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Effects of agitation rate and dissolved oxygen on xylose reductase activity during xylitol production at bioreactor scale

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Abstract

The study of xylose reductase (XR) - one of the key enzymes in the production of xylitol - is important in the fermentation process to have maximum efficiency in the bioconversion of xylose to xylitol in lignocellulosic hydrolysate. The aim was to evaluate the effect of agitation rate and dissolved oxygen at 7 L bioreactor scale on the production of xylose reductase (XR) from *Candida tropicalis* during the bioconversion of xylose into xylitol in the non-detoxified oil palm empty fruit bunch (OPEFB) hydrolysate. The highest xylose consumption (95.5%) and the maximum xylitol production (5.46 $g.L^{-1}$) were presented under 30% dissolved oxygen and 50 rpm. The maximum XR activity (0.646 U mg^{-1} protein) was obtained after 144 h of fermentation and at the same conditions of dissolved oxygen and agitation rate mentioned above. The oxygen availability influences the XR activity of *C. tropicalis* and the xylitol production, observing a xylitol yield factor ($Y_{P/S}$) of 0.27 $g.g^{-1}$ and volumetric productivity (Q_p) of 0.33 $g.L^{-1} h^{-1}$. At lower dissolved oxygen regardless of the agitation conditions evaluated, an increase in xylitol production was evidenced.

Keywords: *Candida tropicalis*; xylitol; xylose reductase; dissolved oxygen; non-detoxified hydrolysate.

Practical Application: The conversion of xylose to xylitol is suitable when there is a gradually increase of xylose reductase.

1 Introduction

Hemicellulose, the second most abundant polysaccharide in nature, is used to generate value-added products such as xylitol; a penta-hydric alcohol of xylose widely used in the food industry as well as in products for oral care, pharmaceuticals and cosmetics (Arruda et al., 2011). The use of lignocellulosic material such as oil palm empty fruit bunch (OPEFB) is an alternative for the production of this sugar alcohol. During hydrolysis, the polymers present in the hemicellulosic fraction are broken to xylose, a sugar converted to xylitol by fermentation (Kresnowati et al., 2016).

The xylitol yield and substrate consumption must be high and production costs low for the xylitol industrial application. Low cost xylitol production involves the use of a xylose source without the need for detoxification, yeast cell recycling, high yield of xylose to xylitol, high productivity, less energy input, easy post-processing to purify xylitol and use of fermentation media from industrial by-products (Yewale et al., 2017). Several factors have been studied to achieve an industrial application of the biotechnological xylitol production, such as the age of the inoculum, the initial xylose concentration (Mussatto & Roberto, 2005; Xu et al., 2019), pH (Manaf et al., 2017), carbon source, nutrients, temperature, aeration and bioreactor operation mode (Silva et al., 2007; Martínez & Santos, 2012: Albuquerque et al., 2014; Dasgupta et al., 2017). One of the biotechnological trends for the xylitol production is the development of recombinant strains with high production potential of xylitol, such as Saccharomyces with Candida XYL1 gene, because the first species is more tolerant in terms of xylose fermentation, toxicity and growth tolerance in the presence of inhibitors of hemicellulosic hydrolysates (Prakasham et al., 2009). However, there are few studies of xylitol production using native yeast strains of the Candida genus. In our knowledge, metabolic engineering methods and production of these recombinant strains have not been performed or documented recently (Dasgupta et al., 2017). Furthermore, the strains used in this study have not had any genetic transformation and are native strains (Yokoyama et al., 1995).

Dissolved oxygen is one of the most important factors affecting xylitol production, especially when Candida is used for the bioconversion of D-xylose (Albuquerque et al., 2014). During the fermentation process, Candida metabolizes xylose into xylitol in the presence of key enzymes that participate in a metabolic pathway such as xylose reductase (XR; EC1.1.1.21); an intracellular enzyme located in the cytoplasm, which has the ability to reduce xylose to xylitol in the first phase of the

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metabolic pathway in the presence of NADPH as a cofactor (Cocotle-Ronzon et al., 2012). The XR activity has also special scientific attention for its application in the fermentation of lignocellulosic substrates for the production of ethanol and xylitol (Zhang et al., 2019).

