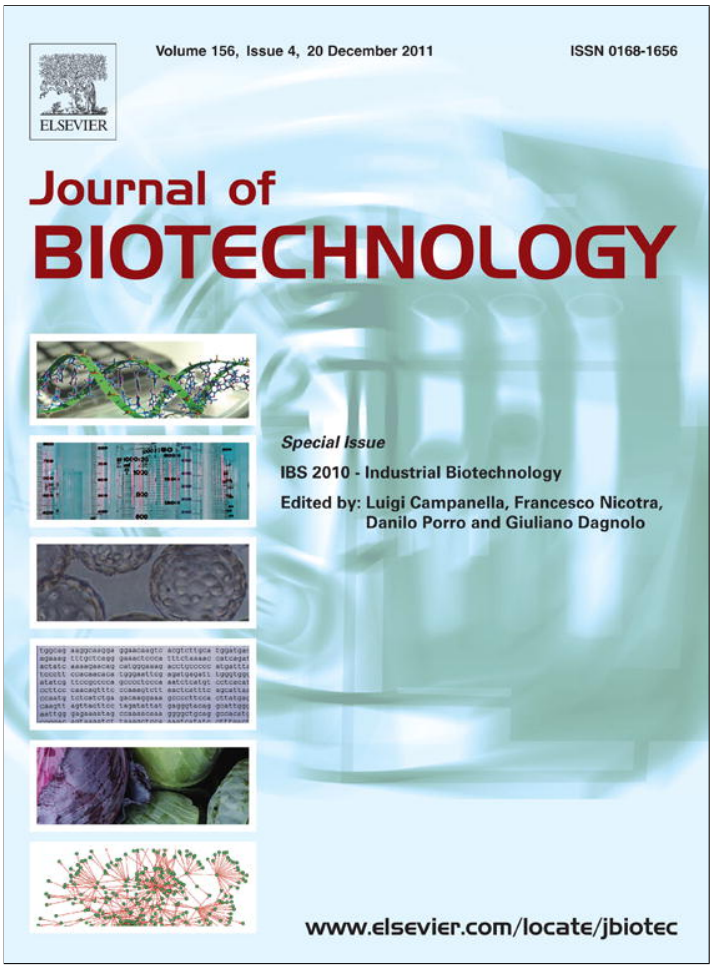

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Kinetics of production and characterization of the fucose-containing exopolysaccharide from *Enterobacter* A47

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ABSTRACT

A fucose-containing exopolysaccharide (EPS) was produced by the bacterium *Enterobacter* A47 using glycerol byproduct from the biodiesel industry. The analysis of kinetic data suggested a partially growth associated EPS synthesis model. Although the EPS was composed of fucose, galactose and glucose at all cultivation stages, their relative proportion has varied considerably during the run. At the beginning (24 h), glucose was the main component (82.4 wt.%), being fucose and galactose minor components (5.0 wt.% and 10.9 wt.%, respectively), while at the end (96 h) it was composed of 26.0 wt.% fucose, 28.9 wt.% galactose and 43.7 wt.% glucose. The acyl groups content and composition have also changed, reaching their maximum content (19.2 wt.%) at the end of the run. Moreover, the molecular weight has increased linearly during the run (from 8×10^5 to 5×10^6). The changes observed in EPS composition and molecular weight have also had an impact upon the polymer's intrinsic viscosity, as shown by its linear increase from 3.95 to 10.72 dL g^{-1}. The results suggest that the culture might have synthesized at least two distinct EPS, with different sugar composition and average molecular weight, which predominated at different cultivation stages.

1. Introduction

Polysaccharides' distinct physical–chemical properties, such as water retention capacity, rheology (e.g. emulsifying, thickening and gelling agents) and film forming capacity, allow their application on several industries (e.g. food, cosmetic and pharmaceutical) (Moreno et al., 1998). Plants, crustacean, algae, as well as a wide range of microorganisms, represent sources of a wide diversity of natural polysaccharides. In the last years, the interest on this sort of polymeric biomaterials has increased due to their environmentally friendly features as biodegradable, biocompatible and value-added products (Kumar et al., 2007).

Microorganisms usually have higher growth rates than plants, crustacean and algae, being microbial production of polysaccharides more amenable to process manipulation, allowing for improved yields, productivity and properties (Alves et al., 2010). On the other hand, industrial microbial production is limited by the high cost of the most commonly used carbon sources (e.g. glucose, fructose, sucrose) (Kumar et al., 2007). This limitation can be overcome by replacing those traditional substrates by low cost carbon sources, such as agro and industrial wastes or byproducts (Kumar et al., 2007). Several industrial processes, essentially biodiesel production, generate large quantities of glycerol as byproduct. Since this glycerol contains several impurities, it cannot be used in many of the traditional glycerol applications unless costly purification steps are performed (Freitas et al., 2009). Hence, it is necessary to develop alternative processes to convert this crude glycerol, into higher value products. The use of glycerol byproduct as carbon source for microbial cultivations may contribute for the reduction of production costs, thus making those bioprocesses more cost effective.

