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Rheological and morphological characterization of the culture broth during exopolysaccharide production by *Enterobacter sp.*

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ABSTRACT

Enterobacter sp. was grown on glycerol byproduct from the biodiesel industry for the production of a value-added exopolysaccharide (EPS). The culture broth was characterized in terms of its morphological and rheological properties throughout the cultivation run. Microscopic observations revealed the formation of cell aggregates surrounded by the EPS at the beginning of the cultivation run, while, at the end, aggregates were reduced and an EPS matrix with the cells embedded in it was observed. The apparent viscosity of the culture broth increased over time, which was attributed to the increase of the EPS concentration in the first period of the cultivation run. However, in the final stage, the creation of new polymer interactions within the complex culture broth was likely the reason for the viscosity increase observed, since there was not a significant variation of the EPS concentration, average molecular weight or chemical composition. The broth presented a Newtonian behavior at the beginning of the run, changing to pseudoplastic as the EPS concentration increased, and revealed to follow the Cox–Merz rule.

1. Introduction

The maximum possible productivity and product concentration that can be obtained in any biochemical process are limited by the transport phenomena occurring during it, which are influenced by the mixing degree and hydrodynamic conditions in the process. These parameters are related to the fluid flow characteristics that determine mass (oxygen, carbon source and other nutrients) and heat transfer rates in bioreactors (Bandaiphet & Prasertsan, 2006). Hence, the study of the rheological properties of culture broths is the key to improved yield of the desired microbial products.

Cultivation broths containing unicellular microorganisms of simple shape should behave as Newtonian fluids. However, in many microbial cultivation the broths are much more complex and deviations from Newtonian behavior are significant. In many of them, the broth exhibits different types of behavior, depending on their stage of development during the cultivation. In a number of industrially important cultivation processes the broth develops pseudoplastic behavior. Examples of such behavior include industrially important microbial polysaccharides, such as xanthan gum that is produced by *Xanthomonas campestris* (Candia & Deckwer, 1999), pullulan that is produced by *Aureobasidium pullulans* (Furuse, Amari, Miyawaki, Asakura, & Toda, 2002) and dextran that is produced by *Leuconostoc mesenteroides* (Landon, Law, & Webb, 1993).

In order to improve EPS production, it is necessary to keep the highly viscous broth well mixed, which is problematic, especially at a large scale. In view of this, the study of the rheological properties of microbial culture broths is essential to improve EPS yield and productivity. Rheological properties provide a sensitive analytical means for the characterization of cellular mass changes and provide a clue to the relationship between cellular structure and biochemical activity in a microbial cultivation.

The viscosity of microbial culture broths may be influenced by physical, as well as biological parameters, including the cultivation medium used, the size of both cells and cell aggregates formed, biomass concentration, morphological parameters and the products being secreted into solution (Al-Asheh, Abu-Jdayil, Abunasser, & Barakat, 2002). However, in most cases, the change in flow behavior in such microbial processes is attributed to the increasing extracellular polymer concentration being produced, with negligible contribution from the cells (Landon et al., 1993).

In this work, *Enterobacter sp.* was grown on glycerol byproduct from the biodiesel industry for the production of an extracellular polysaccharide. The culture broth was characterized regarding its rheological and morphological properties throughout the cultivation run. A special attention was driven to evaluate the changes

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experienced by the bacterial cells during growth and EPS production on glycerol byproduct. In addition, the culture broth rheology was related to polymer concentration and average molecular weight, as well as to the cell concentration and morphology.

2. Materials and methods

2.1. Cultivation conditions

Enterobacter sp. was grown on a slightly modified Medium E* (2009a), supplemented with glycerol byproduct to give a concentration of 25 g L^{-1}. The cultivation was performed in triplicate experiments.

Glycerol byproduct, supplied by SGC Energia, SGPS, SA (Portugal), had a glycerol content of 89% and residual contents of methanol (0.04%), organic material (0.4%), ashes (6.8%) and water (3.5%).

EPS production was performed in a 10 L bioreactor (BioStat B-plus, Sartorius), with controlled temperature and pH of 30.0 ± 0.1 °C and 6.80 ± 0.05, respectively. The bioreactor was operated in a batch mode during the first day of cultivation and, afterwards, in a fed-batch mode, by supplying the bioreactor with cultivation Medium E*, with a glycerol concentration of 200 g L^{-1}, at a constant rate of 20 mL h^{-1}. The aeration rate (0.125 vvm, volume of air per volume of reactor per minute) was kept constant throughout the cultivation, and dissolved oxygen concentration (DO) was controlled by automatic variation of the stirrer speed (400–800 rpm) provided by two six-blade impellers. During the fed-batch phase, the DO was maintained below 10%.

Throughout the cultivation, culture broth samples were recovered from the bioreactor and centrifuged (18,000 × *g*, 15 min) for cell separation. The cell-free supernatant was stored at −20 °C for the determination of glycerol and ammonium concentrations. Cell dry weight (CDW), glycerol and ammonium concentrations were performed as described by Freitas et al. (2009a).