The XR study and its correlation with the dissolved oxygen concentration and the agitation rate in non-detoxified OPEFB hydrolysate at bioreactor scale has not been reported so far. For this reason, it is necessary to explore which fermentation conditions lead to maximum efficiency in bioconversion of xylose to xylitol in OPEFB hydrolysate through the XR activity study. Thus, the objective of this work was to evaluate the influence of dissolved oxygen and agitation rate on the XR activity at bioreactor scale (7 L) during the fermentation of non-detoxified OPEFB hydrolysate for xylitol production using *C. tropicalis*.

2 Materials and methods

2.1 Materials

The oil palm empty fruit bunch (OPEFB) was supplied by Palmares del Oriente S.A.S. from Villanueva, Casanare, Colombia. The OPEFB was prepared to be hydrolyzed and used in bioreactor-scale fermentations as described in previous studies (Manjarres-Pinzon et al., 2017).

2.2 OPEFB diluted-acid hydrolysis

OPEFB diluted-acid hydrolysis was performed in 500 mL Erlenmeyer flasks with a solid/liquid ratio of 1:8, 40 g of the OPEFB and 320 mL of 2% diluted sulfuric acid at 121 °C as described in previous studies (Manjarres-Pinzon et al., 2017). The inhibitor concentrations in the hydrolysate, such as hydroxymethylfurfural, glucose, acetic acid, and xylose were 0.023, 1.033, 11.078 and 30 $g.L^{-1}$, respectively (Rahman et al., 2007; Ferrer et al., 2013).

2.3 Microorganism and pre-inoculum preparation

C. tropicalis cells were maintained with a culture age of 48 h on PDA agar at 30 °C. The pre-inoculum was prepared by transferring colonies of yeast cells to 250 mL Erlenmeyer flasks with 100 mL of yeast extract, peptone and xylose medium (YPX) with a concentration of 20 $g.L^{-1}$ of each of these compounds. The flasks were incubated on a rotary shaker at 200 rpm for 24 h at 30 °C. Then, cells were recovered by centrifugation (2500 rpm, 20 min). The suspension of the cells obtained (about 5 $g.L^{-1}$) was used to inoculate the fermentation medium on a bioreactor scale (Sene et al., 2011; Hernández-Pérez et al., 2016; Moraes et al., 2020).

2.4 Fermentation process

Batch cultures were carried out in a 7 L Applikon bioreactor with agitation module (Applikon ADI310, Delf, NL) and Alpha-DO2000PPG dissolved oxygen control (Eutech Instruments, Delf, NL). The dissolved oxygen sensor had a pulsed polarographic electrode of nitrogen and oxygen as required by the system. Moreover, the agitation rate was controlled in the control panel as a set-point so that the rotor connected to the Rushton turbines was always at the agitation rates established in the experimental design. The fermentations were performed with 2.5 L of non-detoxified OPEFB hydrolysate, which was sterilized together with the autoclave bioreactor at 121 °C for 20 min at 15 psi. The hydrolysate had an initial xylose concentration ranging between 20 and 22 $g.L^{-1}$, the inoculum concentration used was around 5 $g.L^{-1}$, the pH and temperature of all cultures were kept at 5.6 and 30 °C, respectively. The agitation rate (50, 75 and 100 rpm) and the dissolved oxygen - DO (30 and 80%) during 164 h of fermentation were evaluated; samples were taken every 24 h in order to determine cell growth, xylose consumption, xylitol production, and XR activity (Mareczky et al., 2016; Rao et al., 2016).

2.5 Enzymatic extracts

Cells were collected by centrifugation at 2500 rpm for 30 min. A volume of 1000 µL of cell mass was taken for cell breakdown using a mortar with liquid nitrogen. Subsequently, 3 mL of phosphate buffer at pH 7.2 were added. This solution was undergone to a second centrifugation at 2500 rpm for 20 min. The supernatant was used for XR determinations (Yokoyama et al., 1995; Arruda et al., 2011).