Microbial polysaccharides can be divided into intracellular, structural and extracellular polysaccharides. Extracellular polysaccharides or exopolysaccharides (EPS) are secreted by the cells, either as a capsule that remains associated with the cell surface or as a slime which is loosely bound to the cell surface (Kumar et al., 2007). EPS have easier extraction processes, which is an advantage comparing to other natural polysaccharides (e.g. plants or algae cell-wall constituents).

EPS are composed of monomers such as neutral sugars and/or acidic or amino-sugars, commonly containing non-sugar components, such as acyl groups (e.g. acetyl, pyruvil, succinyl). The most common sugar residues in EPS structures are glucose and galactose. However, certain EPS have an increased value due to their content

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Nomenclature

S	Glycerol concentration ($g\,L^{-1}$)
N	Nitrogen concentration ($g\,L^{-1}$)
X	Biomass concentration ($g\,L^{-1}$)
P	Concentration of exopolysaccharide ($g\,L^{-1}$)
$Y_{X/S}$	Yield of biomass on glycerol ($g\,g^{-1}$)
$Y_{P/S}$	Yield of exopolysaccharide on glycerol ($g\,g^{-1}$)
$Y_{X/N}$	Yield of biomass on nitrogen ($g\,g^{-1}$)
D	Dilution rate (h^{-1})
V	Volume (L)
K_S	Glycerol half saturation constant ($g\,L^{-1}$)
K_N	Nitrogen half saturation constant ($g\,L^{-1}$)
m_S	Maintenance coefficient on glycerol ($g\,g^{-1}\,h^{-1}$)
α	Yield of product synthesis per biomass produced ($g\,g^{-1}$)
β	Specific rate of non-growth associated product synthesis ($g\,g^{-1}\,h^{-1}$)
μ	Specific rate of biomass growth (h^{-1})
μ_{max}	Maximum specific rate of biomass growth (h^{-1})
ν_S	Specific rate of glycerol uptake ($g\,g^{-1}\,h^{-1}$)
ν_N	Specific rate of nitrogen uptake ($g\,g^{-1}\,h^{-1}$)
ν_P	Specific rate of exopolysaccharide production ($g\,g^{-1}\,h^{-1}$)

in some rare sugars, which occur rarely in Nature (Vanhooren and Van Damme, 2000). One of those rare sugars is fucose. It has been reported that fucose-containing polysaccharides possess biological activity that potentiate their use in medical or cosmetic areas, for example, as anti-carcinogenic and anti-inflammatory agents or as moisturizing and anti-aging additives, respectively (Cescutti et al., 2005; Péterszegi et al., 2003; Guetta et al., 2003).

Fucose-containing EPS have been reported to be produced by several bacterial genera, including *Klebsiella*, *Clavibacter*, *Escherichia* and *Enterobacter*. Examples of *Enterobacter* fucose-containing EPS producing strains include: *Enterobacter amnigenus* that produces a heteropolymer containing glucose, galactose, fucose, mannose, glucuronic acid and pyruvil (Cescutti et al., 2005); *Enterobacter* sp. that secretes an acidic EPS in which glucose, mannose, rhamnose and fucose monomers are present in a molar ratio of 3.3:3.0:2.6:1 (Shimada et al., 1997); and *Enterobacter cloacae* that produces an EPS containing glucose, galactose, glucuronic acid, fucose and acetyl in the molar ratio of 5:4:4:11:1 (Meade et al., 1994).

Formerly, we have reported that in a nutrient medium containing glycerol byproduct from the biodiesel industry as the sole carbon source, the bacterium *Enterobacter* A47 (DSM 23139) produced an EPS composed of fucose, glucose and galactose (Alves et al., 2010). A preliminary polymer characterization in terms of its chemical composition, molecular weight and intrinsic viscosity was performed (Freitas et al., 2011). In this work, we characterize cell growth and EPS synthesis kinetics with the aid of a simple kinetic model. Furthermore, the progress of the polymer's chemical composition, molecular weight and intrinsic viscosity along the cultivation run was analyzed with the objective of describing the behavior of the culture during its growth on glycerol and EPS synthesis.

2. Materials and methods

2.1. Fucose-containing EPS production

2.1.1. Microorganism and media

Enterobacter A47 (DSM 23139) (Freitas et al., 2011) was grown on a slightly modified Medium E* (pH 7.0), supplemented with glycerol byproduct to give a concentration between 25 and 50 $g\,L^{-1}$, as described by Freitas et al. (2009). Glycerol byproduct (with a glycerol content ca. 89%) was supplied by SGC Energia, SGPS, SA, Portugal.

Inoculums for bioreactor experiments were prepared by incubating the culture in Medium E* supplemented with glycerol byproduct (40 $g\,L^{-1}$), in shake flasks, for 72 h at 30 °C, in an incubator shaker (150 rpm).

