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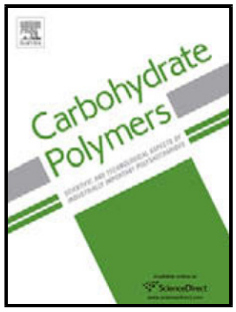

Cost-effective optimized scleroglucan production by *Sclerotium rolfsii* ATCC 201126 at bioreactor scale. A quantity-quality assessment

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PII: S0144-8617(20)31678-7

DOI: https://doi.org/10.1016/j.carbpol.2020.117505

Reference: CARP 117505

To appear in: *Carbohydrate Polymers*

Received Date: 27 July 2020

Revised Date: 24 November 2020

Accepted Date: 8 December 2020

Please cite this article as: Valdez AL, Delgado OD, Fariña JI, Cost-effective optimized scleroglucan production by *Sclerotium rolfsii* ATCC 201126 at bioreactor scale. A quantity-quality assessment, *Carbohydrate Polymers* (2020), doi: https://doi.org/10.1016/j.carbpol.2020.117505

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Cost-effective optimized scleroglucan production by *Sclerotium rolfsii* ATCC 201126 at bioreactor scale. A quantity-quality assessment

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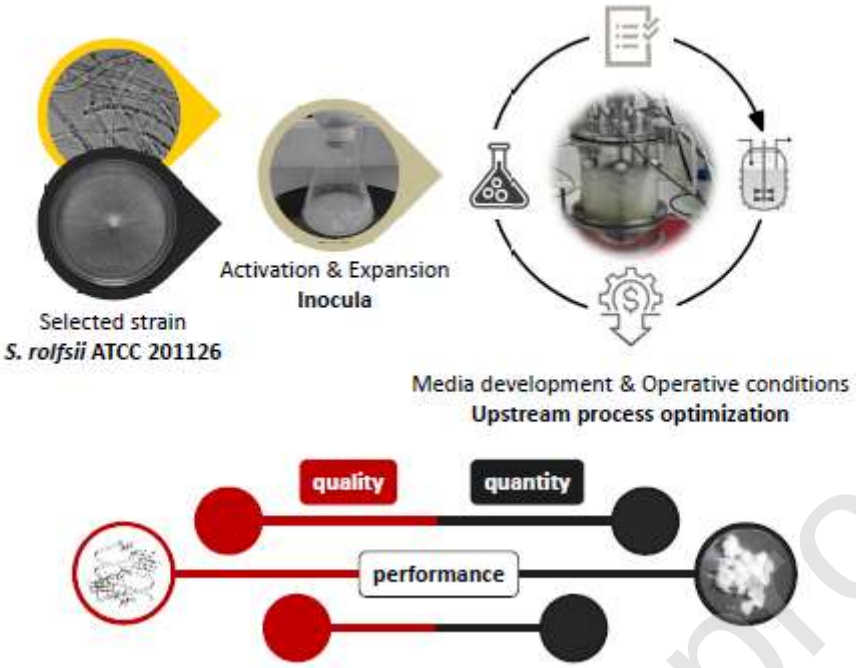
Graphical abstract



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HYPOTHESIS

Nutritional and operative conditions may not be valid for every scleroglucan producer. A polyphasic EPS assessment (amount, properties & performance) would be the best way towards non-biased decisions.

Highlights

- Yield-competitive EPS production involved 80 g sucrose L^{-1} and 2.25 g $NaNO_3$ L^{-1}
- Validated conditions at bench-top bioreactor were 200 rpm, 0.5 vvm, 28 °C, pH_i 4.5
- pH_i at 5.5 without control alternatively helps to reduce biomass production
- Controlled pH=4.5 affects EPS purity, M_W and rheological features; 31 °C affects M_W
- Unbiased evaluation should ponder EPS titre, production costs, quality and features

ABSTRACT

Exopolysaccharide (EPS) secretion by *Sclerotium rolfsii* ATCC 201126 in submerged cultures, already identified as high-osmolarity responsive, was assessed by reducing C-source without compromising EPS yields. A designed medium with 80 g sucrose L^{-1} ($MOPT_{80}$) was tested at 3 L-bioreactor scale at different temperature, agitation, aeration and pH (uncontrolled *vs*. controlled) values. Optimal operative

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conditions (200 rpm, 28 °C, 0.5 vvm and initial pH -pH_i- 4.5) were validated, as well as the possibility to work at pH_i 5.5 to reduce biomass production. Purified EPSs produced in $MOPT_{80}$ at optimal and other valid operative conditions exhibited refined grade (<1 % proteins and ash, 3–4 % reducing sugars, 87–99 % total sugars). EPS purity, M_W and rheological parameters led to discourage pH controlled at 4.5. Relatively constant M_W ($6–8\times10^6$ Da) and outstanding viscosifying ability were found. Polyphasic EPS analysis (titre, purity, macromolecular features and rheological fitness) would support to properly select production conditions.

Keywords *Sclerotium rolfsii* · Scleroglucan · Practical yield · Operative conditions · EPS properties

1. Introduction

Scleroglucan is a neutral and hydrosoluble β-1,3-β-1,6-glucan secreted by selected *Sclerotium* species. We have already demonstrated that pure (~90–98 %, w/w) *S. rolfsii* ATCC 201126 scleroglucan, at low concentrations (2 g L^{-1}), is able to yield highly viscous solutions with pseudoplastic non-Newtonian behaviour. These solutions are able to retain viscosity at high temperatures (100 °C/60 min) and within a wide range of pH (0–13). Its neutral nature has been related to the ability to keep pseudoplasticity when their solutions are salted with NaCl (up to 20 % w/v), KCl, $CaCl_2$, $MgCl_2$ and $MnCl_2$ (Fariña, Siñeriz, Molina, & Perotti, 2001; Viñarta, Delgado, Figueroa, & Fariña, 2013a).

These outstanding properties reveal the promising potential of scleroglucan for several biotechnological and biomedical applications (Castillo, Valdez, & Fariña, 2015; Giavasis, 2014; Haug & Jonat, 2011; Sletmoen & Stokke, 2008; Survase, Saudagar,

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Bajaj, & Singhal, 2007; Viñarta, François, Daraio, Figueroa, & Fariña, 2007). Particularly in the current global pandemic context and due to its immunomodulatory and immune enhancing activity, the use of β-glucan polysaccharides has been recommended in China (combined with western medical treatment) to treat COVID-19 patients (Cao et al., 2020). Taking into account the relevant spectrum of scleroglucan applications, the possibility to obtain high-quality preparations at bioreactor scale has become a strategic goal.

Concerning culture medium composition for scleroglucan production, it is well known that a high C:N ratio stimulates exopolysaccharide (EPS) biosynthesis (Fariña, Siñeriz, Molina, & Perotti, 1998; Survase et al., 2007). The optimized culture medium used up to date in our laboratory, MOPT, with 150 g sucrose L^{-1} and 2.25 g $NaNO_3$ L^{-1}, led to 26 g scleroglucan L^{-1} (Fariña et al., 1998). However, the high residual sucrose concentration at the end of batch (100 g L^{-1}) led us to further questioning the economic benefits of this strategy.

Besides culture medium, operative conditions also constitute a quite essential issue to control this type of bioprocess. A temperature of 28 °C was reported as optimal for scleroglucan production (Giavasis, Harvey, & McNeil, 2005), whilst the best temperature for biomass production in *S. rolfsii* has been described between 30 and 32 °C (Fariña, Siñeriz, Molina, & Perotti, 1996; Wang & McNeil, 1995a). With regard to pH, batch processes with no automatic control of this parameter and whose initial pH is set around 4–5, use to show a decrease to 2–3 mainly due to oxalate production (Fariña et al., 1998). Despite the implied extra costs for automatic pH control, other authors have yet reported this strategy for scleroglucan production (Tan, Lyu, Zeng, & Zhou, 2019).

