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Exopolysaccharyde production by *Cryptococcus laurentii* SD7 using molasses and corn steep liquor as substrates

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ABSTRACT. Microbial polysaccharides are of great biotechnological and commercial interest and have wide application in the food, cosmetic and medicine industries. Exopolysaccharide (EPS) production by the yeast *Cryptococcus laurentii* SD7, isolated from fresh water molluscs, was studied using agro-industrial byproducts as substrates in the submerged fermentation. The Central Composite Design (CCD) 2^3 was used to study the influence of pH, different concentrations on sugarcane molasses and corn steep liquor (CSL), for 48 hours. According to the results, the highest EPS production occurred at the initial pH 5 and at 8.4% concentration of sugarcane molasses, which were statistically significant variable at 10% ($p < 0.1$). The concentration of CSL had no influence in the studied range, thus, it can be used lowest concentration (0.3%). The time course of EPS production showed that while cell growth peaked within 48 hours, the highest EPS production (6.61 g L^{-1}) occurred at 168 hours, with a productivity of about 0.04 g L^{-1} h^{-1}. The pH of the medium remained approximately constant throughout the fermentation process. The yeast *C. laurentii* SD7 showed great potential for EPS production at a low cost and with sustainable substrates.

Keywords: Biopolymer; fungi; agroindustrial substrates.

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Introduction

Microbial polysaccharides are of great biotechnological and commercial interest and have wide application in the food, cosmetics, and medicine industries due to their emulsifying, thickening, flocculating, stabilizing, anti-oxidizing, and antimicrobial properties (Zhou et al., 2016; Yildiz & Karatas, 2018; Adebayo-Tayo & Fashogbon, 2020; Rana & Upadhyay, 2020). Some studies have also reported the medical applications of microbial EPS (Exopolysaccharides or Extracellular Polymeric Substances) as an anticoagulant having properties very similar to those of synthetic heparin with the advantages of being natural molecules, working as an anti-cholesterol, reducing serum levels of LDL, and anti-tumor activity (Patel & Prajapati, 2013; Hu et al., 2020; Saadat, Khosroushahi, Movassaghpour, Talebi, & Gargari, 2020). Bioremediation is another area that implies the use of EPS where they act as biosurfactants by adsorbing hydrocarbons and facilitating their recovery from the contaminated environment. However, the ability of EPS to perform any of these activities depends on its structure and composition (Zhou et al., 2016).

EPS can be obtained from plants, animals, and algae. However, the microbial production of polysaccharides is much easier and advantageous because of the short life cycle of microbes that allows quick production under controlled environmental conditions (Silva et al., 2006; Özcan & Öner, 2015). Polysaccharides synthesized by microorganisms can be found in the cell cytoplasm, being used as an energy reserve, in the cell wall as one of the structural and morphological components, and can be secreted in the extracellular medium as well. The last ones are called exopolysaccharides or extracellular polymeric substances (EPS) (Kumar, Mody, & Jha, 2007).

Although EPS are not essential for survival, they are secondary metabolites that protect the microorganisms against phagocytosis, osmotic stress, and dehydration along with favoring the formation of biofilms, as well as being associated with virulence (Kumar et al., 2007; Gientka, Błażejak, Stasiak-Różańska, & Chlebowska-Śmigiel, 2015; Prasannath, 2013).

Among the EPS producing microorganisms, several genus of yeasts, such as *Cryptococcus* sp., *Hansenula* sp., *Rhodotorula* sp., *Lipomyces* sp., *Bullera* sp., *Aureobasidium* sp., and *Sporobolomyces* sp., have shown potential for large scale production of EPS with several functional properties (Pavlova, Koleva, Krachanova, & Panchev, 2004; Pavlova et al., 2011; Hamidi et al., 2020). *Cryptococcus laurentii* is a non-pathogenic yeast species that has been described as an excellent producer of EPS. Studies have shown that the EPS produced by this particular species are of great commercial significance such as emulsifier and stabilizer in water/oil emulsions, absorption of heavy metals, and even in reducing serum cholesterol and triglyceride levels (Pavlova et al., 2011; Kuncheva et al.; 2013; Smirnou et al., 2014; Rusinova-Videva, Kambourova, Alipieva, Nachkova, & Simova, 2019).