2.1.2. Bioreactor operation

The 2 L bioreactor (BioStat B-plus, Sartorius) containing 1.3 L of Medium E* (Freitas et al., 2009) supplemented with glycerol byproduct (concentration ca. 40 $g\,L^{-1}$) was inoculated with the culture (400 mL). The bioreactor was operated as described by Freitas et al. (2011). Briefly, it was operated in a batch mode during the first day of cultivation and, in a fed-batch mode, for the next three days, by supplying the bioreactor with cultivation Medium E*, with a glycerol concentration of 200 $g\,L^{-1}$, at a constant rate of 4.5 $mL\,h^{-1}$. Temperature and pH were controlled at 30 ± 0.1 °C and 7.00 ± 0.05, respectively. The aeration rate (0.125 vvm, volume of air per volume of reactor per minute) was kept constant throughout the cultivation, and the dissolved oxygen concentration (DO) was controlled by automatic variation of the stirrer speed (200–800 rpm) provided by two 6-blade impellers. During the fed-batch phase, the DO was maintained below 10%.

2.1.3. Analytical techniques

Culture broth samples were centrifuged at 13,000 × *g*, for 15 min, for cell separation. The cell-free supernatant was stored at −20 °C for the determination of glycerol and ammonium concentrations, and for the quantification of the EPS produced. The cell pellet was used for the gravimetric determination of the cell dry weight (CDW), after washing with deionized water (resuspension in water, centrifugation at 13,000 × *g*, for 10 min, and, finally, resuspension in water and filtration through 0.20 μm filters).

Glycerol concentration in the cell-free supernatant was determined by high performance liquid chromatography (HPLC) with an Aminex HPX-87H column (BioRad), coupled to a refractometer. The analysis was performed at 50 °C, with sulphuric acid (H_2SO_4 0.01 N) as eluent, at a flow rate of 0.6 $mL\,min^{-1}$. Ammonium concentration was determined using a potentiometric sensor (Thermo Electron Corporation, Orion 9512). The viscosity of the culture broth samples was measured using a controlled stress rheometer (Haake RS-75, Germany) equipped with a cone and plate geometry (diameter 3.5 mm, angle 2°), as described by Alves et al. (2010).

2.1.4. EPS extraction

The culture broth recovered from the bioreactor at the end of the cultivation was diluted with deionized water (1:2, v/v) for viscosity reduction and the bacterial cells were removed by centrifugation (13,000 × *g*, 1 h). The cell-free supernatant was subjected to thermal treatment (70 °C, 1 h) to inactivate bacterial enzymes that might cause polymer degradation during the subsequent purification steps. The treated supernatant was centrifuged (13,000 × *g*, 1 h) to remove any remaining cell debris and denatured proteins. Finally, it was dialyzed with a 10,000 MWCO membrane (SnakeSkin™ Pleated Dialysis Tubing, Thermo Scientific), against deionized water (48 h, 4 °C) and freeze dried.

2.2. Analysis and characterization of process kinetics

A simple mathematical model was employed for cell growth and product synthesis kinetics characterization. Growth kinetics

was assumed to follow the Monod model with potential limitation of glycerol (S) and ammonia (N):

$$\mu = \mu_{\max} \cdot \frac{S}{K_S + S} \cdot \frac{N}{K_N + N} \quad (1)$$

Overall glycerol consumption results from glycerol taken up for biomass synthesis ($\mu/Y_{X/S}$), glycerol taken up for EPS synthesis ($\nu_{EPS}/Y_{EPS/S}$) and energy spent for maintenance processes (m_S) (see nomenclature for definition of model parameters):

$$\nu_S = \frac{\mu}{Y_{X/S}} + \frac{\nu_P}{Y_{P/S}} + m_S \quad (2)$$

EPS synthesis was assumed to be partially associated to cell growth and described by the following equation:

$$\nu_N = \frac{\mu}{Y_{X/N}} \quad (3)$$

Finally, ammonia uptake was assumed to be associated with biomass synthesis:

$$\nu_P = \alpha \cdot \mu + \beta \quad (4)$$

These kinetic equations result into the following set of material balance equations for a stirred-tank bioreactor operated in batch/fed-batch mode:

$$\frac{dX}{dt} = (\mu - K_d - D) \cdot X \quad (5)$$

$$\frac{dS}{dt} = -\nu_S \cdot X + D \cdot (S_0 - S) \quad (6)$$

$$\frac{dN}{dt} = -\nu_N \cdot X + (N_0 - N) \quad (7)$$

$$\frac{dP}{dt} = -\nu_P \cdot X - D \cdot P \quad (8)$$

$$\frac{dV}{dt} = D \cdot V \quad (9)$$

with X, S, N and P the concentrations of biomass, glycerol, ammonia and EPS in the reactor respectively, $D = F/V$ the dilution rate ($D = 0\ h^{-1}$ in the batch phase), F the inlet feed rate in the fed-batch phase, V the culture volume and subscript index '0' denoting concentration in the inlet feed stream.

2.2.1. Kinetic parameters estimation

Kinetic parameters were estimated for each cultivation experiment performed using an in-house developed program for MATLAB (Mathworks, Inc). Parameters were estimated in the sense of least squares employing the Levenberg–Marquardt algorithm (*fmincon* MATLAB function). Model differential equations were integrated using a 4th/5th order Runge–Kutta solver (*ode45* MATLAB function). The final residuals and Jacobian matrix served to calculate an approximation to the Hessian matrix assuming that the final solution is a local optimum. The Hessian matrix enabled to calculate the parameters covariance matrix and parameters 95% confidence intervals. See Dias et al. (2005) for more details.

