Xylitol production from lignocellulosic whole slurry corn cob by engineered industrial *Saccharomyces cerevisiae* PE-2

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GRAPHICAL ABSTRACT

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ABSTRACT

In this work, the industrial *Saccharomyces cerevisiae* PE-2 strain, presenting innate capacity for xylitol accumulation, was engineered for xylitol production by overexpression of the endogenous *GRE3* gene and expression of different xylose reductases from *Pichia stipitis*. The best-performing *GRE3*-overexpressing strain was capable to produce 148.5 g/L of xylitol from high xylose-containing media, with a 0.95 g/g yield, and maintained close to maximum theoretical yields (0.89 g/g) when tested in non-detoxified corn cob hydrolysates. Furthermore, a successful integrated strategy was developed for the production of xylitol from whole slurry corn cob in a presaccharification and simultaneous saccharification and fermentation process (15% solid loading and 36 FPU) reaching xylitol yield of 0.93 g/g and a productivity of 0.54 g/L.h. This novel approach results in an intensified valorization of lignocellulosic biomass for xylitol production in a fully integrated process and represents an advance towards a circular economy.

1. Introduction

Concerns regarding the progressive exhaustion of the fossil resources, and the resultant environmental and economic problems, have created the necessity to replace the current petroleum-based economy. Lignocellulosic biomass, being the most abundant and renewable biomass available on earth, has been receiving growing attention as a substitute for the fossil fuels (Budzianowski, 2017). Lignocellulose

presents a recalcitrant structure, mainly composed of cellulose (glucose monomers), hemicellulose (hexose and pentose sugars) and lignin; and the attainment of fermentable sugars from these biomasses requires pretreatment and hydrolysis steps that also result in the release of microbial inhibitory compounds (Zabed et al., 2016). In order to efficiently replace the petroleum based industry, the lignocellulose potential as a substrate for biofuels and value added chemicals must be fully exploited. In fact, the cellulosic fraction has already been extensively

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studied for the production of biofuels, nevertheless the attainment of a sustainable lignocellulose-based bioeconomy should include the valorization of the hemicellulose fraction for the production of value added products (Budzianowski, 2017).

Xylitol, a sugar alcohol, has been identified as one of the 12-top value added compounds to be attained from biomass (Petersen and Werpy, 2004). It is a natural sweetener used as a sugar substitute in food and pharmaceutical industries, as it presents advantageous properties, such as, low energy content, insulin-independent metabolism, anticarcinogenicity, among other pharmacological properties (de Albuquerque et al., 2014). Furthermore, xylitol can also be used in the chemical industry as an intermediate for the synthesis of polymers (Isikgor and Becer, 2015). Currently, xylitol is industrially produced through chemical hydrolysis and hydrogenation of xylan, an expensive and laborious process. Furthermore, even using lignocellulose as a raw material, it is not an environmental-friendly process, resulting in a growing interest in the microbiological production of xylitol (Mohamad et al., 2015).

Several yeast, such as *P. stipitis* (*Scheffersomyces stipitis*), *Debaryomyces hansenii*, *Kluyveromyces marxianus* and *Candida* spp, are naturally capable of consuming xylose through a xylose reductase/xylitol dehydrogenase (XR/XDH) pathway, which converts xylose into xylitol, and subsequently to xylulose. Xylitol is a common by-product of this pathway, mainly resultant of the co-factor imbalance between the reaction catalyzed by the NAD(P)H-dependent xylose reductase (normally with preference for NADPH) and the NAD⁺-dependent xylitol dehydrogenase (de Albuquerque et al., 2014). Considering these, yeast that are naturally capable of xylose consumption have been extensively studied for xylitol production (Dasgupta et al., 2017; Kumar et al., 2018; López-Linares et al., 2018), nevertheless the yields of xylitol from xylose are limited by the use of xylose for yeast growth and maintenance energy. In this sense, the use of *S. cerevisiae*, (a GRAS micro-organism naturally incapable of xylose metabolism) with increased expression of enzymes with xylose reductase activity has emerged as a solution to increase xylitol yields from xylose (Chung et al., 2002; Govinden et al., 2001; Hallborn et al., 1991; Kim et al., 2017; Kogje and Ghosalkar, 2016; Lee et al., 2000; Meinander and Hahn-Hägerdal, 1997), since it allows an easier control of the sugars directed for yeast metabolism. Furthermore, the use of *S. cerevisiae* strains isolated from industrial harsh conditions presents another advantage in terms of increased tolerance towards the presence of lignocellulosic-derived inhibitors, in comparison to laboratorial strains and non-Saccharomyces yeast (Pereira et al., 2014). In recent studies, the industrial *S. cerevisiae* PE-2, isolated from a first generation bioethanol plant, was found to be naturally prone to xylitol accumulation when expressing the XR/XDH pathway from *S. stipitis* (even using a NADH-preferable XR mutant). This accumulation was partially reverted by the deletion of *GRE3* (Costa et al., 2017; Romani et al., 2015), a gene that codifies an unspecific aldose reductase (using NADPH as co-factor), previously reported to be responsible for xylitol accumulation in *S. cerevisiae* (Traff et al., 2001).