EPS quantification was carried out by extracting it from the culture broth as reported by Freitas et al. (2009b). Briefly, the culture broth was diluted with deionised water for viscosity reduction and centrifuged (20,000 × *g* for 1 h) for cell separation. The biopolymer in the cell-free supernatant was precipitated by the addition of cold acetone (3:1), dissolved in deionised water and freeze-dried.

The determination of glycerol, ammonium, CDW and EPS concentration was performed in replicate analysis.

2.2. EPS chemical characterization

The extracted EPS was characterized in terms of its sugar composition, acyl groups, inorganic and protein contents, as described by Freitas et al. (2009b). The EPS average molecular weight was determined by SEC-MALLS, as detailed in the work of Hilliou et al. (2009). For this analysis, the EPS extracted with acetone was further purified by performing a second acetone precipitation followed by dialysis with a 3500 MWCO membrane (SnakeSkinTM Pleated Dialysis Tubing 68035—Thermo Scientific), against deionised water, for 48 h. The dialysis solution contained 6 ppm sodium azide to avoid biological degradation of the samples. The purified EPS obtained after dialysis was also characterized in terms of its chemical composition, namely, its sugar and acyl groups composition and its contents on inorganic material and protein, as described above.

2.3. Microscopic observations

Culture broth samples collected throughout *Enterobacter sp.* cultivation run were observed with an Olympus BX51 microscope in phase contrast mode. The EPS was also visualized by its negative staining with China ink, using a technique based on the work of Hahn, Lünsdorf, and Janke (2004). Briefly, broth samples were spread out in a slide and stained with a drop of China ink (Pelikan). The microscopic observation was performed in phase contrast, with low light intensity.

2.4. Rheology

Culture broth samples were loaded in the cone and plate geometry (diameter 4 cm, angle 2°) of a controlled stress rheometer (ARG2, TA Instruments Inc., New Castle, DE, USA) and the shearing geometry covered with paraffin oil in order to prevent water loss. The samples were equilibrated at 30 °C for 10 min, and demonstrated by the record of time independent dynamic moduli measured at 1 Hz with 0.1 shear strain amplitude. A frequency sweep with a 0.1 strain amplitude was then performed to measure the frequency dependence of the storage (G') and loss (G'') moduli at 30 °C. Flow curves were determined using a steady-state flow ramp (torque was imposed using a logarithmic ramp) in the range of shear rate from around 1 to 700 s^{-1}. The shear rate was measured point by point with consecutive 60 s steps of constant shear rate. The viscosity was recorded for each point to obtain the flow curves.

3. Results and discussion

3.1. Typical Enterobacter sp. growth and EPS production

A typical cultivation run of *Enterobacter sp.* is presented in Fig. 1, in which cell growth on glycerol byproduct and EPS production are shown over time. Cell growth was suppressed within less than 1.0 day of cultivation by imposing nitrogen limiting conditions (<0.1 g NH_4^+ L^{-1}). Ammonium concentration was thereafter kept at a residual value (below the detection limit), even though the feeding solution containing 0.9 g NH_4^+ L^{-1} was fed to the bioreactor at a constant flow rate (20 mL h^{-1}), while the dissolved oxygen concentration was controlled at 10% by the automatic variation of the stirring speed between 400 and 800 rpm.

A maximum biomass concentration of 6.00 ± 0.12 g L^{-1} was reached within about 1.0 day of cultivation. Afterwards, there was a decay of the CDW that was gradually reduced to half its maximum value by day 7.0 (Fig. 1). This decay may be related to a loss of cell viability caused by the nitrogen and oxygen limiting conditions imposed in the bioreactor. Since bacterial cells were not multiplying at this stage, the volume withdraw from the bioreac-

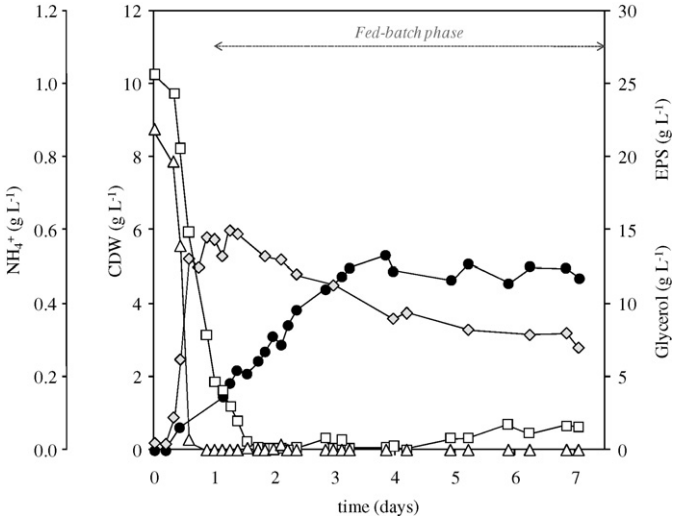

Fig. 1. Time course of the cultivation of *Enterobacter sp.* on glycerol byproduct: (□) glycerol, (△) ammonium, (◇) CDW and (●) EPS.

tor for sampling, concomitant with the continuous introduction of feeding medium, led to a net reduction of the CDW.