2.3. Fucose-containing EPS characterization

2.3.1. Chemical composition

The analysis of the EPS composition in terms of its sugar composition and acyl groups was performed as described by Freitas et al. (2011). Briefly, EPS dried samples (~5 mg) were hydrolyzed with trifluoroacetic acid (TFA) and the hydrolyzate was used for the identification and quantification of the constituent sugar and acyl groups residues.

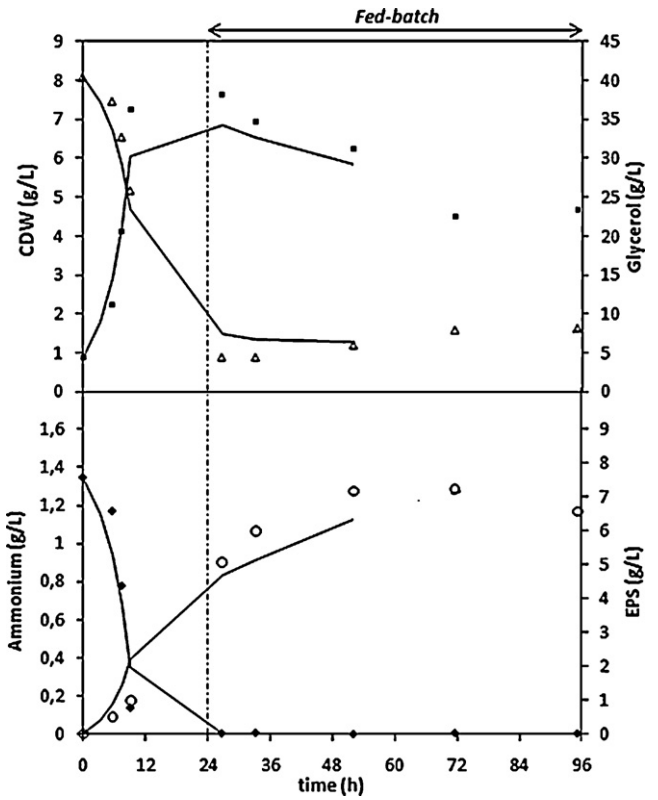

Fig. 1. Experimental (symbols) and modeling results (continuous lines) showing the glycerol (△), ammonia (♦), biomass (■) and EPS (○) kinetics with time. The mean square errors were 0.194 g L^{-1}, 4.09 g L^{-1}, 0.020 g L^{-1} and 0.181 g L^{-1} for biomass, glycerol, ammonia and EPS respectively.

2.3.2. Molecular weight

EPS solutions were analyzed by Size Exclusion Chromatography as described by Freitas et al. (2011).

2.3.3. Intrinsic viscosity

The determination of the intrinsic viscosity of the purified polymer was performed using an automatic viscosity measuring unit AVS 450 (Schott-Gerate, Germany), with an Ubbelhode capillary viscometer (Ref. 53013/Ic, Schott-Gerate, Germany) immersed in a water bath at constant temperature (25 °C) as described by Freitas et al. (2011).

3. Results and discussion

3.1. Fucose-containing EPS production

EPS production was carried out in a 2 L bioreactor fed with Medium E* and glycerol byproduct as carbon source. After a short adaptation period (~7.5 h) *Enterobacter* A47 entered an exponential growth phase that ended within 24 h, when the ammonium concentration became limiting (under 0.1 g $NH_4^+L^{-1}$). The fed-batch phase was initiated at that time with the addition of mineral medium with a high glycerol byproduct concentration (200 g L^{-1}), at a constant rate (4.5 mL h^{-1}).

Fig. 1 shows concentration profiles of biomass, EPS, glycerol and ammonium during cultivation period. The culture attained a maximum cell dry weight around 7.68 g L^{-1}, at the end of batch phase (~24 h). Subsequently, a decrease of the cell dry weight (CDW) was observed (Fig. 1), which may be related with a loss of cell viability due to ammonium and oxygen limiting conditions imposed in the bioreactor. In addition, CDW decline may also be a result of dilution of biomass concentration caused by volume withdrawn from

Table 1
Maximum yields and Michaelis–Menten constant for glycerol uptake.

	Values	Value source
$Y_{X/S}$	$0.49 \pm 0.045\ g\,g^{-1}$	Estimated
$Y_{EPS/S}$	$0.24 \pm 0.04\ g\,g^{-1}$	Estimated
$Y_{X/N}$	$5.45 \pm 0.36\ g\,g^{-1}$	Estimated
K_S	$\sim 0\ g\,L^{-1}$	Lin (1976)
K_N	$0.0021 \pm 0.0168\ g\,L^{-1}$	Estimated
μ_{max}	$0.361 \pm 0.02\ h^{-1}$	Estimated
α	$0.12 \pm 0.09\ g\,g^{-1}$	Estimated
β	$0.041 \pm 0.009\ g\,g^{-1}\,h^{-1}$	Estimated
m_S	$0\ g\,g^{-1}\,h^{-1}$	Fixed
K_d	$0\ h^{-1}$	Fixed

the bioreactor for sampling with concomitant continuous feeding of fresh medium and pH control solutions, on a stage cells were no longer multiplying (Alves et al., 2010).