There are many studies that aimed at the microbial production of EPS on an industrial scale; however, the cost-benefit of these bioprocesses is still a problem. This is because some microorganisms do not produce EPS in satisfactory quantities and the carbon sources such as glucose and sucrose used traditionally are expensive (Gientka et al., 2015; Ruiz et al., 2015; Zhou et al., 2016; Sardari et al., 2017). As a result, the agro-industrial substrates have aroused more interest in the production of EPS, since they are low-cost sustainable substrates (Santos, Kotovicz, Barana, & Almeida, 2012; Ruiz et al., 2015; Santos et al., 2018).

Brazil is the largest producer and exporter of sugar cane, contributing about 40% of the global production due to its favorable climate and large territorial extension. The main byproduct of the sugar industry is molasses that is obtained during the crystallization process of sugar. Some studies revealed that for every tonne of sugar produced, about 40 to 60 kg of molasses are generated as a byproduct (Oliveira Filho, Mattos, Pereira, & Baptista, 2019), thus being an abundant and renewable carbon source that can be used as a substrate for fermentation processes. Therefore, several studies have shown interest in using molasses in the fermentative processes for the production of biomolecules such as bioethanol, single cell oil, pigments, and microbial polysaccharides (Arshad, Abbas, & Iqbal, 2019; Bento, Carvalho, Reis, & Castro, 2020; Dias, Reis, Santos, & Silva., 2020; Wang, Kim, Lee, Kim, & Joe, 2020).

Many studies have earlier described that molasses have a high potential for the production of EPS, as it contains 30–60% of sucrose in its composition, in addition to glucose and fructose (Xu, Rao, Xu, & Liu, 2015; Soukoulis & Tzia, 2018; Palmonari, Cavallini, Sniffen, Fernandes, & Mammi, 2020). Additionally, it also contains vitamins, minerals, proteins, and amino acids that can perfectly replace the use of the conventional cost-intensive sources (Rebelato, Madaleno, & Rodrigues, 2013; Saxena & Tanner, 2013; Soukoulis & Tzia, 2018).

Corn steep liquor (CSL) is another agro-industrial byproduct that is obtained from the processing of corn. It comprises a large percentage of proteins and amino acids, along with other components, that act as a great source of nitrogen for the microorganisms in the bioprocesses, including the production of microbial polysaccharides (Saxena & Tanner, 2013; Sharma, Prasad, & Choudhury, 2013; Amado, Vazquez, Pastrana, & Teixeira, 2017; Hofer, Hauer, Kroll, Fricke, & Herwing, 2018). The use of CSL has been widely used in the fermentation processes due to its low cost, in comparison to the other conventional nitrogen sources such as yeast extract and peptone. However, its use in EPS production processes has not yet been well explored. Therefore, the objective of the present study was to delineate the potential of the yeast *C. laurentii* SD7 in the production of EPS by submerged fermentation, using sugarcane molasses and CSL as the substrates.

Material and methods

Molecular identification of the yeast

DNA extraction was performed according to Sampaio et al. (2001) and Almeida (2005). Molecular characterization was performed by sequencing the D1/D2 region of the 26S rDNA gene using the NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers (O'Donnel, 1993). For the polymerase chain reaction (PCR), the following reagents and concentrations were used: 10 ng of DNA, 1× enzyme buffer for *Taq* DNA polymerase (Sigma-Aldrich), 3.7 mM $MgCl_2$, 0.6 pmol μL^{-1} dNTPs (Sigma-Aldrich), 0.4 pmol μL^{-1} each primer (Biomers), and 5 U of *Taq* DNA polymerase (Applied