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Especially for high-viscosity cultures, the most critical optimization factors tend to be aeration and agitation rates (Giavasis et al., 2005; Survase et al., 2007). Under low stirring speed, scleroglucan could remain adhered to mycelial walls, particularly large biopolymer molecules, whereas very low-M_W EPS is released (Castillo et al., 2015; Rau, Gura, Olszewski, & Wagner, 1992). Contrastingly, very high stirring rates could damage both fungal morphology and biopolymer macromolecular structure (Rau et al., 1992).

Aeration, additionally favours good mixing and does not usually exert negative effects on polysaccharide molecular weight (Giavasis et al., 2005). However, different authors have suggested that a high aeration rate resulted in increased biomass production along with decreased scleroglucan production (Survase et al., 2007). Meanwhile, other reports described that when dissolved oxygen tension (DOT) reached almost zero, the growth was poor but a specific stimulation of scleroglucan production occurred (Rau et al., 1992; Schilling, Rau, Maier, & Fankhauser, 1999). Nevertheless, the stimulating effect of limiting DOT on EPS production may be difficult to understand, since biopolymer synthesis is an energy-dependent process, and in aerobic microorganisms this energy relates to oxygen supply.

Based on the aforementioned reasoning and background, this work first aimed at studying the possibility to reduce culture medium sucrose concentration without compromising scleroglucan production yields by *S. rolfsii* ATCC 201126. Subsequently, the influence of different operative conditions on scleroglucan production and recovery, as well as on EPS characteristics, was also assessed. Results were intended to provide a comprehensive view of the most adequate culture conditions at bioreactor scale for obtaining a satisfactory EPS production in terms of quantity, properties and industrial convenience.

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2. Material and methods

2.1. Strain preservation and activation

S. rolfsii ATCC 201126 was activated in Czapek malt agar medium (containing, in g L^{-1}: malt extract, 40; sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.05; agar, 20; pH 4.5) for 48 h at 30 °C. A mycelium-covered agar plug of the active strain was then transferred to PM_{20} agar medium (containing, in g L^{-1}: sucrose, 20; $NaNO_3$, 3; K_2HPO_4, 1; yeast extract, 1; citric acid$\cdot 1H_2O$, 0.7; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.05; agar, 20; pH 4.5), and incubated for 48 h at 30 °C.

2.2. Influence of sucrose concentration on EPS production yield

Two culture media traditionally used during different stages of scleroglucan research in our laboratory have been PM (with 20 g L^{-1} sucrose, 3 g L^{-1} $NaNO_3$, Fariña et al., 1996), mainly used for inoculum preparation (48 h), and MOPT (with 150 g L^{-1} sucrose, 2.25 g L^{-1} $NaNO_3$, Fariña et al., 1998), for scleroglucan production at bioreactor scale (72 h). Since along these years these two media have been systematically applied for *S. rolfsii* ATCC 201126, in this work different C/N ratios (reported as one of the main factors affecting EPS production) were tested with the basis of each composition, but increasing sucrose concentration.

Therefore, production medium with basal components as PM_{20} except for the C-source was supplemented with increasing concentrations of sucrose: 50, 80 and 120 g L^{-1} (PM_{50}, PM_{80} and PM_{120}, respectively). Identical sucrose concentrations were comparatively tested, but based on the composition of the optimized production medium by Fariña et al. (1998), MOPT (containing, in g L^{-1}: sucrose, 150; $NaNO_3$,

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2,25; $K_2HPO_4 \cdot 3H_2O$, 2; yeast extract, 1; citric acid$\cdot 1H_2O$, 0.7; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.05; pH 4.5), and resulting media were designated as $MOPT_{50}$, $MOPT_{80}$ and $MOPT_{120}$, respectively.

Inocula were prepared from PM_{20} active plates from which, mycelium-covered agar plugs (7 mm-diam.) were used at 10 % ratio in liquid sterile culture medium and were then homogenized with a hand blender. Seed cultures were incubated at 250 rpm and 30 °C for 48 h. After that, they were used to inoculate the different culture media which were incubated at equal conditions for 72 h.

2.3. Operative conditions for scleroglucan production at bioreactor scale

Inocula were prepared and incubated as previously described. After 48 h of cultivation, they were used for inoculating the bioreactor at 10 % v/v. A 5-L stirred-tank reactor fitted with four baffles and two 6-bladed Rushton turbine impellers (New Brunswick Scientific Co.) with a working volume of 3 L of $MOPT_{80}$ culture medium was used. The following conditions were maintained during 72 h for EPS production: air flow rate, 0.5 vvm; stirrer speed, 200 rpm; temperature, 28 °C; initial pH (pH_i) 4.5 - not controlled (taken as basal settings). Additional conditions were also evaluated: temperature, 25 and 31 ºC; stirrer speed, 150 and 250 rpm; air flow rate, 0.25 and 0.75 vvm; initial pH without control, 3.5 and 5.5, and controlled pH at 3.5, 4.5 and 5.5.

In all the studied conditions, samples were periodically taken along cultivation in order to construct kinetic growth and EPS production curves.

2.4. Sample processing

After pH measurement, samples were three-fold diluted with distilled water, homogenized by the use of a hand blender (medium speed, 30 seconds), neutralized

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with either 1 N NaOH or 1 N HCl, and heated to 80 °C for 30 min. Once the processed samples were centrifuged (11,293×*g*, 20 min, 15 °C), the resulting pellets and supernatants were separately recovered, and the following analytical determinations were performed.

For biomass determination, pellets were washed with distilled water and dried at 105 °C until constant weight. The EPS was precipitated by adding ethanol 96 % v/v, (1:1), and after 8 h at 4 °C, it was recovered by vacuum filtration using pre-weighed Whatman No. 1 filter paper and dried at 105 °C until constant weight. From the filtrate, the remaining reducing sugars were determined by the 3,5-dinitrosalicylic acid (DNSA) assay (Miller, 1959).

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2.5. Scleroglucan downstream processing after bioreactor scale production

At the end of cultivation at bioreactor scale, all culture broths were independently recovered and purified according to the complete protocol previously described by Fariña et al. (2001). After 72 h of cultivation, all culture broths were recovered from bioreactor and three-fold diluted with distilled water, hand-blender homogenized, neutralized with 1 N NaOH, and heated to 80 °C for 30 min. After centrifugation (29,774×g, 20 min, 15 °C), the supernatant was collected and precipitated at 1:1 (v/v) proportion with ethanol 96 % v/v (8 h at 4 °C). Precipitated scleroglucan was recovered with the aid of a fine sieve (Macotest ASTM Nº 60). For the purification step, it was re-dissolved in distilled water (400 rpm, 60 °C, 12 h). Once cooled at room temperature, ethanol 96 % v/v (5-8 °C) was added (1:1, v/v) and after good mixing and precipitation, the EPS was recovered as above. Precipitate was then subjected to a second re-dissolution and re-precipitation cycle. Finally, the scleroglucan precipitate was lyophilized and milled.

2.6. Characterization of EPSs obtained at bioreactor scale

2.6.1. Humidity determination

A defined amount of each purified EPS sample (~ 1 g) was placed in pre-weighed aluminium trays and left to dry at 105 °C until constant weight. All samples were analysed in triplicate.

2.6.2. Ash determination

Around one gram of each dry EPS was weighed and placed in previously tared crucibles. Subsequently, it was subjected to an incineration temperature of 600 °C for 30 min. Determinations were carried out in duplicate.

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2.6.3. EPSs solutions preparation

Humidity content of each EPS sample was used to correct the amount required for EPS stock solutions at 2 g L^{-1}. The EPSs were hydrated at 25 °C (24 h, magnetic stirring). Subsequently, temperature was increased (60 °C) and stirring continued until complete dissolution.

2.6.4. EPSs centesimal composition

Reducing sugars content was quantified by DNSA (Miller, 1959). Total sugars content was estimated according to the phenol-sulfuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein determination was performed using a BIO-RAD® Bradford reagent according to the manufacturer's instructions (Bradford, 1976). All results are average from triplicate determinations and were expressed as percent (%, w/w).