Concomitant with cell growth, glycerol concentration in the culture broth decreased from the initial 25.74 ± 0.14 to $4.67 \pm 0.05\,g\,L^{-1}$ by the time that the fed-batch phase was initiated (Fig. 1). From that time on, glycerol concentration was maintained below $2.0\,g\,L^{-1}$, even though it was being continuously fed to the bioreactor at a volumetric rate of $12\,g\,L^{-1}\,day^{-1}$. This result indicates that the glycerol entering the bioreactor was efficiently being used by the culture and hence it did not accumulate in the broth.

EPS synthesis was initiated during cell growth, but increased production was observed during the stationary growth phase (Fig. 1). The EPS attained a maximum concentration of $13.28 \pm 0.74\,g\,L^{-1}$, after about 4.0 days of cultivation. Apparently, during the last 3.0 days of the cultivation there was no further improvement on EPS production, since its concentration in the culture broth remained almost constant. Considering the time window of effective EPS production (1.0–3.8 days), the productivity was $3.64\,g\,L^{-1}\,day^{-1}$ and the net yield of EPS on glycerol was $0.47\,g\,g^{-1}$. In other experiments performed with *Enterobacter sp.* grown on glycerol under different operating conditions, both the final EPS concentration and its productivity were improved. The cultivation for EPS production takes 4.0–7.0 days, with a final production of 12.0–18.0 g L^{-1} of EPS and maximum productivities of 3.6–4.8 g L^{-1} day^{-1} (data not shown). The productivity values achieved are in the range of those presented for xanthan gum by García-Ochoa, Santos, Casas, and Gómez (2000) (3.1–12.2 g L^{-1} day^{-1}), but higher than those reported by Rottava et al. (2009) (1.46–2.4 g L^{-1} day^{-1}). The productivity values are also in the range of those achieved for gellan gum (3.75–11.6 g L^{-1} day^{-1}, Banik & Santhiagu, 2006) production, but are considerably higher than the ones obtained for bacterial alginate (0.43–1.53 g L^{-1} day^{-1}) (Peña, Trujillo-Roldán, & Galindo, 2000) and that referred for the production of a different EPS by *Enterobacter cloacae* WD7 (1.68 g L^{-1} day^{-1}) (Prasertsan, Wichienchot, Doelle, & Kennedy, 2008), using glucose or sucrose as substrates.

3.2. EPS physicochemical characterization

The glycosyl composition analysis of the purified EPS produced by *Enterobacter sp.* from glycerol byproduct revealed that it was mainly a heteropolysaccharide composed by neutral sugars: fucose, galactose and glucose. The relative proportion of the sugar monomers in the purified EPS has suffered small changes throughout the cultivation run. In fact, there was a reduction of the glucose content from 46 to 38%, between days 1.0 and 7.0, while the contents on fucose and galactose have increased from 21 to 25% and from 27 to 32%, respectively.

The content of acyl groups in the purified EPS has also shown to increase during the cultivation time, reaching a maximum value of $11.71 \pm 0.83\%$ of the polymer's mass by day 7.0. The most abundant acyl groups present in the EPS were acetyl ($6.80 \pm 0.66\%$), pyruvil ($3.90 \pm 0.15\%$) and succinyl ($1.01 \pm 0.12\%$). There were other unidentified acyl groups present in the polymer, but their content was lower. The presence of pyruvil and succinyl confers the EPS an anionic character.

The extracted polymer, obtained after only one precipitation step from the cell-free fermentation broth, also contained other non-sugar constituents that were probably remnants of the culture broth, namely, proteins and inorganic residues. The EPS protein content was between 7.6 and 15.9 wt%. As shown by its pyrolysis at 550 °C, the EPS had a total inorganic residues content of 32.5%. Part of these residues were probably present in the extracted polymer as counterions for the anionic acyl groups, but most of them represented salts that co-precipitated with the EPS during the recovery procedure. This was confirmed by purifying the EPS by dialysis, which allowed for the reduction of the inorganic content to only 7.2%.

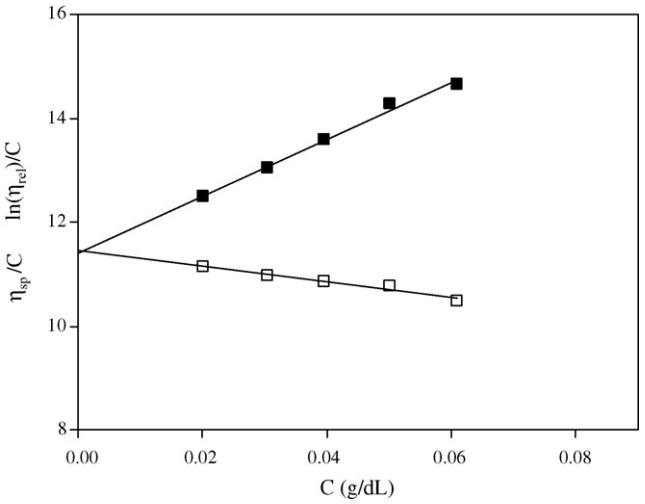

Fig. 2. Determination of the intrinsic viscosity in 0.1 M NaCl using the Huggins (full symbols) and Kraemer (open symbols) equations.