Despite the promising results already obtained with recombinant *S. cerevisiae* for xylitol production (Kim et al., 2017; Lee et al., 2000; Oh et al., 2013), only few work focus on lignocellulosic hydrolysates (Kogje and Ghosalkar, 2016, 2017) and there are no studies focusing on the valorization of both cellulosic and hemicellulosic fractions of the lignocellulosic biomass. Considering this, and the *S. cerevisiae* PE-2 potential for xylitol accumulation, this strain was used as chassis in this study to express the xylose reductase from *P. stipitis*, both the wild type and a mutant with preference for NADH, and to overexpress the endogenous *GRE3* gene, to: (1) evaluate xylitol production in terms of enzyme and co-factor preference, (2) develop an efficient strategy for xylitol production through the valorization of corn cob whole slurry.

Table 1

Yeast strains, plasmids and primers used in this work. Upper case sequences correspond to sequences complementary to the template, and lower case sequences correspond to homologous recombination sites with the desired digested vectors.

<i>S. cerevisiae</i> strains	Relevant Genotype	Reference
CEN.PK 113-5D	MATa; ura3-52	Van Dijken et al. (2000)
PE-2	Diploid; Isolated from bio-ethanol plants in 1994	Basso et al. (2008)
PE-2-XR _{wt}	PE-2, p417-kan	This work
PE-2-XR _{mut}	PE-2, p418-kan	This work
PE-2-GRE3	PE-2, p417-kan-GRE3	This work
CEN.PK 113-5D	CEN.PK 113-5D, p417-kan-GRE3	This work
Plasmids	Relevant Features	
p417	pYPK0_TEF1p_PsXYL1_TDH3t, URA3	Pereira et al. (2016)
p418	pYPK0_TEF1p_PsXYL1(N272D)_TDH3t, URA3	Pereira et al. (2016)
p417-kan	pYPK0_TEF1p_PsXYL1_TDH3t, KanMX4	This work
p418-kan	pYPK0_TEF1p_PsXYL1(N272D)_TDH3t, KanMX4	This work
p417-kan-GRE3	pYPK0_TEF1p_ScGRE3_TDH3t, KanMX4	This work
Primers	Sequence	
KanMX4_FW	ctcagcttaaggagatttggctcatgag CACATACGATTTAGGTGACACTATAGAAC	
KanMX4_RV	catctttgacagcttatcatcgataagctCGACTCACTATAGGGAGACC	
GRE3_TEF1_FW	ggaacgccaggttgccactttctcactagtaaaATGTCCTCACTGGTTAC	
GRE3_TDH3_RV	taaatcctgatcggtttgtctgcacagatggcgcgTCAGGCCAAAAGTGGGG	

2. Materials and methods

2.1. Strains and plasmid construction

The yeast strains used in this study are listed in Table 1. *Escherichia coli* NZy5α (NZYTech) was used for plasmid propagation and maintenance. Yeast DNA transformation was carried out using the LiAC/SS carrier DNA/PEG method (Gietz and Schiestl, 2007). Yeast strains were maintained at 4 °C on YPD plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar). For recombinant yeast strains, liquid and solid YPD media were supplemented with 150 mg/L and 200 mg/L of geneticin (G418), respectively.