2.6.5. Relative molecular weight estimation

Molecular mass of pure freeze-dried EPSs was determined by gel permeation chromatography with a Gilson HPLC system equipped with a Waters Ultrahydrogel linear column (7.8 mm × 30 cm; range 500–8×10^6 Da; Sigma, St. Louis, Mo.) with 0.1 M $NaNO_3$ as the eluent at a flow rate of 0.6 mL min^{-1} (Mozzi et al., 2006). Calibration (r^2 = 0.997) was performed using narrow-band pullulan standards (Shodex Standard P-82, Lot No. 30901, Showa Denko, 5650–710500 g/mol) as previously described (Viñarta et al., 2013b), dissolved (0.5 mg mL^{-1}) in the mobile phase (0.1 M $NaNO_3$) and filtered (0.45 μm) prior to injection. Scleroglucan solutions (2 mg mL^{-1}) were prepared in the mobile phase according to the protocol above (Section *2.6.3*) and injected (20

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μL). Polysaccharides were detected with a Gilson RI 132 differential refractive index detector, and determinations were carried out in triplicate.

2.6.6. Apparent viscosity measurements

Rheological properties of EPS aqueous solutions were assessed by using a rotational viscometer Myr VR 3000 with an APM-001 small sample adapter, equipped with a TL5 spindle at shear rates (γ) ranging from 0.396 to 79.2 s^{-1} (25 °C). For equipment calibration, a known viscosity standard (Cannon® certified viscosity standard for rotational viscometers) was used.

Based on the scleroglucan pseudoplastic behaviour, the Ostwald-de-Waele rheological model ($\eta = K \cdot \gamma^{(n-1)}$) was applied for each sample measurement dataset in order to determine the values of K (consistency coefficient) and n (flow behaviour index) parameters, which allowed EPSs comparisons (Fariña et al., 2001).

3. Results and discussion

There is not a general rule or established sequence for bioreactor production optimization; it depends on the process under consideration. EPS production processes, in addition, are particularly complex because different factors require especial care: shear stress, type of agitation and aeration, bioreactor configuration and stagnant zones, etc. Moreover, if EPS is produced by fungi, production yields are normally related to a given morphology, which may vary with bioreactor design and operative conditions (Seviour, McNeil, Fazenda, & Harvey, 2011).

In this study, optimization started with sucrose concentration with the aim of reducing the costs and increasing the efficiency of the process, with no detriment of EPS production yields. In second place, EPS-production optimal temperature was

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studied based on the previous physiological knowledge about the strain (Castillo et al., 2015; Fariña et al., 1996). Considering this background, the interlaced relationship between biomass and EPS production, and that EPS is a result of enzyme-driven biosynthetic machinery, temperature was evaluated to select optimal conditions. Subsequently, since viscous fermentations are particularly affected by agitation, this aspect deserved especial attention, as it relates to good mixing, heat and mass transfer phenomena and homogenization. Once optimal stirring speed was set, aeration became the focus of attention, as controversial results have appeared in the literature concerning this condition and its influence (Rau et al., 1992; Wang & McNeil, 1995c). Finally, since some reports suggested the use of either initial pH set points different from 4.5 or controlled-pH strategies (Tan et al, 2019; Wang & McNeil, 1995b) for increasing EPS production, the incidence of pH was also investigated.

3.1. Influence of sucrose concentration on EPS production and yield values at shake-flask scale

Fariña et al. (1998) previously reported the highest scleroglucan production by *S. rolfsii* ATCC 201126 when using 150 g sucrose L^{-1}. However, since practical yields ($Y_{p/C\ added}$) tended to be reduced because of the high remaining sucrose concentration in culture broths, an interest in reducing the medium initial sucrose concentration arose. Therefore, with the aim of achieving a best balance between EPS titres and C-source harnessing, different sucrose concentrations were herein tested.

As displayed in **Table 1**, within the same group of culture media, either PM or MOPT, a tendency to obtain higher EPS titres as the C-source concentration increased was observed, supporting the fact of high osmolarity as a positive effector for scleroglucan biosynthesis. Since EPS production did not proportionally augmented as

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sharply as the steep in the added sucrose, this turned into lower practical yields ($Y_{p/C_{added}}$).

Regarding growth in both culture media (PM or MOPT), biomass overtook EPS production at the lowest C level (50 g sucrose L^{-1}). Above this concentration, values were statistically similar for the different media and sucrose concentrations tested. In all cases, pH values after 72 h of culture were very similar ($P>0.05$, Tukey's test).

The use of lower C-source concentrations without significantly compromising EPS production was the main selection premise at this stage. Considering the range of highest EPS concentrations that were statistically similar ($P>0.05$, Tukey's test), culture media leading to the highest EPS production at lowest sucrose incorporation were selected (PM_{80} and $MOPT_{80}$). Thereafter, for the same sucrose concentration (80 g L^{-1}), the culture medium with the lowest N-source concentration was chosen: i.e. $MOPT_{80}$ with 2.25 g $NaNO_3$ L^{-1} *vs*. PM_{80} with 3 g $NaNO_3$ L^{-1}.

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Table 1

Influence of sucrose concentration on growth and EPS production by *S. rolfsii* ATCC 201126

Production media	**Final pH**	**Biomass (g L^{-1})**	**EPS (g L^{-1})**	**$Y_{p/C\ added}$*** **(%)**
PM_{50}	2.87 ± 0.06[a]	11.44 ± 0.88[a]	9.34 ± 1.15[b]	18.68
PM_{80}	2.84 ± 0.11[a]	12.10 ± 0.61[a]	13.05 ± 0.49[a]	16.31
PM_{120}	3.20 ± 0.12[a]	12.71 ± 0.44[a]	16.11 ± 1.13[a]	13.42
$MOPT_{50}$	3.22 ± 0.12[a]	12.88 ± 1.67[a]	9.72 ± 1.08[b]	19.44
$MOPT_{80}$	3.22 ± 0.32[a]	13.17 ± 1.60[a]	12.64 ± 1.29[a]	15.80
$MOPT_{120}$	3.13 ± 0.08[a]	12.02 ± 0.78[a]	14.49 ± 1.07[a]	12.07

Values (at shake-flask scale, 200 rpm, 30 ºC for 72 h of cultivation) are mean of three independent assays ± standard deviation (SD). For the same column, different superscripts indicate significant differences ($P<0.05$, Tukey´s test). * $Y_{p/C\ added}$: grams of EPS produced per gram of added C-source (practical yield).

Finally, as compared to previous reports by Fariña et al. (1998) with the same strain ($Y_{p/C\ added}$ = 17 %, with 150 g sucrose L^{-1}), the herein presented results would support the reduction in initial sucrose concentration around by half (80 g L^{-1}) without seriously compromising the practical EPS yield (~ 16 %, $MOPT_{80}$). On the other hand, results from Survase, Saudagar, & Singhal (2006) showed the ability of *S. rolfsii* MTCC 2156 strain to achieve higher EPS yield (21 %) at 80 g sucrose L^{-1}, but working at a higher N-source concentration (3 *vs*. 2.25 g $NaNO_3$ L^{-1} in $MOPT_{80}$). Unsurprisingly, strain-responsive variability would be a common fact when evaluating scleroglucan production optimization.

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3.2. Evaluation of operative conditions for scleroglucan production at bioreactor scale

3.2.1. Basal operative conditions

The $MOPT_{80}$ culture medium was selected to evaluate EPS production at bioreactor scale under basal operative conditions: 28 °C, 200 rpm, 0.5 vvm, pH_i 4.5 - without control. As expected, and mimicking the behaviour at shake-flask scale, the kinetic of EPS production (**Fig. 1**) exceeded biomass production almost throughout the 72 h of cultivation.