Biosystems), with the final volume adjusted to 50 µL with sterile ultra-pure water. The genomic DNA of *Saccharomyces cerevisiae* (10 ng) was used as a positive control (obtained collection of microorganisms LABGen). The amplification reactions were performed in a peqstar 96× Universal Gradient thermal cycler (PeqLab, Erlangen, Germany) and were programmed for an initial denaturation at 94ºC for 3 min., followed by 28 cycles of denaturation (94°C, 30 s), annealing (58°C, 1 min.), extension (72°C, 2 min.), and a final extension at 72°C for 5 min. The amplified products were resolved on a 0.8% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. The PCR fragments were then purified using a GFX PCR kit (Amersham Biosciences, Piscataway, NJ, USA), and the amplicons were later sequenced on an automatic sequencer ABI 310 (Applied Biosystems, Foster City, CA, USA) at the Molecular Genetics Laboratory of *Universidade Federal do Recôncavo da Bahia*. The second round of the sequencing was performed using an ABI PRISM 3100 genetic analyzer (Applied Biosystems) by ACTGene Molecular Analysis Ltda (Rio Grande do Sul, Brazil). The DNA sequences thus obtained were then compared with the available genomic sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) at http://www.ncbi.nlm.nih.gov (Altschul, Gish, Miller, Myers, & Lipman, 1990).

Fermentation Experiments

The fermentation experiments with *Cryptococcus laurentii* SD7 were carried out in 125 mL Erlenmeyer flasks containing 25 mL of (%): $(NH_4)_2SO_4$ 0.2; KH_2PO_4 0.1; $MgSO_4.7H_2O$ 0.05; $CaCl_2$ 0.01; NaCl 0.01; yeast extract 0.1. The inoculum consisted of 10% of the fermentation medium volume after 24 hours of growth. The fermentation experiments were carried out in 125 mL Erlenmeyer flasks according to the Central Composite Design 2^3 (CCD) matrix as described by Rodrigues and Iemma (2009), and the studied variables were the concentration of molasses (1.6 to 8.4%), pH (1.6 to 8.4) and corn steep liquor (0.16 to 0.84%), and in total 17 assays were carried out (Table 1). The results obtained from the experimental model were analyzed using the Statistica software version 7.1.

EPS determination

Whole cell cultures were centrifuged at 5000 G-force/RCF for 20 min. to separate the yeast cells from the supernatant. The exopolysaccharides in the cell-free supernatant were precipitated with 2 vol. of 96% ethanol at 4ºC for 24 hours. The resultant supernatant was discarded, and the pellet was dried at 80ºC and weighed.

Cellular growth

The cell growth was determined by measuring the turbidity of the diluted sample at 600 nm using a standard absorbance curve against the dry cell.

Validation of model

The validation tests were further carried out to confirm if the increase in the concentration of molasses could increase the production of the EPS. In order to accomplice this, 0.3% of corn steep liquor and pH 5.0 were used, and the concentrations of sugarcane molasses were varied (Figure 3). The tests were carried out in triplicate and the results obtained were evaluated by means of analysis of variance (ANOVA) and Tukey test at 5% significance by the software R.

EPS production kinetics and cell growth

To assess the influence of different cultivation times on the production of EPS, the optimized condition was established and fermentative tests were performed as described for the experimental design. The tests were performed in triplicate and samples were evaluated between 6 and 168 hours. The results were analyzed using the Scott Knott test at 5% significance.

Results

Molecular identification

A 601 bp length coding for the D1/D2 region of strain the SD7 showed 99% identity with the sequences of the D1/D2 domain of *Cryptococcus laurentii* (Figure 1).