For the construction of *S. cerevisiae* PE-2 strains expressing a native *P. stipitis* xylose reductase (XR) and a NADH-preferable xylose reductase mutant (XR-N272D) from *P. stipitis*, the URA3 marker of the p417 and p418 vectors (Table 1) was replaced by the kanMX marker by in vivo homologous recombination: the kanMX geneticin resistance cassette was amplified from plasmid pUG6 (Guldener et al., 1996) with the primers KanMX4_FW and KanMX4_RV and co-transformed with the p417 and p418 vectors digested in the *NheI* restriction site (Fig. 1). Transformants were selected on YPD plates containing 200 µg/mL of G418. The resulting vectors were named p417-kan and p418-kan and the *S. cerevisiae* PE-2 strains carrying them were given the name PE-2-XR_{wt} and PE-2-XR_{mut}, respectively (Table 1). Additionally, to overexpress the *GRE3* gene, a plasmid containing this gene was constructed by homologous recombination from the described above p417-kan. Briefly, the p417-kan plasmid was digested with *AatII*, to remove most of the XR_{wt} gene sequence, and co-transformed with the *GRE3* gene, amplified with primers GRE3-TEF1-FW and GRE3-TDH3-RV from chromosomal DNA of PE-2 strain, originating the plasmid p417-kan-GRE3 (Fig. 1). *S. cerevisiae* PE-2 and CENPK.113-5D transformants containing this plasmid were selected in YPD plates containing 200 µg/mL of G418, and were named PE-2-GRE3 and CENPK.113-5D-GRE3, respectively. The correct recombination between the DNA molecules was confirmed by colony PCR.

Fig. 1. Schematic representation of plasmids construction.

2.2. Inoculum

Yeast cells for inoculation were grown overnight at 30 °C and 200 rpm in Erlenmeyer flasks filled with YPD medium to 40% of their total volume. The cell suspension was collected by centrifugation for 5 min at 3000 rpm, 4 °C and suspended in 0.9% (w/v) sodium chloride solution, in order to achieve a final concentration of 400 g fresh yeast/L. The fermentation experiments were conducted with the concentrated cell suspension, with a cellular concentration from 9 to 11 g fresh yeast/L (corresponding to 3–5 g of dry yeast/L).

2.3. Fermentation experiments

Batch experiments were carried out in complete YP medium (20 g/L peptone and 10 g/L yeast extract) with 30 g/L of xylose and 20 g/L of glucose as carbon source, in 250 mL Erlenmeyer flasks (working volume 40 mL) at 30 °C and 200 rpm.

Fed-batch fermentations were performed in complete YP medium with 30 g/L of glucose and different concentrations of xylose: 64.6 g/L, 126 g/L and 159 g/L and in corn cob hydrolysate medium (supplemented with 20 g/L of peptone and 10 g/L of yeast extract). These experiments were conducted in a 3.7 L Bioengineering's RALF bioreactor (working volume 2 L for synthetic media and 1.5 L for corn cob hydrolysate) at 30 °C, 400 rpm and 2 vvm aeration rate (Lee et al., 2000; Oh et al., 2013). The bioreactor was equipped with a condenser cooled with water to prevent evaporation. The pH-value of 5 was automatically adjusted by addition of NaOH (5 M) or HCl (5 M) solutions. After glucose in the medium was completely depleted, a glucose stock solution of 300 g/L was continuously fed at a flow rate of 4.8 mL/h. Samples were withdrawn at desired times and stored at 4 °C after centrifugation for further analysis of sugars (glucose and xylose) and xylitol and ethanol. Biomass concentration in the media was measured by dry cell weight.

2.4. Corn cob hydrolysate: autohydrolysis pretreatment

Corn cob was collected, milled and submitted to hydrothermal treatment (autohydrolysis) under non-isothermal conditions ($T_{\max}$ of 205 °C, corresponding to a severity of 3.85) based of previous works (Garrote et al., 2008; Rivas et al., 2006) in a 2 L stainless steel reactor (Parr Instruments Company) equipped with Parr PDI temperature controller (model 4848) at liquid to solid ratio of 8 g distilled water/1 g of corn cob oven dry. After treatment, the resulting solid and liquid phases (whole slurry) were separated by filtration. Solid phase (pretreated corn cob) was recovered and washed for Solid Yield (SY) determination. Corn cob and pretreated corn cob were analysed for chemical composition following standard methods described by NREL protocols (NREL/TP-510-42618-42622-4218). Composition of hydrolysates (sugars, acetic acid and furan compounds) was analysed by HPLC.