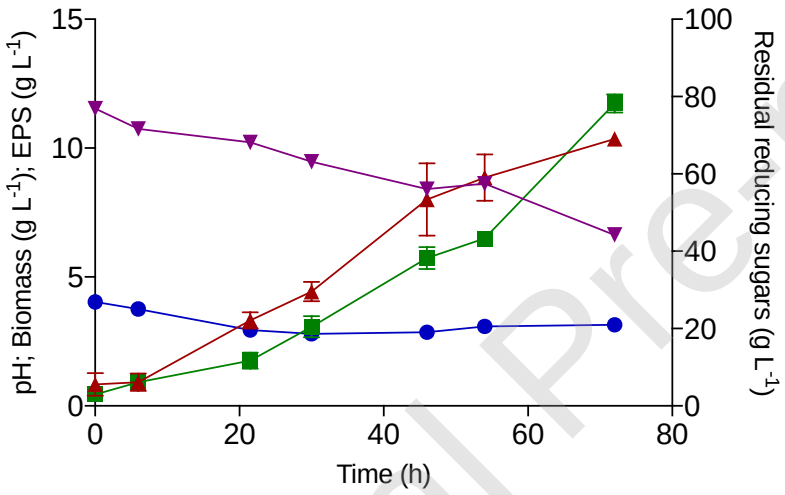

Fig. 1. Kinetics of growth and EPS production by *S. rolfsii* ATCC 201126 at bench-top bioreactor scale (3 L-working volume) in $MOPT_{80}$ culture medium. Basal operative conditions were set to: 0.5 vvm, 28 °C, 200 rpm, pH_i 4.5 (not controlled). ● pH; ■ Biomass; ▲ EPS; ▼ Residual reducing sugars.

The highest values for EPS and biomass production at 72 h of cultivation were 10.36 and 11.74 g L^{-1}, respectively, whilst the practical yield ($Y_{p/C\ added}$) reached 12.95 %. Both EPS production and yield were lower as compared to those observed at shake-flask scale (**Table 1**). This behaviour at different scales has been previously documented (Seviour et al., 2011).

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3.2.2. Influence of cultivation temperature

At 72 h, EPS production values at 28 (basal: 10.36 g L^{-1}, **Fig. 1**) and 31 °C (10.34 g L^{-1}, **Fig. 2-B**) did not show statistically significant differences (P>0.05, Tukey's test). However, when temperature was reduced to 25 °C (**Fig. 2-A**), EPS production became drastically reduced to 5.49 g L^{-1}. Concerning growth, biomass at 31 °C (**Fig. 2-B**) was 13.65 g L^{-1}, a value significantly higher (P<0.05, Tukey's test) than those obtained at 28 °C (basal: 11.74 g L^{-1}, **Fig. 1**) and at 25 °C (11.25 g L^{-1}, **Fig. 2-A**). This fact indicated that higher temperature would preferentially favour growth of *S. rolfsii* ATCC 201126.

At 25 °C, in spite of the fact that fungal growth reached values similar to those obtained at 28 °C, the greater increase in biomass concentration occurred between 41–48 h, whilst at the higher tested temperatures (28 and 31 °C), the most significant increase in biomass reasonably took place earlier: between 21–30 h at 28 °C (**Fig. 1**) and 22–35 h at 31 °C (**Fig. 2-B**). These results are clearly related to a lower growth rate and a slower metabolism when cultivation is performed at 25 °C. At sub-optimal incubation temperature (25 ºC, **Fig. 2-A**) most of the generated energy was likely directed towards its own growth, with an increased biomass at the expense of a stopped EPS production.

Fungal growth contributes *per se* to the viscosity of culture broth, thus developing a pseudoplastic non-Newtonian behaviour (Fariña et al., 2001; Gibbs, Seviour, & Schmid, 2000). Then, for EPS recovery, a lower biomass concentration might ease polymer separation, meaning lower dilutions and therefore lower solvent expenses (Fosmer & Gibbons, 2011). Based on this above rationale, 28 °C was selected as the suitable temperature for scleroglucan production by *S. rolfsii* ATCC 201126 at bioreactor scale. Similar findings were reported by Wang & McNeil (1995a) and Giavasis et al. (2005).

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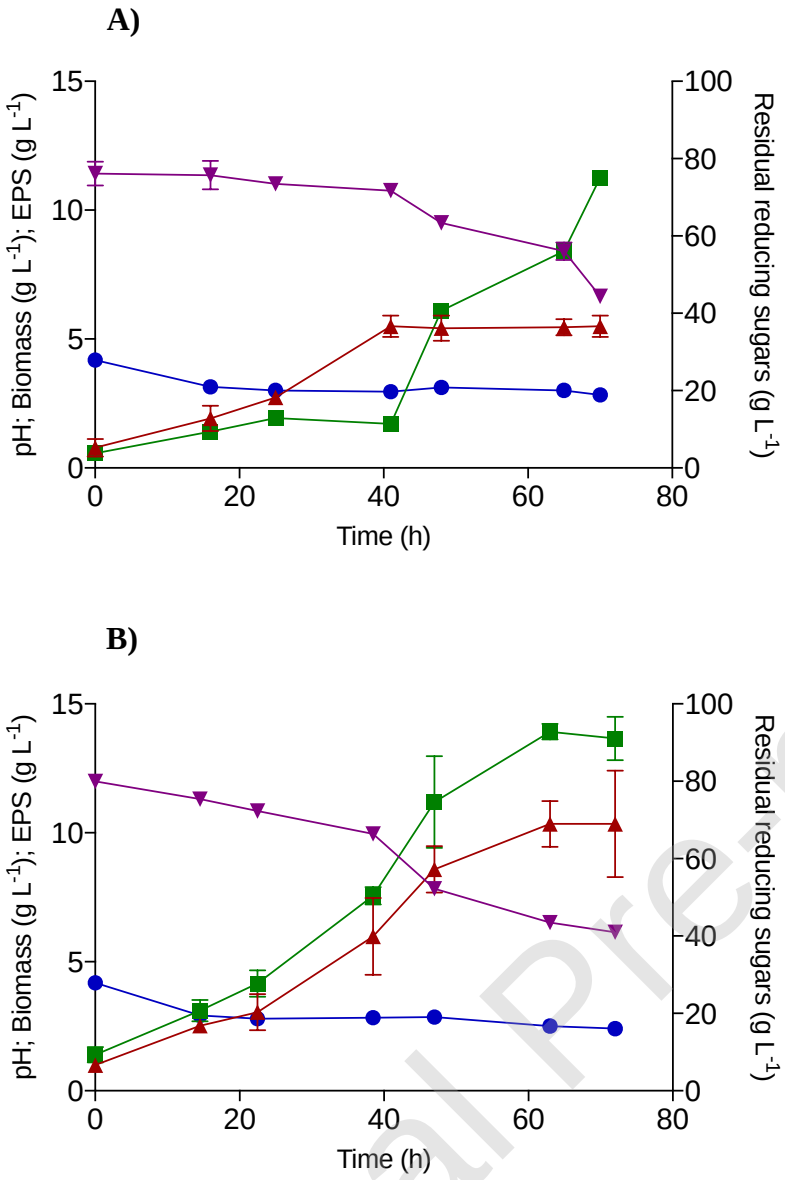

Fig. 2. Kinetics of growth and EPS production by *S. rolfsii* ATCC 201126 at bench-top bioreactor scale (3 L-working volume) in $MOPT_{80}$ culture medium, at different incubation temperatures: 25 ºC **(A)** and 31 ºC **(B)**. Further operative conditions were set to: 0.5 vvm, 200 rpm, pH_i 4.5 (not controlled). ● pH; ■ Biomass; ▲ EPS; ▼ Residual reducing sugars.

3.2.3. Influence of stirring speed

Our evidences up to date indicate that a critical and delicate balance should be maintained concerning agitation rate. A 200-rpm stirring speed, selected as basal condition (**Fig. 1**) and traditionally used in our previous works (Valdez, Babot, Schmid, Delgado & Fariña, 2019), was herein compared to 150 and 250 rpm (**Fig. 3**).