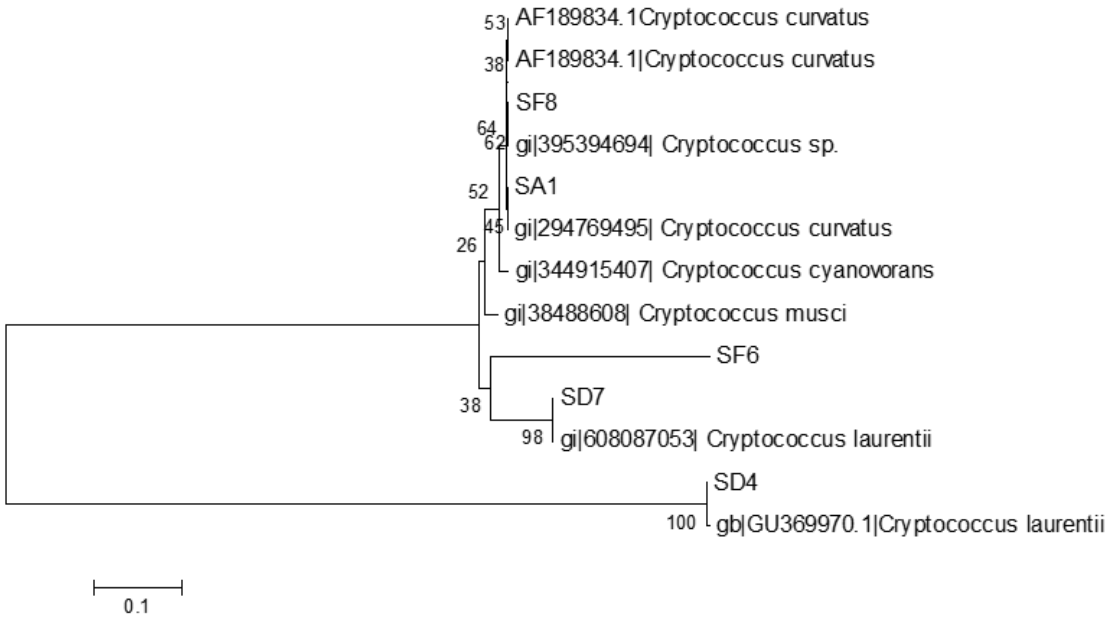

Figure 1. Maximum likelihood phylogenetic tree inferred from the alignment of the D1/D2 region sequences of the yeast rDNA subunit constructed in the MEGA 6 program. The best model selected by the program was the K2: Kimura 2-parameter + Gama model with partial deletion of missing data and confidence level of 1,000 bootstrap repeats.

Effects of various parameters on the EPS production by *Cryptococcus laurentii* SD7

The tests 6, 10, 13, and 17 are the conditions where EPS production was above 2.0 g L^{-1} (Table 1). The common conditions in these tests were 5.0% of molasses, between 0.5% and 0.7% of corn steep liquor and acidic pH (between 3 and 5). Test 10 showed the highest EPS production as 2.65 g L^{-1}.

Table 1. Matrix of Central Composite Design 2^3, coded and real values of the independent variables and the response (EPS production) by *Cryptococcus laurentii* SD7 in submerged fermentation.

Runs	Coded variables			Real variables			
	X_1	X_2	X_3	Sugarcane molasses (%)	pH	Corn steep liquor (%)	EPS production (g L^{-1})
1	-1	-1	-1	3	3	0.3	1.39
2	1	-1	-1	7	3	0.3	1.80
3	-1	1	-1	3	7	0.3	1.14
4	1	1	-1	7	7	0.3	1.55
5	-1	-1	1	3	3	0.7	1.76
6	1	-1	1	7	3	0.7	2.04
7	-1	1	1	3	7	0.7	1.20
8	1	1	1	7	7	0.7	1.70
9	-1.68	0	0	1.6	5	0.5	1.07
10	1.68	0	0	8.4	5	0.5	2.65
11	0	-1.68	0	5	1.6	0.5	1.12
12	0	1.68	0	5	8.4	0.5	1.54
13	0	0	-1.68	5	5	0.16	2.23
14	0	0	1.68	5	5	0.84	1.56
15	0	0	0	5	5	0.5	1.82
16	0	0	0	5	5	0.5	1.88
17	0	0	0	5	5	0.5	2.07