The purified polymer's average molecular weight was in the range of 0.9×10^7–1.3×10^7 (standard deviation: 1.2×10^5–3.3×10^5). The purified polymer was rather homogeneous, as shown by the polydispersity index that ranged between 1.40 and 2.30 (standard deviation: 0.11–0.18).

The intrinsic viscosity of the purified polymer recovered at the end of the cultivation run was determined by double extrapolation to zero concentration of the Huggins and Kraemer equations, respectively (Rao, 1999):

$$\frac{\eta_{sp}}{C} = [\eta] + k_H[\eta]^2 C \quad (1)$$

$$\frac{\ln(\eta_{rel})}{C} = [\eta] + k_k[\eta]^2 C \quad (2)$$

where $[\eta]$, η_{sp} and η_{rel} are the intrinsic, specific and relative viscosities, respectively; k_H and k_k are the Huggins and Kraemer coefficients, and C is the polymer concentration. A Cannon Fenske capillary viscometer was used to measure the viscosity of dilute solutions, with a relative viscosity in the range between 1.2 and 2.0 in order to ensure a good accuracy and linearity in the extrapolations to zero concentration (Fig. 2).

The intrinsic viscosity is a direct measure of the flow behavior of macromolecules and an indirect measure of their size and shape, indicating the hydrodynamic volume of individual polymer molecules (Bae, Oh, Lee, Yoo, & Lee, 2008). Polysaccharides have typical intrinsic viscosity values that range from 1 dL/g for compact coil or flexible chains (e.g. dextran) to 20 dL/g for extended chains (e.g. alginate) (Bae et al., 2008). The intrinsic viscosity value in 0.1 M NaCl, obtained for the EPS extracted at the end of the 7.0 days run was 11.4 dL/g, which is indicative of a conformation somewhat between a compact coil and flexible extended chains. The Huggins coefficient obtained ($k_H = 0.42$) suggests some molecular aggregation, since for flexible macromolecules in a good solvent $k_H \sim 0.3$.

3.3. Morphological characterization of Enterobacter sp. culture during EPS production

Microscopic observations of culture broth samples performed throughout the cultivation run are presented in Fig. 3. *Enterobacter sp.* cells are small rods, almost coccoid in shape, at the beginning