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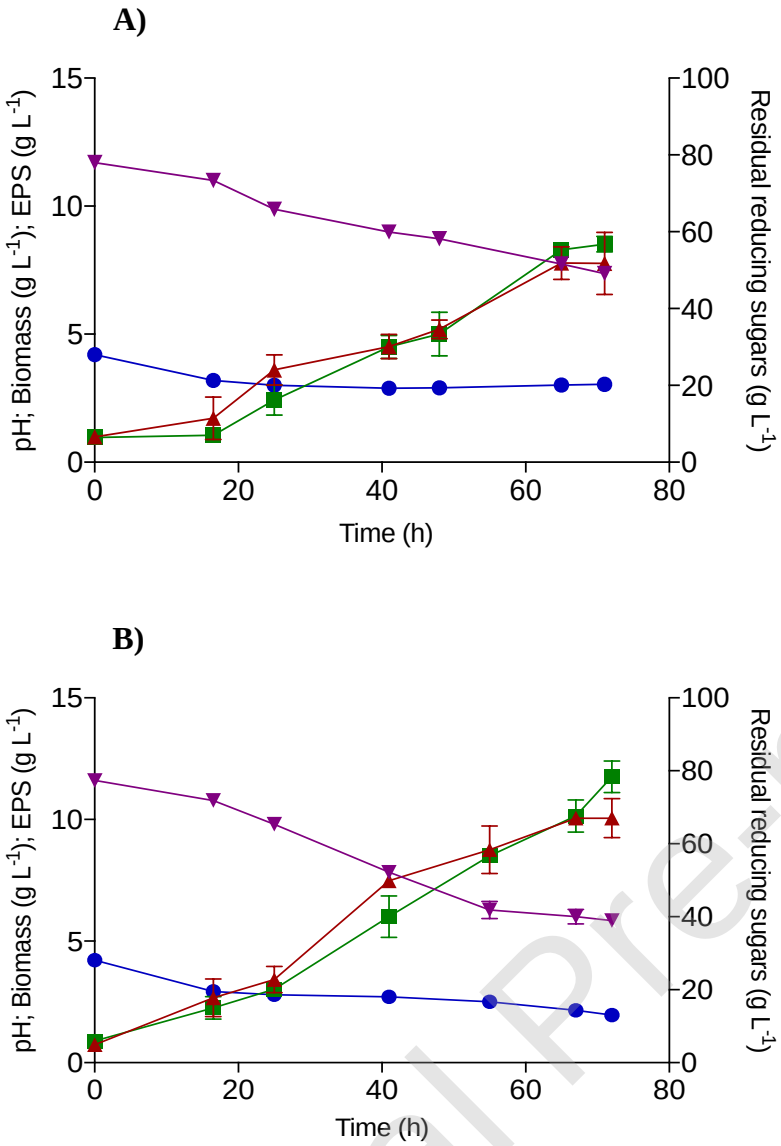

Fig. 3. Kinetics of growth and EPS production by *S. rolfsii* ATCC 201126 at bench-top bioreactor scale (3 L-working volume) in $MOPT_{80}$ culture medium, at different stirring rates: 150 rpm **(A)** and 250 rpm **(B)**. Further operative conditions were set to: 0.5 vvm, 28 °C, pH_i 4.5 (not controlled). ● pH; ■ Biomass; ▲ EPS; ▼ Residual reducing sugars.

Even when a higher agitation speed might be thought as a mean to improve mixing and consequently, growth and EPS production, our results did not show any progress. At 250 rpm (**Fig. 3-B**), both biomass (11.75 g L^{-1}) and EPS production (10.05 g L^{-1}) at 72 h were similar to the basal condition values (**Fig. 1**). High stirring rates may usually lead to degradation of biopolymer macromolecular structure (Rau et al., 1992;

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Survase et al., 2007). For this reason, although titres might be statistically similar, the EPS quality was also evaluated (see section *3.2.6.*).

Residual reducing sugars at the endpoint were lower at 250 rpm (38.94 g L^{-1}) (**Fig. 3-B**) than at the 200-rpm basal condition (44.20 g L^{-1}) (**Fig. 1**). Likewise, the final pH of broth also achieved a significant lower value (1.96 at 250 rpm, **Fig. 3-B**) than the control (3.15 at 200 rpm, **Fig. 1**). This might mean a C-source deviation which, under good aeration conditions, could derive from the stimulation of oxalic acid biosynthesis (Schilling, Henning, & Rau, 2000; Wang & McNeil, 1994).

Conversely, reducing the stirring speed to 150 rpm (**Fig. 3-A**), values of 8.52 g biomass L^{-1} and 7.77 g EPS L^{-1} were reached, both being lower and significantly different from those achieved at 200 rpm ($P<0.05$, Tukey's test).

Summarizing, since the highest EPS production values were reached at both 200 and 250 rpm with no statistically significant differences ($P<0.05$, Tukey's test), the basal 200-rpm condition was selected because of the lower energy demands and costs.

3.2.4. Effect of aeration rate

Both tested alternative aeration rates, 0.25 (**Fig. 4-A**) and 0.75 vvm (**Fig. 4-B**), unexpectedly led to lower growth and EPS production at 72 h of culture, as compared to the basal condition (0.5 vvm, **Fig. 1**).

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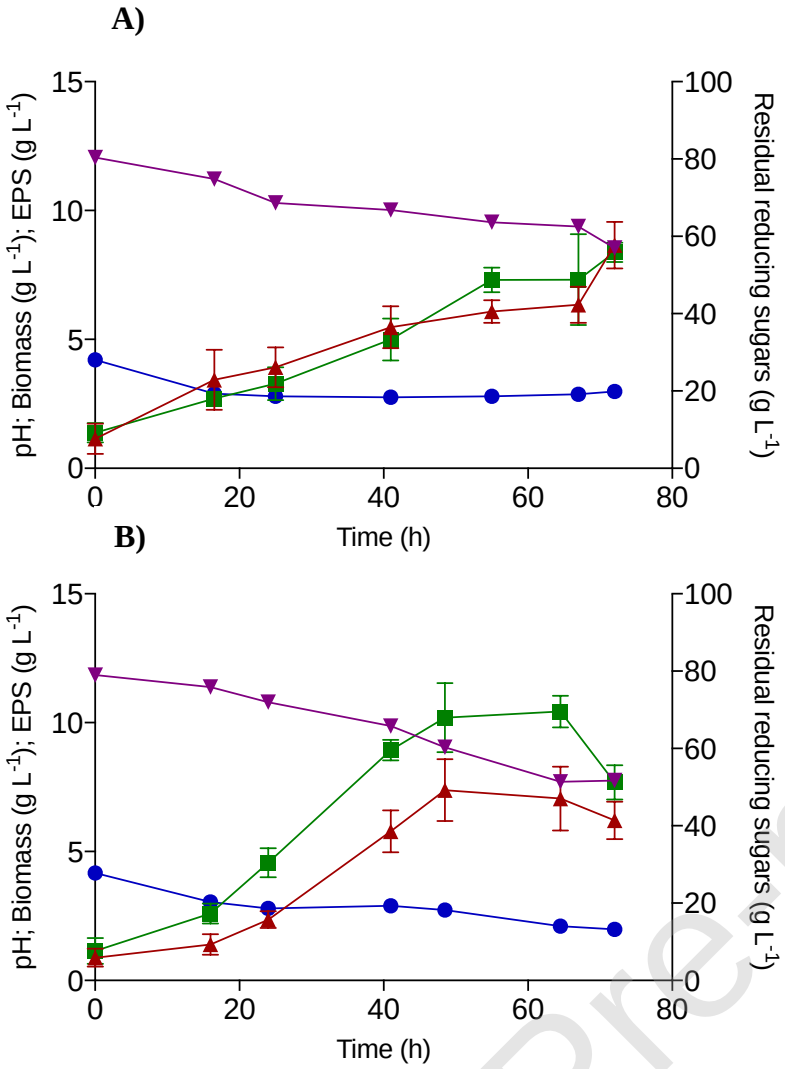

Fig. 4. Kinetics of growth and EPS production by *S. rolfsii* ATCC 201126 at bench-top bioreactor scale (3 L-working volume) in $MOPT_{80}$ culture medium, at different airflow rates: 0.25 vvm **(A)** and 0.75 vvm **(B)**. Further operative conditions were set to: 200 rpm, 28 °C, pH_i 4.5 (not controlled). ● pH; ■ Biomass; ▲ EPS; ▼ Residual reducing sugars.

At 0.25 vvm (**Fig. 4-A**) a decrease in biomass and EPS values (8.38 g L^{-1} and 8.66 g L^{-1}, respectively) was achieved. As previously reported, a lower DOT has been already linked to limited fungal growth (Rau et al., 1992; Schilling et al., 2000; Wang & McNeil, 1995c). These reports also indicated that limited oxygen might stimulate EPS production, which would be contrary to the results herein described. However, since EPS production has been found to start before DOT levels reach limiting values, it becomes unlikely that oxygen limitation triggers scleroglucan biosynthesis by itself (Castillo et al., 2015; Wang & McNeil, 1995c).