2.5. Enzymatic saccharification of pretreated corn cob

Enzymatic saccharification of pretreated corn cob was carried out at 30 °C, 200 rpm in an orbital shaker using a percentage of solids of 5 and 10% at different enzyme loadings (6, 12 and 24 FPU/g). Enzyme used in these assays was Cellic CTec2 (kindly supplied by Novozymes, Bagsvaerd, Denmark). Cellulase and hemicellulase activities of Cellic CTec2 were 122 FPU/mL and 9764 U/mL, determined following the procedure described by (Ghose, 1987) and (Bailey et al., 1992), respectively. Samples were withdrawn and analysed by HPLC to determine the glucose and xylose concentration and glucose yield from experiments. Glucose yield was calculated following the equation:

$$\text{Glucose yield (\%)} = \frac{G}{\frac{180}{162} f B} \times 100 \quad (1)$$

where, G is glucose concentration (g/L), B is dry corn cob biomass concentration (g/L), f is glucan fraction in dry biomass (g per g), 180/162 is the stoichiometric factor that converts glucan to equivalent glucose.

2.6. Presaccharification and simultaneous saccharification and fermentation (PSSF and SSF) assays of corn cob whole slurry

Simultaneous saccharification and fermentation (SSF) assays were carried out in 250 mL Erlenmeyer flasks (working volume 40 mL) at 30 °C and 200 rpm in an orbital shaker, using both solid and liquid phases (whole slurry) from corn cob autohydrolysis as substrates. Autohydrolyzed solid phase (pretreated corn cob) was sterilized at 121 °C for 20 min, whereas the liquid phase (corn cob hydrolysate) was subjected to a second step of acid hydrolysis with 0.5% (w/w) H_2SO_4 for 165 min at 125 °C (Rivas et al., 2006). The obtained hydrolysates, enriched in xylose, were neutralized with CaCO_3 until pH 5 and sterilized by filtration (0.2 μm) and aseptically added to autoclaved solid fraction. SSF assays were carried out using 5% solids at 6 and 12 FPU/g. Presaccharification and simultaneous saccharification and fermentation (PSSF) assays were carried out in 250 mL Erlenmeyer flasks (40 mL of working volume) and in a 3.7 L Bioengineering's RALF bioreactor (1.5 L of working volume). Enzymatic saccharification stage of whole slurry (solid and liquid phases) was carried out for 24 h using 5 or 10% solids at 12 or 24 FPU/g. During these PSSF experiments, fed-batch of 5% solids (supplemented with respective enzyme loading) was conducted to further feed glucose. Fig. 2 shows a schematic representation of corn cob processing for xylitol production carried out in this work.

2.7. Analytical methods

Samples from corn cob analysis, autohydrolysis treatment of corn cob (hydrolysate and pretreated corn cob) and from fermentation assays were analysed for quantification of sugars (glucose, xylose, arabinose), acetic acid, xylitol, furfural, hydroxymethylfurfural (HMF) and ethanol by HPLC utilizing a BioRad Aminex HPX-87H (300 $\times$ 7.8 mm) column, at 60 °C, using 0.005 M sulfuric acid as eluent in a flow rate 0.6 mL/min. The peaks corresponding to sugars, acetic acid, xylitol and ethanol were detected using a Knauer-IR intelligent refractive index detector, whereas furfural and HMF were detected using a Knauer-UV detector set at 280 nm.

2.8. Determination of fermentation parameters

Metabolic yield of xylitol from xylose ($Y_{\text{XL}/\text{XY}}$) was defined as g of xylitol produced/g of xylose consumed. Biomass yield ($Y_{\text{X/S}}$) was expressed as g of dry cell/g of glucose consumed. The xylitol productivity (Q_{P_t} , g/L h) was calculated as follows:

$$Q_{\text{P}_t} = \frac{[\text{XL}]_t}{t} \quad (2)$$

where $[\text{XL}]$ is xylitol concentration at time t divided by time t .