2.6 Analytical methods

Cell concentration was determined by the dry weight method, where 10 mL was centrifuged at 3900 rpm for 20 min at 25 °C, washed twice with distilled water and dried in Petri dishes at 60 °C for 72 h (Niño-Camacho & Torres-Sáenz, 2010). Xylose and xylitol concentration were analyzed using an HPLC system (Shimadzu Prominence, Kyoto, Japan), with a refractive index (RI) detector, equipped with an Aminex HPX-87H column (Biorad) (Manjarres-Pinzon et al., 2017). Samples were prepared in duplicate and filtered through a 25 mm syringe filter with nylon membrane (pore size 0.45 µm) before analysis.

The XR activity in the enzymatic extracts was established by the oxidation of the NADPH cofactor as a co-substrate by decreasing absorbance at 340 nm and 30 °C using a Multiskan Sky Microplate Spectrophotometer (Thermo Scientific, Waltham, MA). The enzymatic reaction was carried out in cuvettes containing a solution of 100 µL of deionized water, 600 µL of the phosphate buffer at pH 7.2, 100 µL of mercaptoethanol, 50 µL of the extract to be analyzed and 50 µL of 3 mM NADPH. The absorbance was measured after 1 min and designated as the pre-xylose absorbance of the extract to be analyzed. Subsequently, 100 µL of a 0.5 M xylose solution were added and the absorbance was measured every 6 s for 60 s. The XR unit (U) was defined as the amount of enzyme that catalyzes the reduction of 1 µmol of NADPH per min. The specific XR activity was expressed as a unit of the enzyme per mg of protein with Equation 1 (Cocotle-Ronzon et al., 2012).

$$\mathbf{Activity} = \frac{1000 * \mathbf{TV} * \mathbf{dA}/\mathbf{dt}}{\varepsilon * \mathbf{V} * \mathbf{CF}} \quad (1)$$

Where TV is the total volume of the cuvette, that in this case corresponds to 1000 µL; V is the cell extract volume (50 µL); ε

represents the molar extinction coefficient of NADPH, which is 6.22 L $mmol^{-1}$; dA/dt is the difference between the experimental speed and the control speed; and CF is the dilution value, which in the case is 1. The protein concentration in the extracts was measured by the Bradford method (Bradford, 1976) using agitated microplates, the same spectrophotometer mentioned above and a standard solution of bovine serum albumin at 595 nm.

2.7 Experimental design

For bioreactor fermentations, a 2^k factorial design was applied taking dissolved oxygen (30 and 80% DO) and agitation rate (50 and 100 rpm). The experimental data were subjected to an ANOVA, with a level of significance of 5% and a Fisher's least significant difference post hoc test (LSD) was used to compare treatments with significant differences. Statistical analysis was performed using Statgraphics plus 5.1. Results were presented as the average of the data and all experiments were performed in duplicate.

3 Results and discussion

3.1 Xylitol production under different oxygen and agitation conditions

The bioconversion from xylose - contained in non-detoxified OPEFB hydrolysate - to xylitol by *C. tropicalis* was evaluated under various oxygen and agitation conditions in a 7L bioreactor (Figure 1a). The strain was able to consume xylose in the 5 treatments evaluated (Table1), indicating that OPEFB hydrolysate contains sufficient carbon sources for cell growth, although this consumption varied considerably between treatments. Xylose is consumed to a greater extent under conditions of 30% DO and 50 rpm showing a consumption of 95.5%, whereas conditions of 80% DO and the same agitation rate showed a xylose consumption of only 69%. The initial xylose (21 $g.L^{-1}$) was consumed to a residual xylose of 0.9 and 6.3 $g.L^{-1}$, respectively (Figure 1a). *C. tropicalis* showed a higher capacity to consume xylose under limited oxygen conditions and low agitation rates, although the latter was not statistically significant at the levels tested during the xylose fermentation. Yeasts require little oxygenation to reduce xylose (Albuquerque et al., 2014). Other studies have reported that microaerobiosis generates O_2 restriction conditions, tending toward the xylitol accumulation and regenerating NADPH, an important cofactor for the bioconversion of xylose to xylitol (Dasgupta et al., 2017).