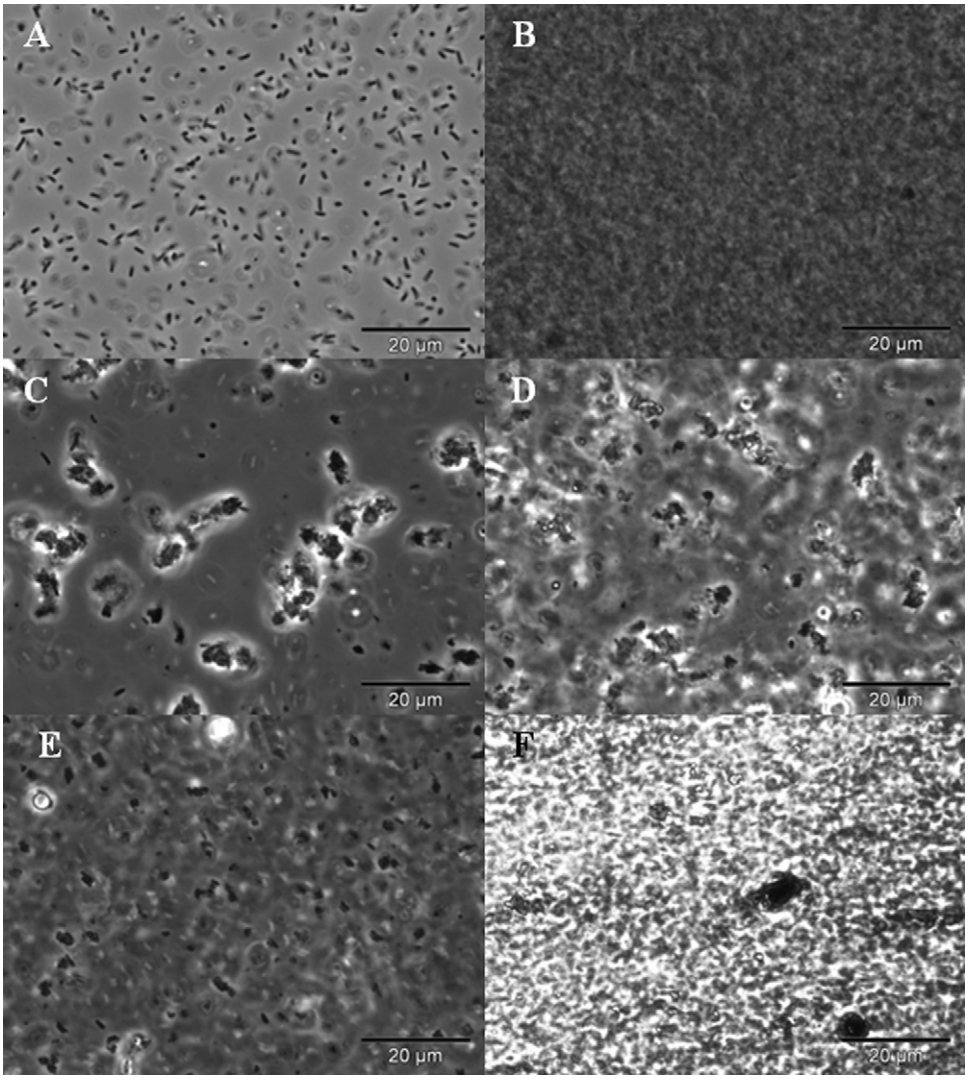

Fig. 3. Microscopic observations of *Enterobacter sp.* broth samples at different cultivation times: 1.0 day (upper images: A and B), 3.0 days (middle images: C and D) and 7.0 days (lower images: E and F), observed in phase contrast (left images) and after staining with China ink (right images).

of the cultivation, being found separately and in pairs. During the cultivation, most of the cells increased in length and a few short chains started to form (Fig. 3A).

For the indirect detection of the EPS in culture broth samples, a negative staining technique (China ink) was used, based on the work of Hahn et al. (2004). When observed with the microscope, under low light intensity, the EPS appears as a light halo or ring around the cells, while the background is stained in black. At the beginning of the cultivation, the broth presented a low cellular density and no EPS was detected. Within 1.0 day of cultivation, CDW attained its maximum concentration (Fig. 1) and *Enterobacter sp.* cells were visualized under the optical microscope (Fig. 3A). Although the amount of EPS produced at this time was still too low to be clearly visualized, China ink staining (Fig. 3B) already showed some lighter areas surrounding the cells, which may attributed to the presence of the EPS.

Around 3.0 days cultivation, the microscopic observations showed that *Enterobacter sp.* cells were clumped together, forming aggregates (Fig. 3C). This cell aggregation behavior has also been noticed for other microorganisms, such as, for example, the bacterium *Pseudomonas aeruginosa* (Al-Asheh et al., 2002) and the microalga *Rhodosorus marinus* (Básaca-Loya et al., 2008). At this cultivation time, EPS production had reached its maximum concentration (Fig. 1), so it was possible to detect its presence by China ink staining (Fig. 3D). The image shows a white halo around cell aggregates, as well as a lighter background than that observed in Fig. 3B.

At the end of the cultivation (day 7), cell aggregates were reduced both in number and size (Fig. 3E). Fig. 3F shows an image of a broth sample taken at 7.0 days of cultivation stained with China ink. As shown by the white background of the image, the EPS matrix was spread out occupying nearly all the optical field, being the cells embedded in it, in contrast with the image taken at day 3.0 (Fig. 3D), where the EPS seems to be somewhat bounded to the cells. This may suggest that, as the EPS was being synthesized (up to day 4.0), it remained loosely attached to the bacterial cells. During the last 4.0 days of the cultivation there was no further significant EPS production and the polymer may have become detached from the cells, thus spreading throughout the broth.

3.4. Rheology of culture broth

3.4.1. Steady-shear behavior

Fig. 4 shows the flow curves of *Enterobacter sp.* culture broth samples at different times of the cultivation run represented in Fig. 1. For all cases, the apparent viscosity was immediately recovered at low shear rates, after subjecting the samples to shear rate values up to 700 s^{-1}. Until 1.0 day of cultivation, the broth exhibited a Newtonian behavior (Fig. 4). Afterwards, it has developed non-

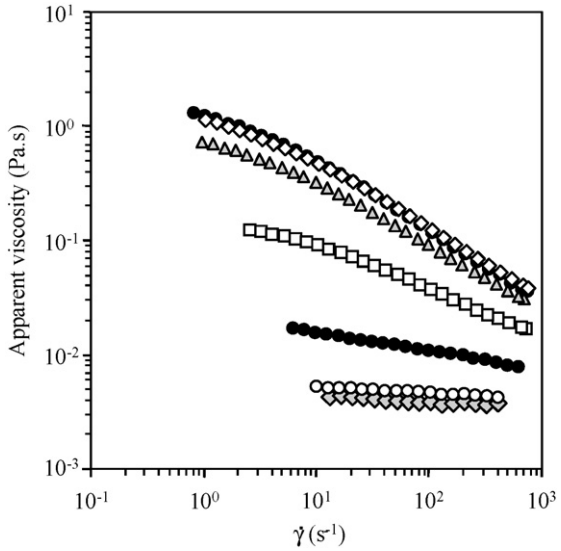

Fig. 4. Flow curves for culture broth samples at different cultivation times: (◇) 0.2 days, (○) 0.9 days, (●) 2.0 days, (□) 4.0 days, (△) 5.2 days, (●) 6.2 days and (◊) 7.0 days. The measurements were made at 30 °C.

Newtonian characteristics acting as a pseudoplastic fluid, showing an increase of shear-thinning as the cultivation time proceeded. The apparent viscosity of the culture broth measured at low shear rates has shown an increase of two orders of magnitude (from 10^{-3} to 10^{-1} Pa s) (Fig. 4). The rise in the broth viscosity and the increasingly non-Newtonian behavior of the culture broth was mostly due to the accumulation of EPS in the aqueous medium. Bacterial cells had a negligible contribution to the changes in the broth rheological properties, since those changes occurred after maximum CDW was reached (day 1). In fact, the flow behavior at the beginning of the experiment (day 0.2), for a very low cell concentration, was identical to that of the culture broth sample with the highest CDW at day 1.0 (Fig. 4).