3. Results and discussion

3.1. Evaluation of strains for xylitol production in batch fermentation

Several factors interfering with the xylitol production have already been identified, such as specific XR activity, transport of xylose into the cell and generation of reduced cofactors (Meinander and Hahn-Hägerdal, 1997). In this sense, the recombinant strains PE-2-XR_{mut}, PE-2-XR_{wt} and PE-2-GRE3, expressing different xylose/aldose reductases, were compared in terms of xylitol production (Fig. 3, Table 2).

Considering that xylose is not naturally consumed by *S. cerevisiae*, the recombinant strains need a carbon source for cell growth and cofactors regeneration (Hallborn et al., 1991). Therefore, to evaluate the

Fig. 2. Flowchart of main steps involved in the corn cob to xylitol process.

xylose consumption and the xylitol production patterns for the different strains, aerobic batch fermentations were performed on medium containing glucose and xylose (Fig. 3, Table 2). In yeast, xylose uptake occurs by facilitated diffusion through the hexose uptake systems (Hamacher et al., 2002), being competitively inhibited by glucose (Subtil and Boles, 2012). Accordingly, all strains showed xylose uptake only after a considerable decrease in glucose concentration for all tested strains (Fig. 3). After glucose depletion, the ethanol produced during the cultivations was re-assimilated and used as co-substrate for co-factors regeneration by yeast, allowing further xylitol production. Although the cell mass production was similar in all experiments (~20 g/L at 48 h), xylose was converted into xylitol at different rates by the strains: PE-2-XR_{mut} produced 12.7 g/L of xylitol, with 16.3 g/L of xylose remaining in the media (Fig. 3a); while the PE-2-XR_{wt} and PE-2-GRE3 strains produced 24.2 and 27.8 g/L of xylitol, respectively, by conversion of almost all xylose present in the media (Fig. 3b and c). The xylitol yield attained by PE-2-XR_{wt} and PE-2-GRE3 was close to the theoretical (Table 2), however PE-2-GRE3 consumed xylose considerably faster and produced higher amounts of xylitol (28 g/L) with a superior productivity of 0.54 g/L·h at 48 h. Both strains PE-2-XR_{wt} and PE-2-GRE3 express an enzyme with higher specificity for NADPH (Kuhn et al., 1995; Watanabe et al., 2007), while the PE-2-XR_{mut} express an NADH-preferable xylose reductase enzyme. It is well known that the pentose phosphate pathway (PPP), an essential metabolic pathway in the glucose metabolism, is a major source of NADPH (Bruinenberg et al., 1983), and in this case, the use of glucose as co-substrate may result in a superior performance of the strains expressing enzymes with NADPH

preference. Furthermore, the fact that XR_{wt} is capable of using both NADH and NADPH, while the aldose reductase encoded by GRE3 uses solely NADPH, may explain the higher production of xylitol by the PE-2-GRE3 strain. Accordingly, the xylitol production from a GRE3-over-expressing *S. cerevisiae* strain, was already described as a better strategy for xylitol production, in comparison with the expression of XR of *P. stipitis*, in the presence of glucose as co-substrate (Kogje and Ghosalkar, 2016). In fact, the use of different co-substrates could result in different mechanisms of co-factors regeneration and consequently different responses by the enzymes. Another study of comparison of *S. cerevisiae* strains harboring XYLI gene of *P. stipitis* or overexpressing GRE3 gene, using ethanol as co-substrate, reported a superior xylitol production by the recombinant strain expressing the XR, justified by a higher regeneration of NADH during ethanol oxidation to acetate and subsequent metabolism in the TCA cycle (Kim et al., 2002).

CEN.PK 113-5D-GRE3 was used as control to confirm the advantage of using as chassis a natural xylitol accumulating background (Costa et al., 2017; Romani et al., 2015), and despite having consumed more than 90% of xylose in the medium, only produced 19.1 g/L of xylitol. Besides and as expected, this laboratorial strain exhibits a slower carbon source consumption (glucose and ethanol) and consequently, a lower yield (0.68 g/g) and xylitol productivity (0.22 g/L·h) when comparing to the high-rate glucose-consuming industrial PE-2 strain (Pereira et al., 2010). Moreover, additional process advantages are expected when using PE-2 as chassis, namely the tolerance to inhibitors present in hydrolysates (Pereira et al., 2014) that enable the use of non-detoxified hydrolysates simplifying significantly the overall process.