The xylitol production was improved with low agitation rates (50 rpm) and limited oxygen conditions (30%), with a maximum production of 5.46 $g.L^{-1}$ followed by 4.9 $g.L^{-1}$, when the oxygen concentration was 80% (Figure 1b). Although the concentrations reached in the xylitol production were similar under different conditions of DO, the limited conditions led to very little residual xylose and had a higher productivity during the fermentation process, indicating a good result in terms of efficiency of economic and fermentation processes. The DO in low conditions increases the possibility of xylitol production, being 30% the best for *C. tropicalis* at 7 L bioreactor scale using non-detoxified OPEFB hydrolysate. DO concentrations of 30% and low agitation rate (50 rpm) favored cell growth; the growth curve had a lag phase of 24 h and then exponential phase started from 48 h of fermentation. The pre-inoculum in the fermentations was carried out in synthetic medium rich in xylose, which was probably affected when inoculating the cells in non-detoxified OPEFB hydrolysate (Kresnowati et al., 2016). The yeasts had to adapt to a new environment with different conditions in nutrients, pH, oxygen concentrations, presence of inhibitors and other sugars, among others. The cells began to be active after 48 h with an exponential phase that lasted approximately 96 h, reaching the maximum biomass concentration (11.2 $g.L^{-1}$) corresponding to 6.2 $g.L^{-1}$ produced during the 144 h of fermentation (Figure 1c). Other studies have reported that during the xylitol fermentation similar biomass values have been achieved using *C. tropicalis* and sugarcane bagasse (Tizazu et al., 2018) or synthetic media (Wang et al., 2015); however, the data are useful to know the kinetic behavior of *C. tropicalis* in non-detoxified OPEFB hydrolysate at 7 L bioreactor scale, although the experimental conditions and the culture media were completely different.

Several studies have reported that xylitol-producing yeasts have a higher xylose intake and xylitol production under limited oxygen conditions (Veras et al., 2017). The best volumetric productivity (Q_p : 0.033 $g.L^{-1}$ h^{-1}) was observed after 144 h of fermentation as shown in Table 2. A lower productivity than that in this study (0.02 $g.L^{-1}$ h^{-1}) was reported for OPEFB hydrolysate and *Debaryomyces hansenii* ITBCC R85 for xylitol production after 96 h of fermentation (Mardawati et al., 2015). In the same way, the highest xylitol yield factor ($Y_{P/S}$: 0.98 g.g-1) was at 30% DO and 100 rpm; nevertheless, 1.8 $g.L^{-1}$ of xylitol and a xylose consumption of 1.98 $g.L^{-1}$ were produced in this treatment during the 168 h of fermentation only; therefore, the product yield was very high (Table 2). Meanwhile, $Y_{P/S}$ with 30% DO and 50 rpm was 0.27 $g.g^{-1}$, exceeding some reported in the literature at bioreactor scale (Mardawati et al., 2015) or studies that have modified yeasts such as *Saccharomyces cerevisiae* to improve the xylose fermentability on synthetic and hydrolyzed medium of lignocellulosic materials during 100 h of fermentation, obtaining $Y_{P/S}$ yields equal to 0.04 $g.g^{-1}$ (Karhumaa et al., 2007).

Table 1. Factors of experimental design in the study.