During the batch phase, the glycerol concentration in the culture broth decreased from the initial $40\ g\,L^{-1}$ to $5\ g\,L^{-1}$ simultaneously with cell growth (Fig. 1). During the fed batch operation glycerol was kept between 4 and $10\ g\,L^{-1}$, despite that it was being continuously fed with a solution containing $200\ g\,L^{-1}$ of glycerol (Fig. 1).

Enterobacter A47 initiated EPS production at the onset of exponential cell growth while the maximum EPS concentration ($7.50\ g\,L^{-1}$) was attained at around 52 h. It should be noted that the final EPS concentration obtained in this study is lower than the value reported in a previous study (Alves et al., 2010) due to differences in the extraction/purification method. The actual method used for EPS purification was dialysis, which enables a complete ash removal and a higher protein removal than the acetone extraction method used by Alves et al. (2010). Additionally, during dialysis loss of some oligosaccharides may occur, which are quantified when the acetone extraction method is used.

3.2. Analysis of process kinetics

A kinetic characterization was obtained by fitting the model Eqs. (1)–(9) to the corresponding experimental data. Modeling results are shown in Fig. 1 and Table 1. The glycerol half saturation constant (K_S) could not be accurately estimated due to the lack of measured data at low glycerol concentrations. An arbitrarily low value was used instead, $K_S = 9.2 \times 10^{-5}\ g\,L^{-1}$, that was reported for glycerol assimilation by bacteria (Lin, 1976). All other parameters were estimated according to the previously described method.

It can be observed that model calculations and experimental data are in good agreement for the calibration dataset (Fig. 1). The mean squared errors (MSE) for biomass, glycerol, ammonia and EPS were $0.194\ g\,L^{-1}$, $4.09\ g\,L^{-1}$, $0.020\ g\,L^{-1}$ and $0.181\ g\,L^{-1}$ respectively. Also, parameter estimates show narrow confidence intervals, strengthening their statistical confidence. This essentially means that the model structure is in conformity with observed experimental data and that measured variables are highly sensitive to kinetics parameters. This model is however an oversimplified phenomenological representation of the process and cannot capture batch-to-batch variability. Its predictive capacity is insufficient for model-based process optimization. It is here rather used as a data analysis tool to extract reliable kinetic parameter values of each individual experiment.

The analysis of kinetic parameter values showed that the maximum cell growth rate was rather high ($\mu_{max} = 0.36 \pm 0.02\ h^{-1}$). The yield $Y_{X/Gly} = 0.49 \pm 0.045$ (w/w) was also high denoting a robust and efficient cell growth process. EPS synthesis seemed to be partially growth associated ($\alpha = 0.046 \pm 0.098$ w/w and $\beta = 0.038 \pm 0.006\ gEPS\,gCDW^{-1}\,h^{-1}$), although most of the EPS was synthesized after cell growth arrest at a specific synthesis rate of $0.038 \pm 0.006\ gEPS\,gCDW^{-1}\,h^{-1}$. Considering only the nitrogen limited fed-batch phase, the volumetric EPS productivity is $0.28\ gEPS\,L^{-1}\,h^{-1}$ and the yield $Y_{EPS/Gly}$ is 0.28 ± 0.035 (w/w).

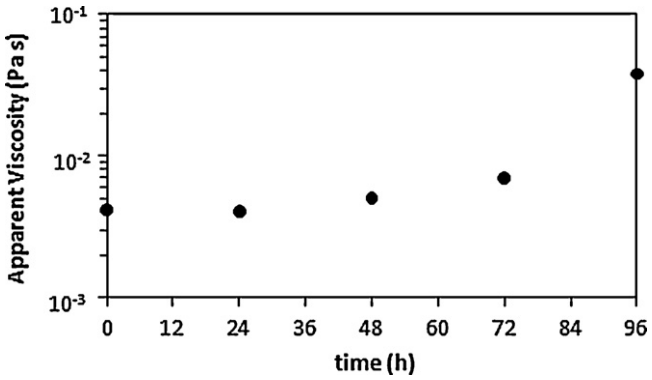

Fig. 2. Culture broth apparent viscosity during the cultivation of *Enterobacter* A47 in glycerol byproduct (shear rate $100\ s^{-1}$).

The EPS productivity value is within the range of productivity values reported for xanthan production by García-Ochoa et al. (2000) ($0.13–0.51\ g\,L^{-1}\,h^{-1}$) and by Zhang and Shen (2010) ($0.04–0.44\ g\,L^{-1}\,h^{-1}$). It is higher than the productivity reported for bacterial alginate $0.014\ g\,L^{-1}\,h^{-1}$ (Chèze-Lange et al., 2002) and also similar to gellan gum production ($0.16–0.48\ g\,L^{-1}\,h^{-1}$) reported by Arockiasamy and Banik (2008).