EPS production increased with an increase in the concentration of sugarcane molasses (Figure 2a). For corn steep liquor, in turn, the concentration range studied did not show any significant effects, suggesting that its variation did not influence the production of EPS (Figure 2b). Therefore, it was possible to work with the lowest concentration of 0.3% of this nitrogen source. Regarding the pH, the maximum EPS production occurred at pH 5, where the EPS production decreased at pH values above and below pH 5 (Figure 2c).

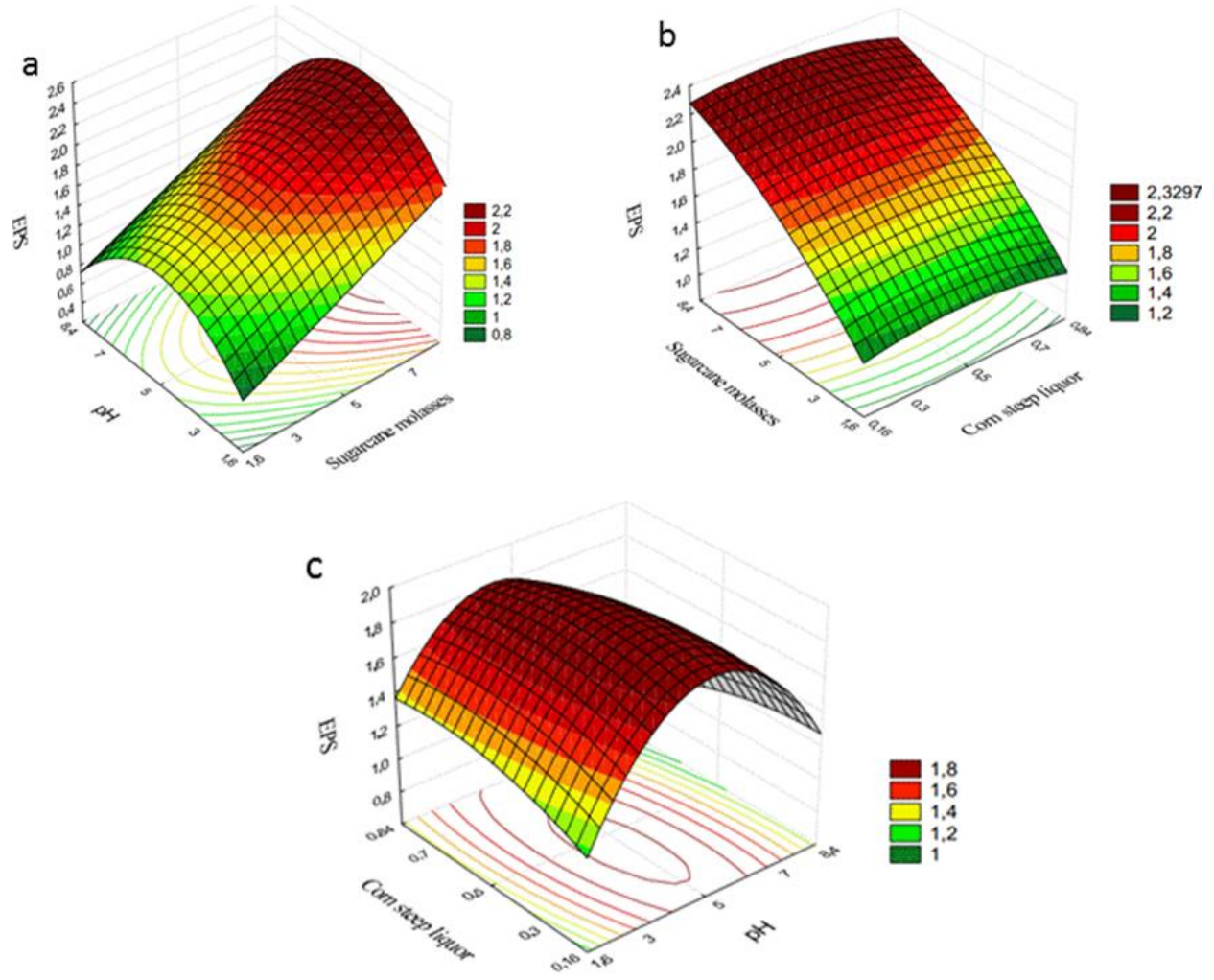

Figure 2. Response surface showing the relationship between: a) pH and sugarcane molasses; b) Sugarcane molasses and Corn steep liquor; c) Corn steep liquor and pH in the EPS production by *Cryptococcus laurentii* SD7.

The analysis of the regression coefficients showed that the only statistically significant variables in the production of EPS ($p < 0.1$) were carbon source and pH (Table 2). The F_{calc} value of 13.2 showed that the results were highly significant, and the correlation between the predicted and observed values was satisfactory (Table 3). This indicated that the results well fitted the model, and the regression coefficient values can be used to generate the model equation (Equation 1):

$$EPS\ (g\ L^{-1}) = 1.851 + 0.312\ X_1 - 0.216\ X_2^2 \quad \text{(Equation 1)}$$