Run #	Factors	
	Agitation rate (rpm)	Dissolved oxygen (%)
1	100	30
2	100	80
3	50	30
4	75	55
5	50	80

3.2 Effect of dissolved oxygen and agitation rate on xylose reductase activity at bioreactor scale

The correlation between the *C. tropicalis* ability to ferment xylose and produce one of the key enzymes in the xylitol production, such as XR under different DO and agitation rates in non-detoxified OPEFB hydrolysate at bioreactor scale has not been reported so far. There are different studies on the behavior of key enzymes in the metabolism of *C. guilliermondii* in rice

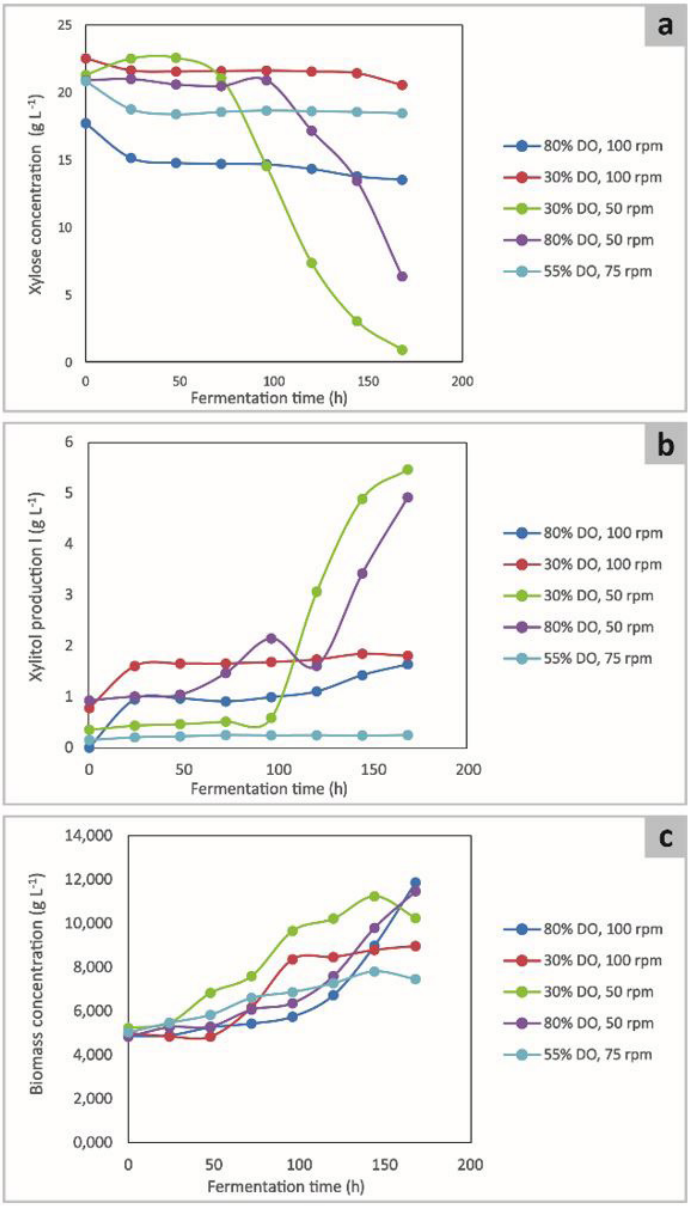

Figure 1. Xylose concentration (a), xylitol production (b) and biomass concentration (c) during fermentation of non-detoxified OPEFB hydrolysate using *C. tropicalis*. The results are presented as the mean value of two replicates.

Table 2. Volumetric productivity (Q_p) and xylitol yield ($Y_{P/S}$) during fermentation of non-detoxified OPEFB hydrolysate using *C. tropicalis*.

Treatment	80% DO, 100 rpm		30% DO, 100 rpm		30% DO, 50 rpm		80% DO, 50 rpm		55% DO, 75rpm	
Fermentation time (h)	Q_p [a]	$Y_{P/S}$ [b]	Q_p [a]	$Y_{P/S}$ [b]	Q_p [a]	$Y_{P/S}$ [b]	Q_p [a]	$Y_{P/S}$ [b]	Q_p [a]	$Y_{P/S}$ [b]
24	0,039	0,368	0,067	0,926	0,018	0,066	0,042	0,794	0,008	0,025
48	0,020	0,329	0,034	0,901	0,010	0,086	0,022	0,386	0,005	0,029
72	0,013	0,302	0,023	0,929	0,007	0,667	0,020	1,284	0,003	0,042
96	0,010	0,325	0,018	1,004	0,006	0,034	0,022	0,170	0,003	0,042
120	0,009	0,326	0,014	0,983	0,026	0,194	0,013	0,183	0,002	0,042
144	0,010	0,361	0,013	0,967	0,034	0,248	0,024	0,336	0,002	0,038
168	0,010	0,391	0,011	0,517	0,033	0,251	0,029	0,275	0,001	0,041
$Y_{P/S\ global}$	-	0,391		0,517		0,251		0,274		0,041

[a]Volumetric productivity (g xylitol L^{-1} h^{-1}). [b]Xylitol yield (g xylitol g^{-1} xylose).