3.3. Broth rheology

As shown in previous work (Alves et al., 2010), there is a significant increase of the broth's viscosity during the cultivation of *Enterobacter* A47. As shown in Fig. 2, the apparent viscosity of the culture broth increased two orders of magnitude during the 96 h cultivation run. During the first 24 h there was no significant increase in broth viscosity, while a gradual raise was observed between 24 and 72 h. During this period the viscosity increase may be related to increase of EPS concentration in the broth, as well as with changes of the biopolymer's composition and molecular weight. The most significant increase of the broth's viscosity was observed during the final day of the run (Fig. 2). This significant increase is not explained by EPS concentration since there was no considerable polymer production during that period. It may be explained by changes in EPS composition and molecular weight occurring simultaneously (Sections 3.4.1 and 3.4.2). Moreover, the establishment of novel or stronger interactions is likely to occur, either among EPS molecules, or between polymer chains and the other components of the cultivation broth, contributing for the viscosity build-up (Alves et al., 2010).

Along the cultivation run the culture broth started developing a non-Newtonian behavior acting as a shear thinning fluid, with the apparent viscosity decreasing with increasing shear rates (Alves et al., 2010). The great increase in viscosity observed during the final day of operation caused a loss of bulk homogeneity in terms of mixing, mass and heat transfer, which resulted in significant gradients of DO, temperature, pH and nutrients concentration across the bioreactor. Hence, in contrast with previous work, wherein the run took 7 days (Alves et al., 2010), in this work the cultivation was terminated at 96 h, because there was no significant EPS synthesis after around 48 h of cultivation, despite the viscosity built up observed.

3.4. EPS physical–chemical characterization

The fucose-containing EPS synthesized by *Enterobacter* A47 is a high molecular weight (5.8×10^6) heteropolysaccharide composed of neutral sugars, namely fucose, galactose and glucose, and

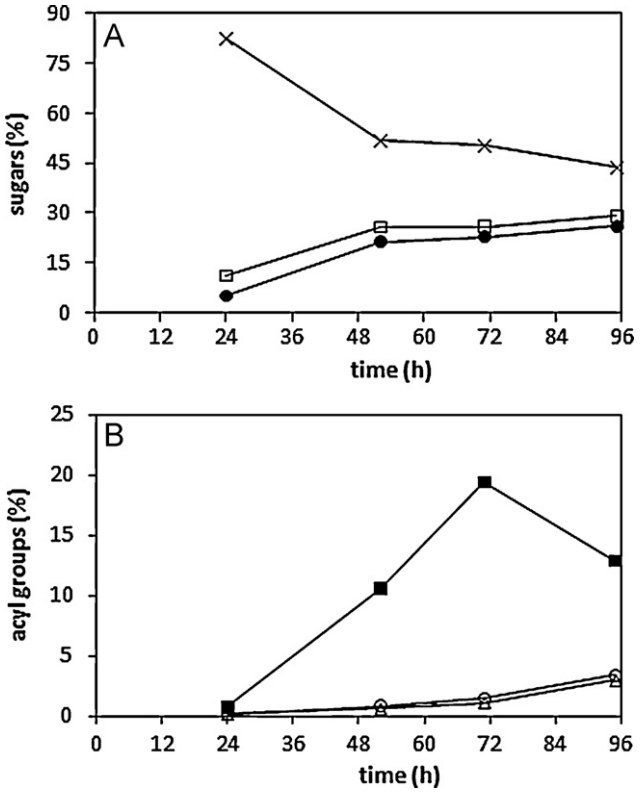

Fig. 3. Profile of the fucose-containing EPS sugar composition ((×) glucose; (□) galactose; (●) fucose) (A) and acyl groups ((■) succinyl; (○) pyruvil; (Δ) acetyl) (B) along the cultivation run.

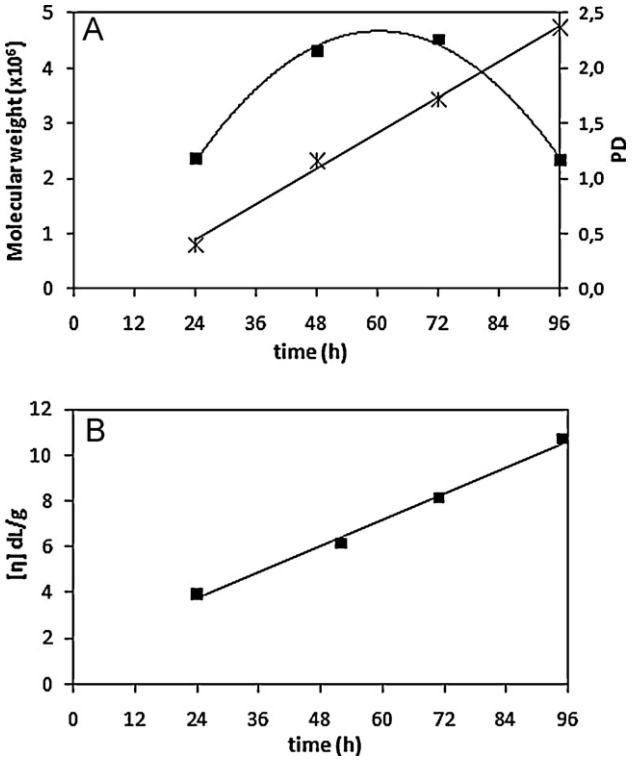

Fig. 4. (A) Evolution of the fucose-containing EPS average molecular weight (stars) and polydispersity (squares) along the cultivation run. (B) Variation of the intrinsic viscosity over time.

acyl groups substituents (Alves et al., 2010; Freitas et al., 2011). In this work, biopolymer composition, intrinsic viscosity and average molecular weight have been analyzed throughout the cultivation run.