Table 2. Regression analysis of EPS production by *Cryptococcus laurentii* SD7 in submerged fermentation using only the variables that were significant (sugar cane molasses and pH).

Factors	Regression coefficients	Standard error	t (14)	*p-value*
Mean	1.850771	0.088691	20.86763	0.000000
Sugarcane molasses (L)	0.311729	0.072908	4.27564	0.000769
pH (Q)	-0.215504	0.074645	-2.88706	0.011943

L = Linear; Q = Quadrático

Table 3. Analysis of variance (ANOVA) for the EPS production by *Cryptococcus laurentii* SD7 in submerged fermentation using sugarcane molasses, pH and corn steep liquor as variables.

Factor	FD	SQ	MD	Fc
Regression	2	1.932	0.966	13.23
Residue	14	1.016	0.073	
Total	16	2.948		

F_{tab}: 2.73; significant at the 10% level; Fc: F calculated; FD: Freedom degree; SQ: Sum of squares; MD: Midlle square.

Validation of model

The increase in the concentration of molasses up to 12% did not lead to the increase in the production of EPS, and there was no statistical difference between the different concentrations of the molasses ($p > 0.05$). Therefore, we concluded the 10% molasses concentration should be used for the successive tests (Figure 3).

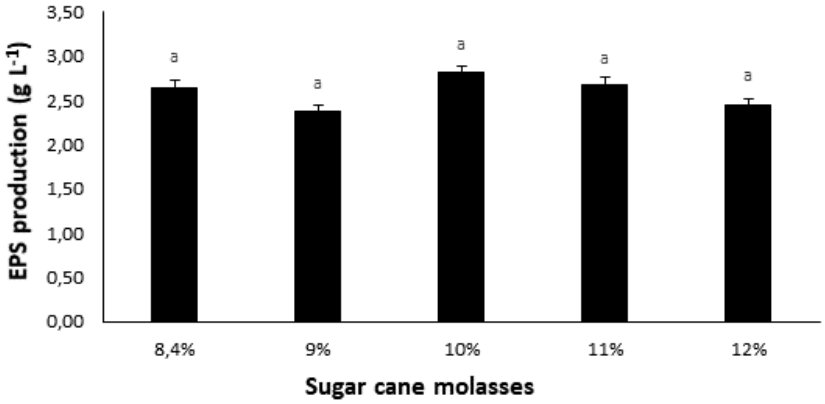

Figure 3. EPS production as a function of variation in the concentration of sugarcane molasses. Equal letters indicate that there was no statistical difference between the results obtained according to the Scott Knott test at 5% significance.