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On the other hand, at 0.75 vvm (**Fig. 4-B**), biomass concentration fell to 7.68 g L^{-1} at the end of batch. This result could be related to reports on *Cordyceps militaris* by Park et al. (2002), who observed biomass morphological changes at different aeration rates that were also related to lower EPS production. However, further studies will be necessary to clarify this aspect for *S. rolfsii* ATCC 201126. Also, EPS showed a moderate drop to 6.21 g L^{-1} at the end of cultivation. A high shear stress at higher aeration rate may have led to EPS fragmentation, thus hampering its normal alcohol-precipitation and recovery. Finally, the low final pH (1.98) reached at 72 h, could imply a C-source derivation towards organic acids production under high oxygen transfer environments (Schilling et al., 2000).

Therefore, the understanding of the influence of dissolved oxygen level in an EPS-producing culture involves certain complexity as it can affect both growth rate and fungal morphology, and these latter have their own impact on the synthesis of metabolites like EPSs (Reddy, Reddy, Pillay, & Singh, 2002; Tang & Zhong, 2003).

This intricate relationship which seems to also depend on the producing microorganism leaves no place for generalization and makes this parameter to require standardization for each particular production process. In this work, analysing all the above-mentioned results and considerations, an aeration rate of 0.5 vvm was selected to continue EPS production at bioreactor scale.

3.2.5. Effect of initial pH

3.2.5.1 Shake-flask scale

Fungal growth and EPS production at different initial pH values are shown in **Fig. 5**. Although biomass and EPS did not show statistically significant differences between pH 3.0–5.5 and 3.5–5.5, respectively ($P>0.05$, Tukey's test), pH_i values between 4.5 and

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5.5 showed the highest EPS titres (~ 10 g L^{-1}), and a tendency to lower biomass production was found beyond pH_i 4.0. Thus, at pH_i 5.5, EPS was shown to surpass biomass concentration, a condition later evaluated in this work as an operational and economic alternative for the process. Meanwhile, at pH_i = 6.0, a sharp drop in both fungal growth and EPS production was observed.

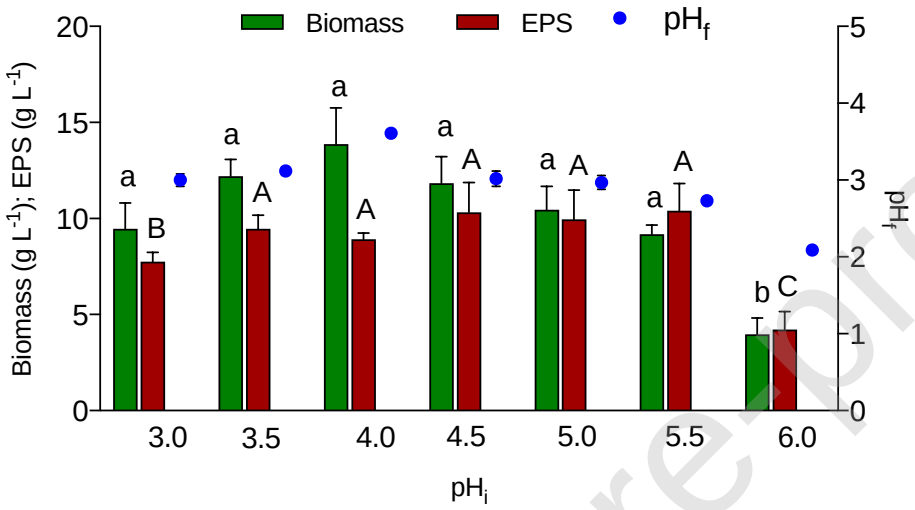

Fig. 5. Growth, EPS production and final pH values at the endpoint (72 h) of scleroglucan production by *S. rolfsii* ATCC 201126 in $MOPT_{80}$ culture medium, at different initial pH values at shake-flask scale. Different lowercase letters (biomass) or uppercase letters (EPS) indicate significant differences ($P<0.05$, Tukey´s test).

The final pH of cultures was also analysed (**Fig. 5**) and a trend towards higher broth acidification as initial pH increased was observed. In agreement, Maxwell (1968) and Wang & McNeil (1994) also described that oxalic acid accumulation in *Sclerotium* genus took place at highest initial pH values.

3.2.5.2 Bioreactor scale

Based on the previous results at shake-flask scale, the effect of setting the initial pH value at 5 (**Fig. 6-A**) and 5.5 (**Fig. 6-B**) without control, at bioreactor scale, was studied. At pH 5.5 (**Fig. 6-B**), the EPS value at 72 h (9.80 g L^{-1}) surpassed biomass

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concentration (6.22 g L^{-1}). This behaviour was similar to the one previously found at smaller scale (**Fig. 5**), and it was consistently observed throughout the production process. On the other hand, at pH_i 5.0 without control (**Fig. 6-A**), biomass values slightly exceeded EPS production, reaching 9.12 g EPS L^{-1} and 11.06 g biomass L^{-1} at the end of process.

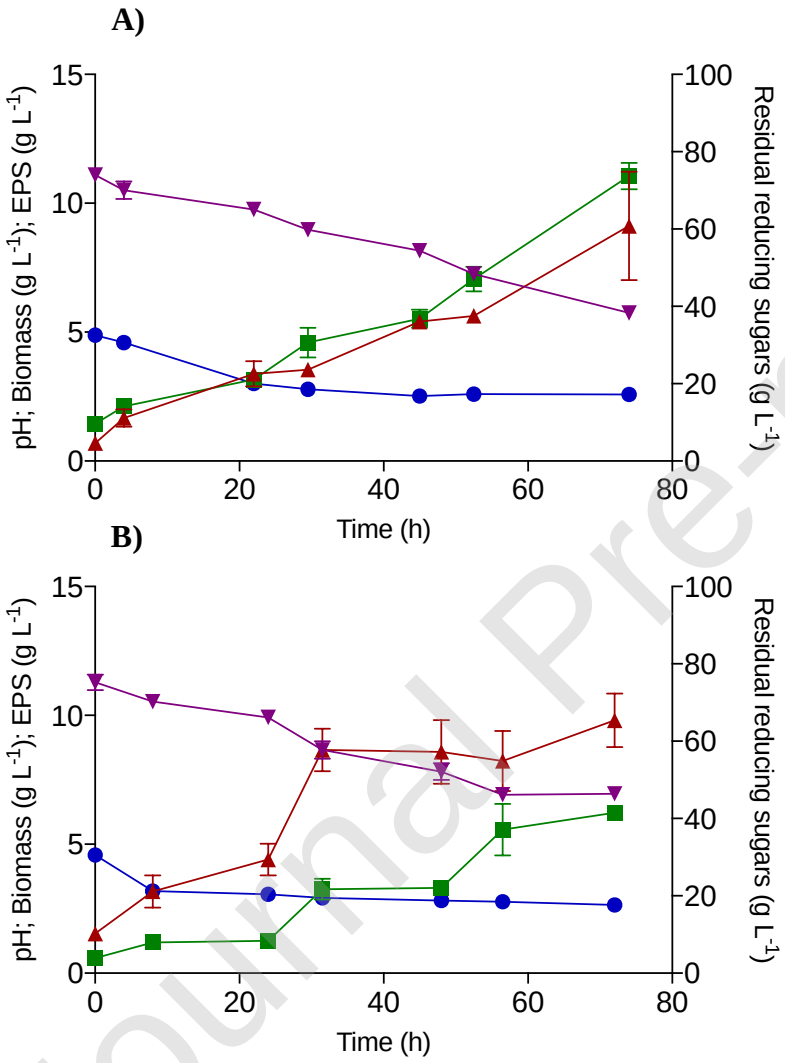

Fig. 6. Kinetics of growth and EPS production by *S. rolfsii* ATCC 201126 at bench-top bioreactor scale (3 L-working volume) in $MOPT_{80}$ culture medium, at pH_i 5 **(A)** and pH_i 5.5 **(B)**, both without control. Further operative conditions were set to: 200 rpm, 28 °C, 0.5 vvm. ● pH; ■ Biomass; ▲ EPS; ▼ Residual reducing sugars.