In each case, the relationship between the shear stress (τ, Pa) and the shear rate ($\dot{\gamma}$, s^{-1}) for the different broth samples could be fitted using the Power law or Ostwald-de-Waele model (Eq. (3)), which is commonly employed, namely by Sanchéz, Jiménez-Aparicio, López, Tapia, and Rodríguez-Monroy (2002) and (Candia & Deckwer, 1999):

$$\tau = k\dot{\gamma}^n \quad (3)$$

where K is the consistency index (Pa s^n) and n is the power law index. The later indicates the degree of non-Newtonian behavior. For $n = 1$, the fluid is Newtonian, while for $n < 1$ it is considered pseudoplastic. Both these parameters have changed throughout the cultivation time (Table 1). The power law index is inversely proportional to the consistency index, decreasing throughout the cultivation run. In fact, at the beginning (up to 1.0 day), $n \approx 1$, thus confirming the Newtonian behavior of the broth at that time. Afterwards, n gradually decreased to a value of 0.4, showing the pseudoplastic behavior that is characteristic of most high molecular weight polymers in aqueous media, and observed in some bioreactor culture broths as well, like those of *P. aeruginosa* (Al-Asheh et al., 2002), *Leuconostoc mesenteroides* (Landon et al., 1993), *Xanthomonas campestris* (Candia & Deckwer, 1999) and *Beta vulgaris* (Sanchéz et al., 2002). However, in the literature are also referred a few systems whose cultivation broths behave differently, such as in the cultivation of *Bacillus cereus*, having a dilatant fluid behavior with an initial yield stress (Al-Asheh et al., 2002).

The consistency index increased over the cultivation time, which is related to the increase of broth viscosity. The increase of

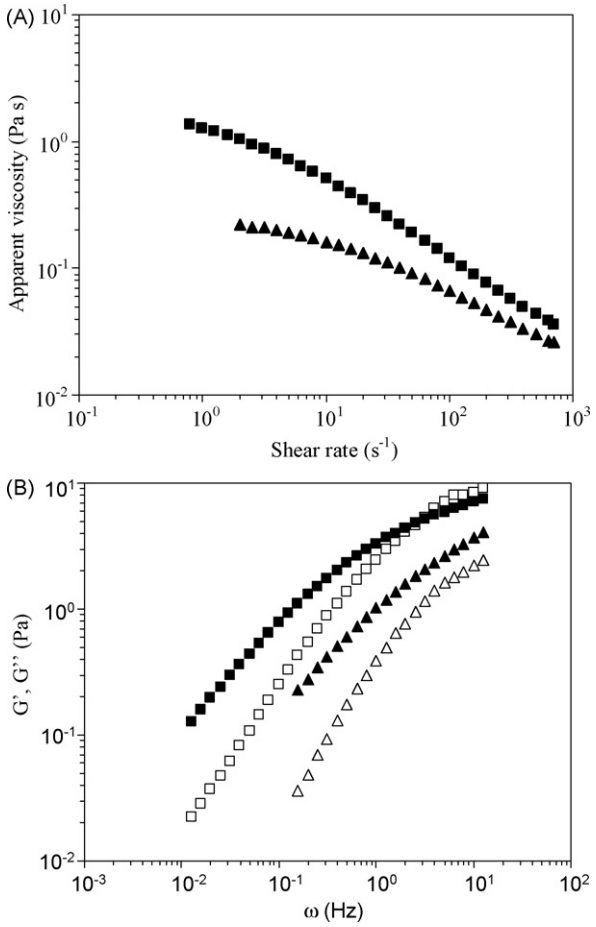

Fig. 5. Steady-state (A) and oscillatory data (B) of the culture broth at day 7.0 (squares), and of an aqueous solution in deionised water of the purified EPS taken at day 7.0 (triangles), both samples having the same effective polymer concentration (0.81 wt%). G'' (full symbols), G' (open symbols).

the consistency index up to 4.0 days of cultivation may be attributed to the increase in EPS concentration (Fig. 1; Table 1). On the other hand, its further increase between days 4.0 and 7.0 cannot be related only to EPS concentration and molecular weight, since they remained nearly constant. Regarding the chemical composition, a slight variation was noticed, namely on the content of pyruvate (from 2.90 to 3.90 wt%) and succinate (from 0.30 to 1.01%), increasing to some extent the molecules negative charge density. Hence, the raise of the broth viscosity and the consistency index after day 4.0 should be essentially related to the formation of new interactions between individual EPS molecules and the other components of the complex media that is the cultivation broth, added by the slightly higher negative groups content. Another factor that probably has contributed to the increase of broth viscosity observed during the last days of the cultivation was the death of some microbial cells. As the cells loose their viability, they release their intracellular components, which might have an influence upon the broth viscosity. These hypotheses are in agreement with the results shown in Fig. 5A and B, where it is presented the steady-state and oscillatory data of the culture broth at day 7.0, and of an aqueous solution in deionised water of the purified EPS recovered at day 7.0, both samples having an identical polymer concentration (0.81 wt%, calculated subtracting the inorganic content). As can be seen, the viscosity of the purified EPS sample is lower, meaning that, the ions and other components of the broth are acting as viscosity enhancers in the presence of EPS molecules. In addition, the mechanical spectrum of the purified EPS sample shows a higher loss modulus (G'')

Table 1
Power law model and Cross model parameters for *Enterobacter sp.* broth samples taken at different cultivation times.