Fig. 3. Performance of the *S. cerevisiae* (a) PE-2-XR_{mut}, (b) PE-2-XR_{wt}, (c) PE-2-GRE3 and (d) CEN.PK 113-5D-GRE3 by the time course of xylose and glucose consumption as well as xylitol and ethanol production.

3.2. Fed-batch fermentations

As observed in the batch fermentations (Section 3.1.), bioconversion stops when the carbon source is depleted, probably due to the lack of reducing power. In this way, to maintain co-factor regeneration and yeast metabolism without catabolite repression of xylose uptake, a glucose-limited fed-batch fermentation strategy has been previously applied for enhancement of xylitol productivity (Bae et al., 2004; Chung et al., 2002; Govinden et al., 2001; Kogje and Ghosalkar, 2016; Lee et al., 2000; Meinander and Hahn-Hägerdal, 1997; Meinander and Hahn-Hägerdal, 1997). Considering that high xylose concentrations may pose as another limitation for xylitol production, the limited-

glucose fed-batch strategy was used in bioreactor to evaluate PE-2-GRE3 xylitol productivity from increasing concentrations of initial xylose (64.6, 126 and 159 g/L) (Fig. 4, Table 2). To attain higher yeast biomass and increase xylitol productivity all fermentations were firstly conducted in batch mode until depletion of the initial 30 g/L of glucose; which was followed by a continuous feeding of low glucose concentrations. The ethanol formed in the batch growth phase remained constant during the fed-batch xylitol production phase while biomass growth was observed even though at a slower rate. In these fed-batch assays, the maximum dry cell mass achieved was of 34.7 g/L (Fig. 4c). In addition, no glucose was detected in the broth during this phase, indicating that glucose gradually supplied was instantly consumed by

Table 2

Main results of batch and fed-batch fermentations of the recombinant *S. cerevisiae* strains in glucose and xylose synthetic media and corn cob lignocellulose hydrolysate (Fed-Batch 4).

Culture	<i>S. cerevisiae</i> strains	Xy _{t0} (g/L)	Xy _{tf} (g/L)	Xy _{l,tf} (g/L)	Y _{Xyol/Xy} (g/g)	Q _{pmax} (g/L·h)
Batch						
1	PE-2-XR _{mut}	30.6 ± 0.8	16.3 ± 0.4	12.7 ± 0.1	0.88 ± 0.03	0.24 ± 0.01
2	PE-2-XR _{wt}	30.6 ± 0.8	5.62 ± 1.30	24.2 ± 0.6	0.97 ± 0.03	0.48 ± 0.03
3	PE-2-GRE3	30.6 ± 0.8	2.57 ± 0.01	27.8 ± 1.4	0.99 ± 0.04	0.54 ± 0.00
4	CEN.PK 113-5D-GRE3	30.6 ± 0.8	2.60 ± 0.55	19.1 ± 0.4	0.68 ± 0.00	0.22 ± 0.01
Fed-Batch						
1	PE-2-GRE3	64.6 ± 0.92	0.86 ± 0.73	62.4 ± 0.02	0.98 ± 0.00	1.44 ± 0.23
2	PE-2-GRE3	126 ± 5.1	1.51 ± 0.09	121.1 ± 3.59	0.98 ± 0.01	1.56 ± 0.15
3	PE-2-GRE3	159 ± 2.08	2.52 ± 0.13	148.5 ± 4.02	0.95 ± 0.01	1.16 ± 0.08
4	PE-2-GRE3	27.2 ± 0.01	5.71 ± 0.01	19.0 ± 0.01	0.89 ± 0.00	0.39 ± 0.01

Xy_{t0} is the xylose concentration at time t₀ = 0 h; Xy_{tf} is the xylose concentration at final time; Xy_{l,max} is the maximum xylitol concentration; Y_{Xyol/Xy} is the xylitol yield from xylose consumed; Q_{pmax} is the maximum productivity achieved in the assays.