The results showed that the highest EPS production occurred after 168 hours of cultivation ($p < 0.05$) and reached 6.61 g L^{-1} on an average, with the productivity of 0.04 g L^{-1} h^{-1} (Figure 4b). The maximum biomass production (3.8 g L^{-1}) occurred at 48 hours and then remained constant until the end of the fermentation period (Figure 4c). There was a continuous decrease in pH from 5 to 4 till the end of the fermentation process (Figure 4a).

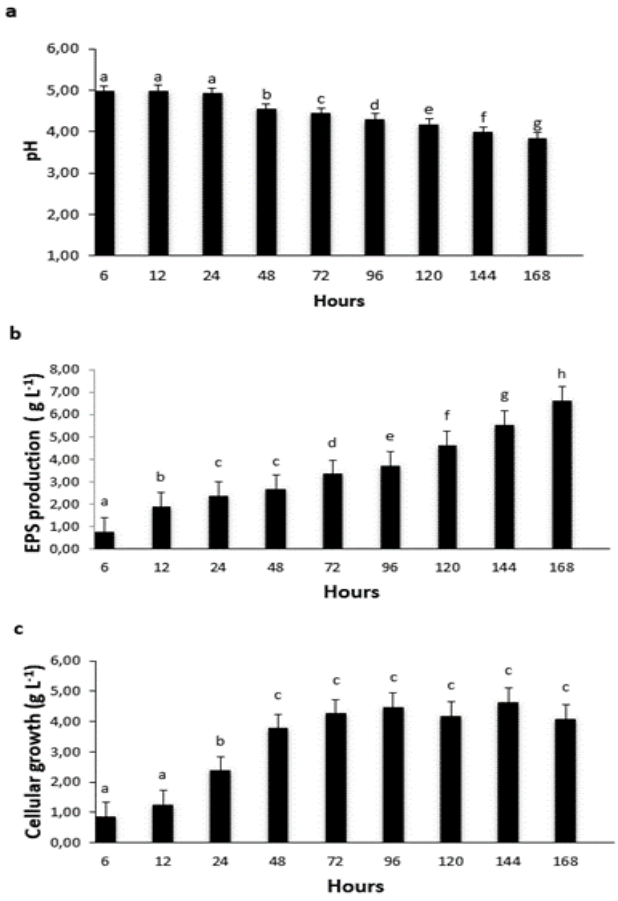

Figure 4. Time-course by *Cryptococcus laurentii* SD7: a) pH, b) EPS production (g L^{-1}) and c) Cellular growth (g L^{-1}). Equal letters indicate that there was no statistical difference between the results obtained according to the Scott Knott test at 5% significance.

Discussion

Among the different carbon sources described earlier, sucrose stands out as one of the best carbon sources for the production of EPS by different species of yeasts, including *C. laurentii* (Pavlova, Panchev, Kratchanova & Gocheva, 2009; Ravella et al., 2010; Hererher, ElFallal, Abou-Dobara, Toson, & Abdelazis, 2018; Silambarasam, Logeswari, Cornejo, & Kannan, 2018). This explained the high yield of EPS by *C. laurentii* SD7, since sugarcane molasses contains on an average of 30 to 60% of sucrose (Mirza & Mushtaq, 2006; Xu & Xu, 2014). Other research works that used molasses as a carbon source also described higher yields of microbial EPS (Ruiz et al., 2015; Moghannem, Farag, Shehab, & Azab, 2018; Razack, Velavutham, & Thangavelu, 2012). Besides being rich in sucrose, molasses also contains amino acids and salts such as phosphates, sodium, calcium, magnesium, and phosphorus (Xu & Xu, 2014; Soukoulis & Tzia, 2018). This combination of macro and microelements probably enhanced the EPS production, thus, making molasses as a good substrate alternative for this purpose.