Cultivation time (days)	EPS (g L^{-1})	η (Pa s)[a]	Power law model[b]		Cross model[c]		
			K (Pa s^n)	n	η_0 (Pa s)	τ(s)	m
0.2	0.0	0.0047	0.004 ± 0.0005	0.984 ± 0.0190	–	–	–
0.9	3.66 ± 0.02	0.0052	0.006 ± 0.0002	0.948 ± 0.0069	–	–	–
2.0	7.73 ± 1.46	0.0147	0.026 ± 0.0013	0.814 ± 0.0083	–	–	–
4.0	12.24 ± 1.01	0.0814	0.263 ± 0.0105	0.577 ± 0.0067	0.198 ± 0.010	0.122 ± 0.024	0.563 ± 0.021
5.2	12.77 ± 0.60	0.2590	1.174 ± 0.0610	0.439 ± 0.0093	1.980 ± 0.045	0.352 ± 0.046	0.677 ± 0.019
6.2	12.64 ± 0.24	0.3910	2.033 ± 0.1231	0.383 ± 0.0105	2.060 ± 0.049	0.492 ± 0.030	0.709 ± 0.009
7.0	11.72 ± 0.25	0.3939	1.947 ± 0.1097	0.407 ± 0.0099	2.070 ± 0.066	0.583 ± 0.057	0.659 ± 0.011

[a] At a shear rate of 15.85 s^{-1}, the associated error was ≤0.5%; the relative deviation errors – $RE = \sum_{i=1}^{n}(|x_{exp,i} - x_{cal,i}|/x_{exp,i})/n$ were [b]$0.0091 \le RE \le 0.1038$ and [c]$0.0114 \le RE \le 0.0192$.

for all frequencies studied, in contrast with the data of the broth sample, for which the storage modulus (G') becomes higher at high frequencies.

The flow curves obtained for broth samples taken at 4.0, 5.2, 6.2 and 7.0 days of cultivation could be fitted by Eq. (4), which is based on the Cross model normally used to describe all stages of the flow curves:

$$\eta_a = \frac{\eta_0}{1 + (\tau\dot{\gamma})^m} \quad (4)$$

where $\dot{\gamma}$ is the shear rate (s^{-1}), η_a is the apparent viscosity (Pa s), η_0 is the zero-shear rate viscosity (of the first Newtonian plateau) (Pa s), τ is a time constant (s) and m is a dimensionless constant, which may be related to the exponent of the power law (n) by $m = 1 - n$. Eq. (4) is obtained from the Cross equation (Cross, 1965) assuming a negligible viscosity of the second Newtonian plateau when compared to η_a and η_0, which is valid in this work since the second Newtonian plateau was never approached.

Eq. (4) fitted quite well the flow curves, and the parameter values obtained are presented in Table 1. The values of the exponent m are consistent with those obtained for n, the exponent of the power law. It is also observed an increase of the time constant τ as the viscosity of the culture broth becomes higher, meaning that more time is needed to form new polymer chain entanglements as they are disrupted by the shear stress imposed. As a consequence, the shear rate corresponding to the transition from Newtonian to shear-thinning behavior moves to lower values as the concentration increases (Fig. 4).

3.4.2. Oscillatory measurements and their correlation to steady-shear data

Fig. 6 shows the angular frequency dependencies of storage (G') and loss (G'') moduli of the broth samples taken at days 4.0, 5.2, 6.2 and 7.0. At low frequencies (terminal zone), values of G'' were much higher than those of G'. This indicates that a liquid-like behavior predominated for all samples. However, at higher frequencies and