3.4.1. EPS composition

As shown in Fig. 3A, the relative proportion of sugar monomers has undergone some changes throughout the cultivation run. Around 24 h of cultivation glucose was the main sugar monomer of the EPS, with a content of 82.4 wt.%. Galactose and fucose were present in much lower amounts (10.9 and 5.0 wt.%, respectively). Between 24 and 52 h, the sugar monomers composition has undergone significant changes, namely a reduction of glucose content from 82.4 to 51.6 wt.%, simultaneously with an increase of the content in galactose and fucose (from 10.9 to 25.6 wt.% and from 5.0 to 21.3 wt.%, respectively) (Fig. 3A). From that time on, the sugar monomer composition of the EPS has changed only slightly, being composed of fucose (26.0 wt.%), galactose (28.9 wt%) and glucose (43.7 wt.%), at the end of the run. The EPS produced in a longer cultivation run (7 days) had identical sugar composition (Freitas et al., 2011). This behavior may reflect bacterial metabolism changes occurring throughout the run. Glucose is converted to glucose-6-P, which is one of the precursors of galactose and fucose (Kumar et al., 2007). Therefore it would be likely to expect that the observed decreased in glucose content in the EPS composition is due to its conversion into galactose and fucose.

The EPS is also composed of non-saccharide components, specifically acyl groups (Fig. 3B). Throughout the cultivation run the total content in acyl groups increased from 0.7 wt.% at 24 h to 19.2 wt.% at the end of the run. The identified acyl groups, in the acid hydrolysate were acetyl, pyruvil and succinyl. Succinyl had the most significant increase throughout the run, attaining its maximum content (19.4 wt.%) at 71 h. Afterwards, it has decreased to 12.9 wt.% at 96 h (Fig. 3B). Acetyl and pyruvil contents gradually increased throughout the entire run, reaching final contents of 2.9 wt.% and 3.4 wt.%, respectively. In previous work, the EPS obtained at the end of a 7 days cultivation had somewhat different contents in succinyl, pyruvil and acetyl (1.1 wt.%, 3.9 wt.% and 6.8 wt.%, respectively) (Freitas et al., 2011), suggesting that further extending the cultivation time results in a polymer with different acyl groups composition. Such changes in the substituent's content and composition have great impact on the polymer's properties, such as solubility and rheology (Rinaudo, 2004). In particular, the EPS anionic character is influenced by its content in pyruvil and succinyl (Freitas et al., 2009).

3.4.2. EPS average molecular weight

The fucose-containing EPS weight average molecular weight (Mw) was estimated by size exclusion chromatography (SEC). As shown in Fig. 4A, both the EPS Mw and the polymer's polydispersity (PD = Mw/Mn being Mn the number average molecular weight) changed during the cultivation run. In fact, the average molecular weight of the polymer formed increased strongly from 8×10^5 to 5×10^6, which is concomitant with the increase of the intrinsic viscosity (Fig. 4A and B).

The bimodal shape of the chromatograms (Fig. 5) seems to suggest that the culture might have synthesized at least two distinct EPS, with different sugar composition and average Mw, and either of them predominated at different cultivation stages. At the beginning (24 h) of the fermentation the lower Mw EPS seems to have prevailed (Fig. 5). Considering that the polymer recovered from the broth at that time was mainly composed of glucose (82.4 wt.%), this EPS might be a glucose homopolymer. The lower polymer content in fucose and galactose (5.0/10.9 wt.%, respectively) may possibly be attributed to the higher Mw peak (Fig. 5). The value of PD, calculated considering a whole peak, only for comparison purposes, was 1.2 (Fig. 4A).

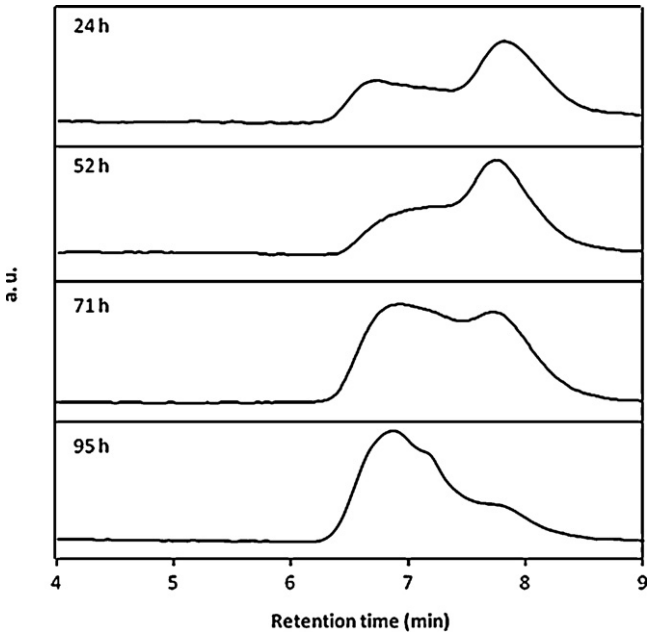

Fig. 5. Mw chromatogram profiles for different cultivation times.