The increase in the EPS production at the highest concentrations of sugarcane molasses, i.e., above 5%, is expected because its synthesis is stimulated by the excess carbon source and, when it is at low concentrations, it is preferably used for the cell growth (Figure 1a and b) (Herenher et al., 2018). For *C. laurentii* SD7, the production of EPS did not follow the cell growth and continued for up to 168h (Figure 3b). The synthesis of the EPS is part of the secondary metabolism, i.e., it is not essential for the survival of the microorganisms. Several strains of *C. laurentii* produce a silty capsule that serves as protection against environmental stresses, such as water loss (Breievorá, , Hromádková, Stratilová, Sasinková, & Ebringerová, 2005; Kumar et al., 2007; Gientka et al., 2015), a situation that may be triggered by the extracellular osmotic pressure at the high concentrations of salts and sugars.

As described for other fungal species, the maximum EPS production by *C. laurentii* SD7 occurred at pH 5.0, and acidification of the medium over time is commonly described in the production of yeast EPS (Pavlova et al., 2009; Poli et al., 2010; Rusinova-Videva, Pavlova, & Georgieva, 2011). However, unlike a significant decrease in the pH normally described earlier (Pavlova et al., 2011; Gientka, Bzducha-Wróbel, Stasiak-Różańska, Agnieszka, & Błażejak, 2016), the pH of the medium with molasses dropped from 5.0 to about 4.0 after 168h of fermentation (Figure 3a). These results were similar to those obtained by Grigorova, Pavlova, and Panchev (1999), who observed that among all the studied carbon sources, molasses was the only one that showed no significant change in the pH. This is probably due to the buffering capacity of amino acids present in the molasses that help maintain a stable pH over a longer period of time (Dumbrepatil, Adsul, Chaudhari, Khire, & Gokhale, 2008). This is interesting because a lower pH variation keeps the medium more stable and closer to the ideal conditions for EPS production. This may explain the continuous synthesis of EPS by *C. laurentii* SD7 for up to 168 hours.

Corn steep liquor is a source of nitrogen widely used in fermentation processes, such as the production of ethanol and organic acids (Saxena & Tanner, 2012; Amado et al., 2017). For *C. laurentii* SD7, the corn steep liquor proved to be a suitable nitrogen source in the production of EPS, especially at low concentrations, due to its rich composition in protein and nitrogen (Nisa et al., 2006; Mirza & Mushtaq, 2006; Sharma et al., 2013).

The use of agro-industrial byproducts has increased the efficiency in the production of EPS in addition to reducing production costs, since the high yields (2.65 g L^{-1} at 48 hours) were obtained in a much shorter period than the previously described ones in the literature. In such studies, yields ranging from 1.0 to 3.0 g L^{-1} on an average, after 6 to 7 days have been reported (Fang & Zhong, 2002; Selbmann, Onofri, Fenice, Federice, & Petrucciolli, 2002; Pavlova et al., 2004; Poli et al., 2010; Gientka et al., 2015).

The factorial planning methodology brings some advantages for obtaining and analyzing the results. As the factors affecting the process are analyzed simultaneously, it is possible to optimize more than one response at the same time. In addition, it allows you to calculate and verify statistical confidence and estimate the reproducibility of results (Rodrigues & Iemma, 2009). Through this methodology, in this work it was possible to observe that two factors influenced the EPS production, carbon source (cane molasses) and pH, and to find the ideal condition to obtain the maximum EPS production. In addition, the information about the source of nitrogen, allowed the use in lower concentrations, which promotes a reduction in the costs of the process with the substrates.

Conclusion

The yeast *C. laurentii* SD7 presented great potential for EPS production. Sugarcane molasses and corn steep liquor were efficient, and their application in the fermentative processes for the EPS production can be a viable alternative in terms of cost and sustainability.

Acknowledgements

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