Although EPS production at bioreactor scale, both at pH_i 5 and 5.5 without control (**Fig. 6-A,B**), was not statistically different to the one reached under basal

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conditions at pH_i 4.5 not controlled (10.36 g L^{-1}, **Fig. 1**), a difference in the final biomass value was observed. At the endpoint, when cultured at pH_i 5.5, biomass was significantly lower (6.22 g L^{-1}) than that obtained under basal conditions (11.74 g L^{-1}, at pH_i 4.5). Therefore, scleroglucan production starting at pH_i 5.5 with no control appeared as a promising alternative to the traditional process (pH_i 4.5), because of the operational advantage of producing less biomass.

In contrast, other authors have recommended working at controlled pH in order to improve scleroglucan production (Tan et al., 2019; Wang & McNeil, 1995b). To check this strategy, different controlled pH conditions (3.5, 4.5 and 5.5) were also tested. The corresponding results are displayed in **Fig. 7**.

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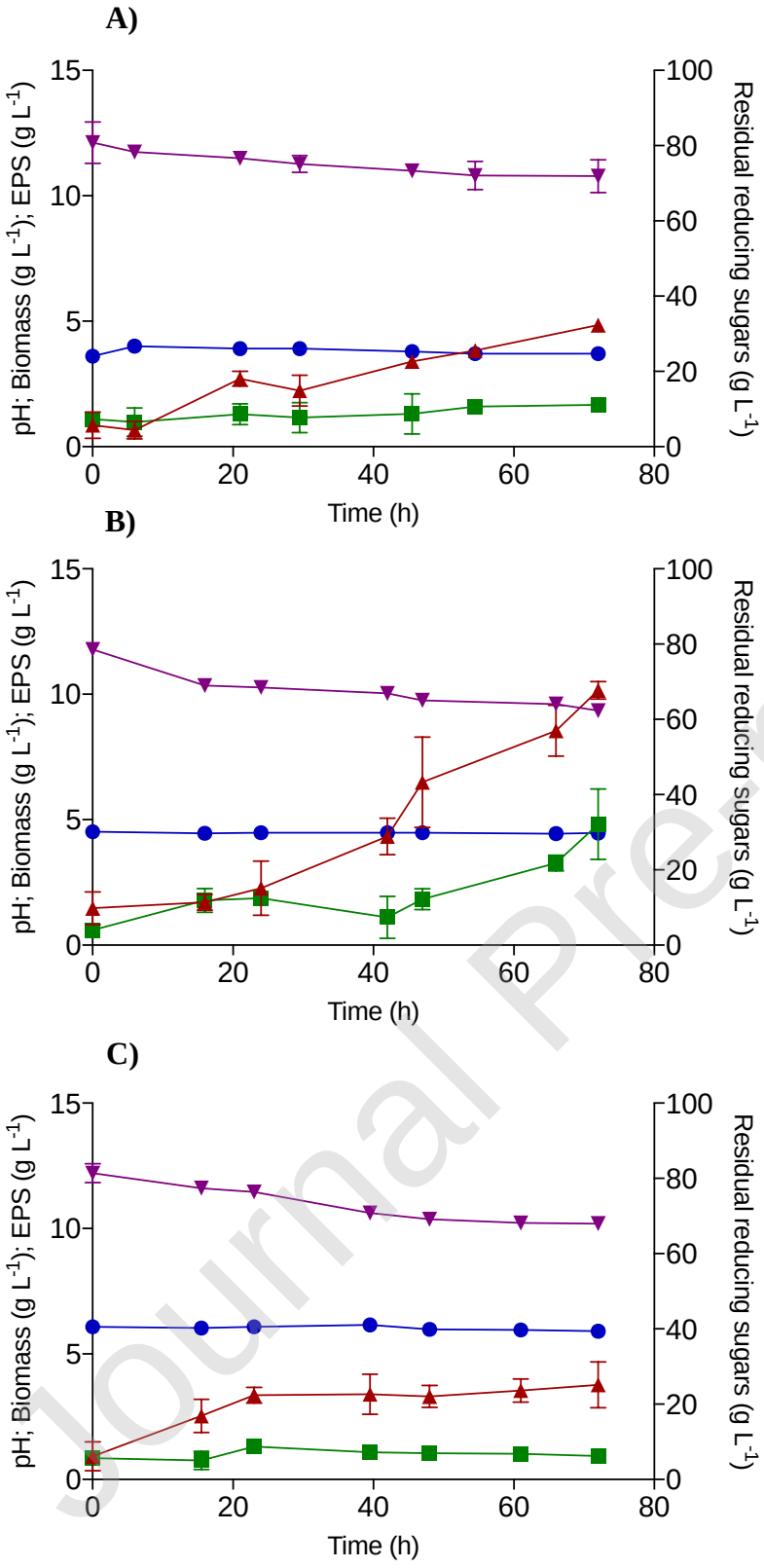

Fig. 7. Kinetics of growth and EPS production by *S. rolfsii* ATCC 201126 at bench-top bioreactor scale (3 L-working volume) in $MOPT_{80}$ culture medium, under controlled pH conditions set to 3.5 **(A)**, 4.5 **(B)** and 5.5 **(C)**. Further operative conditions were set to: 200 rpm, 28 °C, 0.5 vvm. ● pH; ■ Biomass; ▲ EPS; ▼ Residual reducing sugars.

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Growth kinetics and EPS production both with pH controlled at 3.5 (**Fig. 7-A**) and 5.5 (**Fig. 7-C**), showed a similar behaviour: very low biomass (1.67 g L^{-1} and 0.93 g L^{-1}, respectively) and low EPS production (4.85 g L^{-1} and 3.77 g L^{-1}, respectively).

In the literature, results regarding pH effect on scleroglucan production can be contradictory, even using strains of the same genus or species (Tan et al., 2019; Wang & McNeil, 1995b). For this reason, optimization of process parameters should be performed for each selected strain.

In the present work, when pH was controlled at 4.5 (**Fig. 7-B**), the EPS concentration at 72 h was 10.15 g L^{-1} and showed no statistically significant differences ($P>0.05$, Tukey test) with respect the basal condition at pH_i 4.5 not controlled (**Fig. 1**). Meanwhile, the achieved biomass at fixed pH 4.5 (**Fig. 7-B**) was 4.82 g L^{-1}, widely lower than that obtained under not controlled pH conditions.

Therefore, controlling pH at 4.5 showed to have more impact on growth than on EPS production. The strategy of controlled pH at 4.5 (**Fig. 7-B**) did not lead to improvements on EPS production by *S. rolfsii* ATCC 201126, as compared to the batches starting at pH_i 4.5 (**Fig. 1**) and pH_i 5.5 (**Fig. 6-B**), both without pH control. It might yet be noted that fixed pH at 4.5 could facilitate EPS recovery because of the low biomass obtained, as previously discussed. However, since controlling pH also involves extra costs and further handling of the batch with potential contamination risks, the attractiveness and competitiveness of this strategy should be weighed, including the surveillance of EPS quality and performance. This latter aspect will be subsequently discussed.

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3.2.6. Centesimal composition, M_W and rheological properties of purified scleroglucans

Grinding process, which was performed after lyophilisation, allows the sample to be obtained as a fine powder, which turns into improved dissolution properties by increasing the contact surface with solvent. However, milling is not expected to affect macromolecular properties since it is carried out on the dry product at a macroscopic level. It is known that in a process for producing a particulate polysaccharide, the median length of the particles after dry-grinding is controlled by controlling the moisture content of the polysaccharide.

In the present work, humidity percentage of purified EPSs oscillated between 7.43 and 12.18 %, while protein and ash contents were less than 1 % (w/w) in all cases (**Table 2**). The amount of reducing sugars was around 3–4 % (w/w), which represents an expected value for scleroglucans from *S. rolfsii* ATCC 201126 (Viñarta, Yossen, Vega, Figueroa, & Fariña, 2013b).