As discussed above, during the run there were visible changes in the polymer's composition (Fig. 3A) that can probably be correlated with the polymers' average Mw. Concomitant with the reduction of the content in glucose (about 30 wt.%) and the increase of the fucose and galactose contents (about 15 wt.% each), there was a sharp increase on the polymer's Mw from 8×10^5 to 2×10^6 (between 24 and 52 h) (Fig. 4A). Moreover, there was also an increase of the intensity of the peak corresponding to the higher Mw fraction of the polymer and a reduction of the lower Mw peak (Fig. 5). Hence, apparently the high Mw EPS predominated at the end of the run. Glucose was a major sugar component of the polymer at that time, suggesting that the high Mw EPS might be composed of fucose, galactose and glucose. This hypothesis requires a more detailed study of the different Mw fractions of the EPS synthesized by *Enterobacter* A47 at different cultivation times.

The higher heterogeneity of the polymer between 52 and 72 h is also evidenced by the higher PD value obtained (2.2) during that period (Fig. 4A). At the end of the assay the EPS had a value Mw of 5×10^6, and it was rather homogenous, as shown by the low PD of 1.2. The reduction of the PD is evidenced by the narrower chromatogram shape and the loss of the great bimodal character seen in Fig. 5.

These results are in accordance with the increase of the culture broth viscosity (Fig. 2), indicating that besides the influence of the EPS composition on this characteristic, it is also deeply influenced by the raise of the EPS polymerization degree.

The Mw of the fucose-containing EPS recovered from the broth at the end of the 96 h cultivation assay is similar to the value reported for commercial xanthan (5.0×10^6) (Freitas et al., 2011) and higher than that reported by Salah et al. (2010) for xanthan production (8×10^4) by *Xanthomonas campestris* NRRL B-1459 using palm date juice byproduct as carbon source. Commercial bacterial alginate, was reported to have a lower molecular weight (4.3×10^5) (Freitas et al., 2011) than the fucose-containing EPS obtained in this study. Reyes et al. (2003) reported a value Mw of 1.1×10^6 for alginate produced by *Azotobacter vinelandii* in a modified Burk's medium. The Mw of the EPS produced by *Enterobacter* A47 in this study was also comparable to the galactose-rich EPS produced by the bacterium *Pseudomonas oleovorans* ($(1.0–5.0) \times 10^6$), using the same carbon source (Freitas et al., 2009).

3.4.3. Intrinsic viscosity

The polymer's intrinsic viscosity ($[\eta]$) was evaluated along the cultivation run (Fig. 4B). At the end of the run, the EPS presented an intrinsic viscosity around 10.72 dL g^{-1}. This value is in agreement with the ones reported for several commercial polysaccharides, including xanthan and guar gum (5–50 dL g^{-1}) (Arvidson et al., 2006). The EPS intrinsic viscosity and the molecular weight had presented a similar trend, namely, a linear increase along the cultivation run (Fig. 4A and 4B). This linearity is correlated, since the $[\eta]$ is a measure of the molecule's hydrodynamic volume and, consequently, we may expect an increase of $[\eta]$ with the increase of its molecular weight (Bae et al., 2008).

Between 71 and 95 h, the composition in neutral sugars barely varied, as well as EPS concentration. However, the same was not observed with the acyl groups. Their composition had a significant variation, with a great decrease in succinyl and an increase in pyruvil and acetyl content. This fact, can affect the intrinsic viscosity, since the presence of these non-saccharide ionizable components, namely pyruvil and succinyl, can affect the interaction between EPS molecules (García-Ochoa et al., 2000). In other words, the presence of acyl groups affects the hydrodynamic volume, through interaction between molecules and molecule–solvent, which leads to an increase of $[\eta]$.

4. Conclusions

In this work, an exopolysaccharide composed of fucose, galactose and glucose was produced by the bacterium *Enterobacter* A47 in a medium supplemented with glycerol byproduct from the biodiesel industry. The main conclusions from this study were:

- EPS synthesis by *Enterobacter* A47 seems to be partially growth associated, as suggested by the experimental results and confirmed by the developed model.
- The relative proportion of the sugar monomers, as well as the content and composition of the acyl groups substituents, have changed considerably throughout the cultivation run, reaching a quite stable sugar composition at the end of experiment.
- Both the average molecular weight and the intrinsic viscosity of the EPS have increased linearly along the run and these two properties are closely related.
- The changes of the polymer's physical–chemical characteristics could be correlated to the viscosity built up observed in the broth during the assay.
- *Enterobacter* A47 seems to be able to synthesize at least two distinct EPS, with different sugar composition and average Mw, as suggested by the bimodal shape of the SEC chromatograms.

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