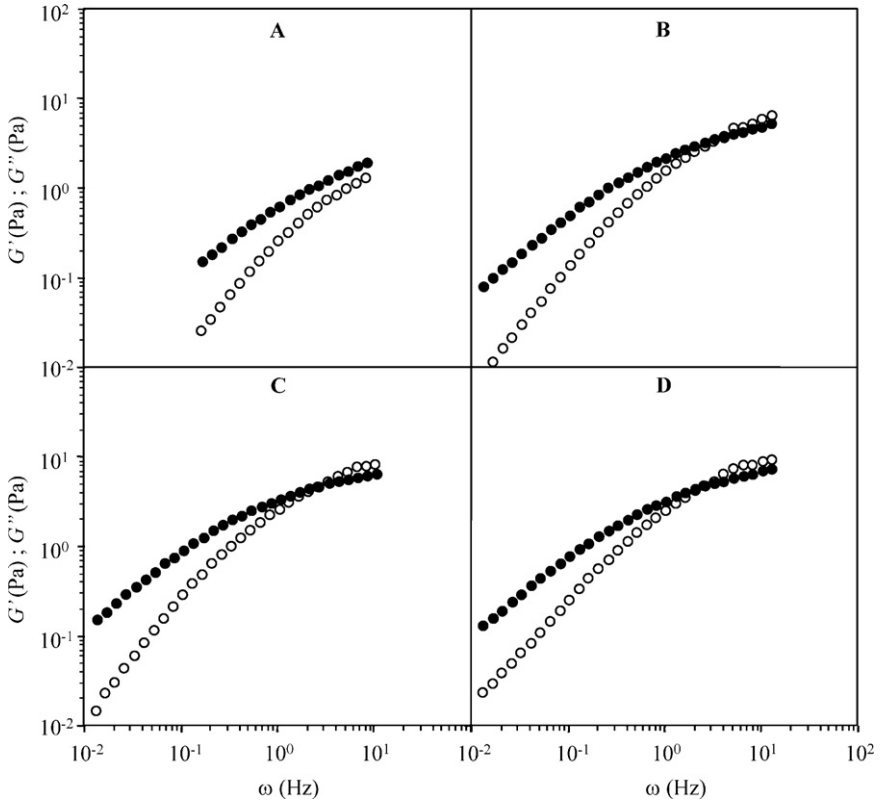

Fig. 6. Storage (open symbols) and loss moduli (full symbols) for broth samples taken at different cultivation times: (A) 4.0 days; (B) 5.2 days; (C) 6.2 days and (D) 7.0 days.

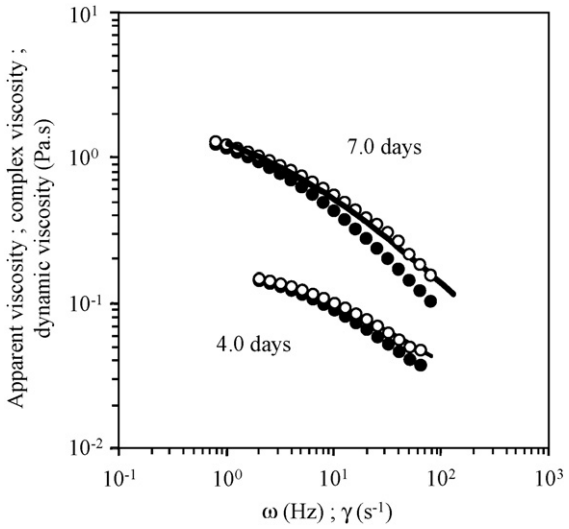

Fig. 7. Cox–Merz plots for the broth samples isolated at days 4.0 and 7.0: (—) apparent viscosity; (○) complex viscosity and (●) dynamic viscosity.

for 5.2, 6.2 and 7.0 days broth samples, a cross-over was detected beyond which the elastic contribution predominated (plateau zone). This "cross-over frequency" (where $G' = G''$) moved to lower frequency values when the concentration increased (Fig. 6B–D), as a consequence of increasing relaxation times.

In the measurement of the viscosity and viscoelasticity of conventional polymeric solutions, the angular frequency dependence of complex viscosity is well superimposed on the shear rate dependence of the apparent viscosity, known as the Cox–Merz model. In this study, the correlation between apparent and complex viscosity was determined using *Enterobacter sp.* broth from different cultivation times. The plots of complex viscosity against angular frequency were completely superimposed to the curve of apparent viscosity against shear rate for all samples. Fig. 7 presents the results obtained for broth samples at 4.0 and 7.0 days. This fact means that, although the culture broth is a complex system composed, not only by a high molecular weight polysaccharide, but also by other components, such as salts, glycerol and cells, it still possesses simple rheological properties. As the cultivation process proceeded, similar types of molecular rearrangements were taking place in the two flow patterns for the applied shear rate and frequency ranges (Xu, Willför, Holmlund, & Holmbom, 2009).

The dynamic viscosity is also presented in Fig. 7, and behaves as in many polysaccharide systems: approaching the zero-shear rate viscosity at low shear rates and diverging from the complex and apparent viscosities as the angular frequency increases. This fact may be attributed to different molecular motions present in the dynamic and steady conditions at high frequency and shear rate (Ferry, 1980).

4. Conclusions

In this study, a rheological and morphological characterization of the culture broth of *Enterobacter sp.* during EPS production was presented. Microscopic observations revealed the formation of cell aggregates surrounded by the EPS at the beginning of the cultivation run, while, at the end, aggregates were reduced both in number and size, and an EPS matrix with the cells embedded in it was observed. The culture broth flow behavior changed from Newtonian in the beginning, to pseudoplastic as the EPS was produced. It has been shown that the viscosity of *Enterobacter sp.* culture broth was not dependent only on EPS concentration, molecular weight and chemical composition, as the broth viscosity continued to increase when polymer production ceased, and when there was not a significant variation of the EPS molecular weight and chemical composition. The increase of viscosity should be essentially related to the formation of new interactions between individual EPS molecules and the other components of the broth. Nevertheless, changes in culture broth viscosity reflected the progress of the cultivation and, hence, rheological data can be used, in some extent, for monitoring the EPS production process.

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