The total sugars content of EPSs was in most cases between 85 and 99 % (w/w) (**Table 2**). This fact revealed a high purity degree, which according to the literature, could be industrially classified as a refined-grade polysaccharide (Survase et al., 2007). Furthermore, these values were higher than those reported for other commercial scleroglucans (Castillo et al., 2015; Survase et al., 2007).

Routinely, an aliquot from scleroglucan solutions is checked at the microscope. Although the appearance is usually very clear and translucent, small fungal fragments may eventually be observed, particularly because after broth homogenization, these fragments could be not completely settled down at the applied centrifuge speed (even set at maximum). The proportion of hyphal fragments, when found, is very low, and are visualized in a dehydrated state, which is expectable after the intensive broth treatment (homogenization, heating), centrifugation, and subsequent alcohol precipitation, re-

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dissolution and re-precipitation, a sequence which is normally repeated twice. The mentioned steps thus would normally lead to infimum non-viable fungal rests, along with a refined-grade polysaccharide.

Scleroglucan M_W values were in the range of 3.31–8.84×10^6 Da (**Table 2**) and showed a good correlation with the value of 5×10^6 Da previously reported for scleroglucan produced by *S. rolfsii* ATCC 201126 in $MOPT_{150}$ at fermenter scale (Fariña et al., 2001). Differing from reports by Schilling et al. (1999) for scleroglucan from *S. rolfsii* ATCC 15205, our results showed that EPS molecular mass was not markedly affected by increasing the stirring speed, whilst aeration rate seemed to have more impact on the EPS quantity than on its quality (**Table 2**).

Several scleroglucan industrial applications depend on its rheological properties, which are directly influenced by its purity, M_W and conformational features. In this work, rheological parameters (*K*, *n*) kept quite stable for EPSs from different conditions, except for controlled-pH strategies (**Table 2**), and similar to those already reported for other scleroglucan samples from *S. rolfsii* ATCC 201126 (Fariña et al., 2001; Viñarta et al., 2013a,b).

On the other hand, the scleroglucan recovered from the pH 4.5-controlled batch, showed both lowest purity value and M_W, accompanied by inferior rheological parameters (lowest *K* and highest *n* values) (**Table 2**). Therefore, despite the amount of recovered EPS at the end of cultivation was similar to that of the basal condition (pH_i 4.5, uncontrolled), this cultivation strategy already reported by other authors (Tan et al., 2019; Wang & McNeil, 1995b), would not be suitable for obtaining a high-quality polysaccharide in *S. rolfsii* ATCC 201126. According to biopolymer M_W and polydispersity index (P_i, Table 2), cultivation at 31 °C also showed to be not suitable.

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Table 2

Maximal EPS production, centesimal composition, relative molecular weight (M_W) and rheological parameters of aq. solutions (2 g L^{-1}) of purified scleroglucans synthesized by *S. rolfsii* ATCC 201126 after 72 h of culture in $MOPT_{80}$ under different bioreactor operative conditions

Operative conditions	Max. EPS (g L^{-1})*	Ash % (w/w)	Red. sugars	Total sugars	Protein	Humidity	M_W - P_i (g mol^{-1}) - (adim.)	Consistency coefficient *K* (mPa·s^n)	Flow behaviour index *n* (adimensional)
Control#	10.36	0.21	3.51	95.73	0.03	10.60	8.31×10^6 - 1.09	1564	0.13
25 °C	6.21	0.30	3.50	87.11	0.04	11.00	7.81×10^6 - 1.07	1733	0.12
31 °C	10.34	0.15	3.85	92.54	0.06	9.27	3.76×10^6 - 1.20	1769	0.13
150 rpm	7.77	0.31	3.90	99.57	0.06	12.18	8.84×10^6 - 1.16	1557	0.13
250 rpm	10.05	0.06	3.36	87.85	ND	7.43	7.22×10^6 - 1.09	1799	0.13
0.25 vvm	8.66	0.19	3.35	86.79	0.06	10.00	7.93×10^6 - 1.15	1492	0.13
0.75 vvm-	6.21	0.18	3.35	87.53	0.01	10.48	7.93×10^6 - 1.07	1652	0.15
pH=3.5§	4.85	0.79	3.88	84.23	0.17	10.61	6.98×10^6 - 1.10	1367	0.14
pH=4.5§	10.15	0.60	4.10	74.12	0.04	9.75	3.31×10^6 - 1.13	885	0.19
pH=5.5§	3.77	0.23	3.42	90.62	0.09	9.25	6.69×10^6 - 1.10	1342	0.18
pH_i 5.0†	9.12	0.13	3.99	99.46	0.19	11.21	6.38×10^6 - 1.11	1590	0.17
pH_i 5.5†	9.80	0.49	3.45	98.10	0.16	12.06	6.98×10^6 - 1.11	1787	0.13

* results at 72 h of cultivation #corresponding to basal operative conditions: 200 rpm, 0.5 vvm, 28 °C, pH_i 4.5 (not controlled) ND: not detectable with the method used. P_i: polydispersity index (adim., adimensional). Values of *K* and *n* were estimated after fitting apparent viscosity measurements at 25 °C *vs*. shear rate (γ) (see *Material and methods*) to the Ostwald-de-Waele rheological model (r^2 = 0.999). § controlled pH † not controlled pH. Standard errors were all below 10%.

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Scleroglucan susceptibility to alkaline treatments (> 0.15-0.2 N NaOH) has been already demonstrated (Fariña et al., 2001; Viñarta et al., 2013a), and it particularly depends on polysaccharide conformational features and coiling degree. According to the microenvironment alkalinisation magnitude during pH adjustment in bioreactor, the effect of OH^- anion could mainly affect scleroglucan interchain H-bonds, thus promoting partial openings up to denaturation, with the consequent loss of pseudoplasticity, particularly on more expanded macromolecules. Thus, the persistent addition of NaOH to keep pH controlled at 4.5, helped by high viscosity, may have generated alkaline microenvironments where pH surpassed this critical denaturation point, thus giving rise to a polymer of lower quality (**Table 2**). This will probably deserve further study as to interpret the observed behaviour.

Based on the above considerations, it seems necessary to emphasize that each production process requires a polyphasic evaluation, where not only the amount of recovered EPS and production costs are considered, but also the quality and features of the purified EPSs from different nutritional, operative and downstream processing conditions. As previously emphasized by other authors (Sletmoen & Stokke, 2008) there are some apparent inconsistencies in the literature which resulted from the lack of knowledge on the relationship between environmental conditions and sample treatment history with molecular organization.

4. Concluding remarks

The herein reported results provide support for producing high scleroglucan amounts by employing about half of the sucrose concentration previously suggested for *S. rolfsii* ATCC 201126, but without significantly compromising the yield of the process. Additionally, in the light of previous work of the group and different reports in

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the literature, the best operative conditions for scleroglucan production were systematically compared and evaluated. This critical and polyphasic assessment allowed to confirm the following operative conditions as optimal for scleroglucan batch culture at bioreactor scale: temperature, 28 ºC; agitation speed, 200 rpm; aeration rate, 0.5 vvm; and pH, 4.5 without control.

Likewise, the present findings along with their discussion and rationale, contributed to the understanding on how each operative condition might affect not only the polymer produced in quantitative terms but also on its physicochemical and rheological properties. Concluding, this study highlights that each EPS production process needs a complete evaluation which ponders not only the amount of EPS recovered and its production costs, but also the quality and properties of the final polysaccharide. In sum, this will help to find adequate industrial and biomedical applications.

CRediT authorship contribution statement

A.L. Valdez: Investigation, Conceptualization, Writing - original draft. **O.D. Delgado:** Investigation, Validation, Supervision. **J.I. Fariña:** Visualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

This study was supported by CONICET (Grants N° PIP 0976 and PIO-UNCa) and FONCYT (Grants N° PCBII CONICET-MINCYT-DFG and PICT-2016-3945).

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Authors would like to thank Lic. Gonzalo Arnau for valuable help with HPLC-SEC analyses.

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