the different temperatures [20].

The TTS for both moduli is shown in Fig. 3, and the respective shift factors are presented in Table 2. TTS principle was
applied successfully, which suggests that the purified EPS solution is thermorheologically simple, where the relaxation times for all mechanisms change identically with temperature [18]. In addition, the range of frequencies was significantly enlarged for the lower temperatures, enabling the estimation of the dynamic moduli for frequency values outside the range measured by the rheometer.

3.2.4. Temperature influence on rheological parameters

To determine the activation energy at a given concentration and shear rate, the apparent viscosity and the zero-shear rate viscosity were studied as a function of the temperature according to the Arrhenius law [21]:

$$\eta_i = A \exp \left( \frac{E_a}{RT} \right)$$  \hspace{1cm} (2)

where $\eta_i$ (Pa·s) represents the apparent viscosity or the zero-shear rate viscosity determined by the Carreau equation, $A$ is the pre-exponential factor, $E_a$ is the activation energy, $R$ is the gas constant and $T$ is the absolute temperature.

The variation of the activation energy with the apparent viscosity for two different shear rates (5 and 13 s$^{-1}$), and for the zero shear rate, is plotted in Fig. 4. The Arrhenius equation fitted quite well the experimental data and, as can be seen, the activation energy decreased as the shear rate increased. For zero shear rate the activation energy was 36.8 ± 5.2 kJ mol$^{-1}$ and for 13 s$^{-1}$ it was

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**Table 2**

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**Fig. 3.** Frequency and temperature superposition of the loss ($G''$) and storage ($G'$) moduli. The solid line represents the data for the reference temperature (25 °C) and the symbols correspond to the other temperatures: (■) 15 °C, (○) 30 °C, (▲) 45 °C, (●) 55 °C, and (▲) 65 °C.

**Fig. 4.** Temperature dependence of: apparent viscosity at $\gamma = 5$ s$^{-1}$ (■) and $\gamma = 13$ s$^{-1}$ (▲); zero-shear rate viscosity estimated by the Carreau model (○). The lines correspond to Arrhenius equation.

**Fig. 5.** Data measured at 25 °C before (open symbols) and after (full symbols) the temperature cycles: (A) flow curves; (B) dynamic moduli and (C) dynamic ($\eta'$) and out of phase ($\eta''$) viscosities.
25.7 ± 3.1 kJ mol⁻¹. In fact, as the shear rate increases, molecular interactions have shorter lifetimes and less energy is needed to promote viscous flow. The activation energy value determined at zero shear rate is quite similar to that estimated for the galactose-rich EPS sample with a similar concentration, 39.3 ± 2.4 kJ mol⁻¹ [6].

Consecutive temperature cycles were also performed, in which the same sample was subjected to different temperatures (from 25 °C to 80 °C) during 10 min. After each heating step, the temperature was reduced to 25 °C, where both oscillatory and steady-state tests were carried out. As can be seen in Fig. 5A, the flow curves were nearly concurrent. The same may be observed for the dynamic data presented in Fig. 5B (G' and G") and C (η' and η″). Besides a small difference at low frequencies, before and after the temperature cycles, the mechanical spectra are rather similar, meaning that the polymer solution did not suffer significant alterations and its rheological properties were maintained. These results indicate that the polymer sample is quite stable under the tested temperature fluctuations.

3.2.5. Cox–Merz rule

The Cox–Merz rule is an empirical relationship which predicts that the magnitude of the complex viscosity should be compared with the apparent viscosity at equal values of frequency and shear rate. It is used to establish relationships between steady shear flow and dynamic rheology. It is especially useful to estimate the complex viscosity for cases in which the oscillatory operating mode is not available. In addition, a qualitative perception of the sample microstructure may be perceived according to its applicability. In general, it is not valid for most particulate dispersions or when there is the formation of large aggregates and gel systems, as reported for several exopolysaccharides, including xanthan, curdlan and Aeromonas gum [22–24].

Fig. 6 presents the apparent and complex viscosities as a function of the shear rate and angular frequency, respectively for several exopolysaccharides, including xanthan, curdlan and Aeromonas gum.

4. Conclusions

The exopolysaccharide (EPS), rich in fucose, produced by Enterobacter A47 using glycerol byproduct, demonstrated to have a shear-thinning behavior, recovering immediately the viscosity when the shear rate was decreased. The Carreau model fitted quite well the steady state data at the different temperatures tested. The mechanical spectra obtained and the application of the Cox–Merz rule suggest the presence of entangled macromolecules in a viscous solution. The Arrhenius equation adequately described the temperature dependence of ηp and the apparent viscosity at different shear rates. As expected, a decrease of the activation energy was noticed with the increase of shear rate due to the lower resistance to viscous flow. The EPS maintained its rheological properties at 25 °C under consecutive temperature fluctuations, even after heated up to 80 °C. This novel polysaccharide produces aqueous solutions that are thermorheologically simple and stable, suggesting its application in processes in which thermal variations may occur (e.g. food processing and oil drilling fluids). It consists on a good candidate to substitute, potentially with a lower cost, some biopolymers already in the market.

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