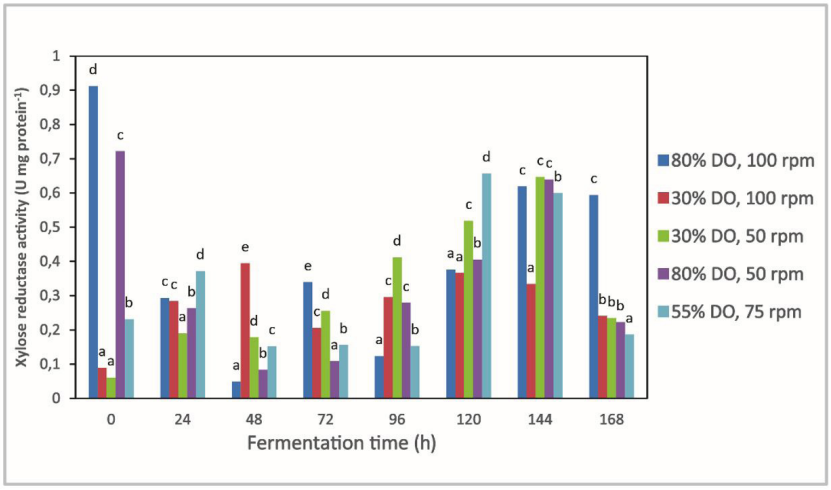

Figure 2. Xylose reductase (XR) enzyme activity during fermentation of non-detoxified OPEFB hydrolysate using *C. tropicalis*. The results are presented as the mean value of two replicates.

hydrolysates considering the initial xylose concentration and medium pH (Gurpilhares et al., 2009), the XR behavior and xylitol dehydrogenase (XDH) during the xylitol production using *C. guilliermondii* FTI 20037 in synthetic medium and different k_{La} values in agitated bioreactor (Branco et al., 2009). Other authors have evaluated the effect of oxygen transfer rate (OTR) on important enzymes in xylose metabolism by *D. hansenii* (Gírio et al., 1994) and the effect of adapting *C. tropicalis* on the XR production in sawdust hydrolysates (Rafiqul & Sakinah, 2014).

XR activity was measured in crude cell extracts of *C. tropicalis* during fermentation of non-detoxified OPEFB hydrolysate under different oxygen (30-80%) and agitation rates (50 and 100 rpm) for 168 h (Figure 2). The maximum XR concentration had a value of 0.91 U mg^{-1} protein with 80% DO and 100 rpm at the beginning of fermentation. However, at these same conditions during the fermentation process the XR values fluctuated widely, no trend was shown and the xylitol concentrations were not the best. This behavior has also been reported in other studies using rice hydrolysate and *C. guilliermondii* (Gurpilhares et al., 2009). High XR at the beginning of fermentation could be attributed to the fact that the cells used came from a pre-inoculum containing xylose (YPX) (Arruda et al., 2011). Moreover, the pre-inoculum used in the treatment with 80% DO and 100 rpm probably had a high XR from the moment in which the bioreactor was inoculated. Normally, cells in a culture are in different stages of growth (asynchronous), which directly influences their metabolic activity (Arruda et al., 2011)

On the other hand, the XR values were gradually increasing to a result of 0.646 U mg^{-1} protein at 144 h with the conditions of 30% DO and 50 rpm, which also presented higher xylitol concentrations (5.46 $g.L^{-1}$). After that time, the XR dropped considerably, probably because limited oxygen conditions restrict cell growth and, thus, XR activity diminishes after reaching its maximum peak (Kklaif et al., 2020). The previous behavior was also reflected in the xylitol production by *C. guilliermondii* FTI 20037 in synthetic medium (Branco et al., 2009). The gradual increase in XR directly affects the xylitol production. A behavior similar to the previous conditions was observed for the treatment with 80% DO and 50 rpm, although its xylitol production was lower than that of 30% DO and 50 rpm; at these process conditions, the XR activity began to increase gradually after 48 h of fermentation, but the xylose consumption throughout the fermentation was 69% much lower compared to the 30% DO and 50 rpm treatment, which xylose consumption was higher than 95%, showing better use of the substrate during the whole fermentation time. Furthermore, the cells take the substrate for growth at high oxygen concentrations, thus, a residual xylose concentration of 6.38 $g.L^{-1}$ after 168 h and a xylitol production of 4.91 $g.L^{-1}$ in the treatment with 80% DO and 50 rpm reflect the importance of having high biomass concentrations to produce xylitol. In the case of the 30% DO and 50 rpm treatment, it showed a lower biomass production than the 80% DO and 50 rpm treatment, but higher xylitol production. This confirms what is reported by some authors, who claim that aeration stimulates the sugar transport in some yeasts and the xylitol production is directly related to cell growth and oxygen consumption (Vallejos & Area, 2017).

In this study, limited oxygen conditions were achieved at 7 L bioreactor scale, demonstrating that xylitol production may not be favored by high XR concentrations (Rafiqul & Sakinah, 2014); but by the behavior of XR enzyme during the whole fermentation, which must have a tendency of increase without fluctuations in time. The metabolism in general is accelerated and the biomass production increases with the increase of available oxygen. Other studies have reported that the increase in k_{La}, which is related to dissolved oxygen in the medium, generates a deviation from metabolism to cell mass production and a decrease in XR during the process, whereas the xylitol production increases with a limited dissolved oxygen condition and generates an increase or keeps the XR amount in the medium (Branco et al., 2009; Kim et al., 1997). Xylitol values in high oxygen conditions may be due to cells tending to produce more amino acids, enzymes and proteins. In this work, the protein value was around 1.7-1.8 mg mL^{-1} in all treatments

where the xylitol concentration was maximum, indicating that the treatments with the highest protein concentration were the best for xylitol production.

On the other hand, under a limited oxygen supply, it is possible to guarantee the minimum conditions in which a fraction of the NADH cofactor is regenerated. Therefore, the difference in activity between XR and XDH allows intracellular accumulation of xylitol to a limit concentration, after which excess xylitol is excreted. The direct xylitol production does not bring any energy gain to the cell, and the NADH accumulation damages their natural metabolism. Accordingly, it becomes clear that reduced amounts of oxygen are needed to maintain minimum vital cellular functions and, consequently, make xylitol production viable (Faria et al., 2002).

4 Conclusions

Non-detoxified OPEFB hydrolysate represents an important carbon source for the *C. tropicalis* growth at 7L bioreactor scale. The bioconversion of xylose to xylitol by these conditions requires studies that correlate operating variables of the bioreactor, such as dissolved oxygen and agitation rate on the XR activity, important in reducing xylose. Limited oxygen conditions and low agitation rates showed that *C. tropicalis* increased XR concentration gradually throughout the fermentation process. It is precisely this increase without fluctuations in time, which could be the metabolic pattern that the strain must have for a good fermentability of xylose and then result in high xylitol concentrations. The best operating conditions were 30% DO and 50 rpm, with a 95.5% xylose intake and a maximum xylitol production of 5.46 $g.L^{-1}$. During the fermentation process, the maximum XR activity (0.646 U mg^{-1} protein) was given after 144 h under the conditions mentioned above. The fermentation process started to be effective after the fourth day of bioreactor inoculation. It is recommended to evaluate other key enzymes such as xylitol dehydrogenase in the future to know the metabolic behavior of the strain under different conditions of dissolved oxygen and agitation rate.

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