Fig. 4. Performance of *S. cerevisiae* PE-2-GRE3 in glucose-limited fed-batch fermentations in YP medium with (a) 65 g/L, (b) 126 g/L and (c) 159 g/L xylose; in (d) corn cob hydrolysate. Xylose and glucose consumption as well as xylitol and ethanol production. The dotted line denoted dry cell weight (DCW). The experimental results were revised for the effects of dilution and sampling on the broth volume.

the yeast. After starting the glucose feed, xylose uptake occurred at constant rate (reaching nearly xylose depletion) and xylitol was produced close to theoretical yields in all the experiments, with 62.4, 121.1 and 148.5 g/L of xylitol being produced from 64.6, 126 and 159 g/L of initial xylose, respectively (Fig. 4). Furthermore, and despite some variation, xylitol productivity does not seem to be diminished by high initial xylose concentrations (Table 2), indicating that there are no saturation of xylose transporter system and no substrate inhibition of the xylose reductase activity.

The xylitol production obtained with this strategy is in the upper range of previous works using recombinant *S. cerevisiae* strains. Oh et al. (2013) with an engineered *S. cerevisiae* for co-utilization of xylose and cellobiose attained 93 g/L of xylitol. Some studies describe a gradual addition of xylose to the culture medium in order to increase xylitol productivity. In fact, Lee et al. (2000), with a recombinant *S. cerevisiae* expressing a xylose reductase from *P. stipitis*, obtained 105 g/L of xylitol with a productivity of 1.69 g/L/h, maintaining a low substrate concentration using a fed-batch strategy with simultaneous addition of xylose and glucose during the bioconversion phase. More recently, Kim et al. (2017) achieved high xylitol productivity in a glucose-limited fed-batch culture with pulsed addition of xylose, producing a maximum of 178 g/L of xylitol. Nevertheless, it should be noted that the strain used expresses the arabinose H⁺ symporter (AraE) from *Bacillus subtilis*, in addition to the *XYL1* gene from *Scheffersomyces stipitis*, which substantially increases xylose uptake (Kim et al., 2017).

In comparison, the PE-2 strain presented in this work, with the sole overexpression of *GRE3*, withstands high substrate loading, efficiently converting high values of initial xylose into xylitol, and presents similar productivities to the ones attained with lower xylose concentrations or

with modifications in the yeast xylose uptake system (Kim et al., 2017; Lee et al., 2000; Oh et al., 2013).

3.2.1. Hydrothermal treatment of corn cob: hemicellulosic hydrolysate

The use of renewable and low cost raw materials as lignocellulosic biomass (including agricultural and forest residues) is mandatory to develop a sustainable bioprocess for xylitol production (Dasgupta et al., 2017; Venkateswar Rao et al., 2016). In this sense, corn cob was selected for its high xylan content (Irmak et al., 2017; Rivas et al., 2002; Ruiz et al., 2013; Venkateswar Rao et al., 2016). The chemical composition of corn cob (expressed in g/100 g wood in oven-dry basis $\pm$ standard deviation based on three replicate determinations) was: 30.89% $\pm$ 0.45 of xylan, 27.32% $\pm$ 0.24 of glucan, 22.92% $\pm$ 0.84 of Klason lignin, 3.52% $\pm$ 0.22 of arabinan, 2.15% $\pm$ 0.03 of acetyl groups and 6.05% $\pm$ 0.22 of extractives. The complex and recalcitrant structure of lignocellulosic materials hinder the access to monomeric sugars (Romaní et al., 2010a). Hydrothermal treatment (also known as autohydrolysis or liquid hot water) followed by dilute acid treatment has been extensively used for the enhancement of cellulose saccharification (Garrote et al., 2008; Romaní et al., 2010b) and to obtain hemicellulosic hydrolysate enriched in xylose (Rivas et al., 2002; Rodríguez-López et al., 2012; Romani et al., 2015). The operational conditions of corn cob processing were selected based on literature (Garrote et al., 2008; Rivas et al., 2002). The solid phase was recovered for the solid yield (56.2 $\pm$ 0.02) determination. Chemical composition of solid phase (expressed in g/100 g of pretreated corn cob in oven-dry basis $\pm$ standard deviation based on three replicate determinations) was: 39.63% $\pm$ 0.75 of glucan, 15.05% $\pm$ 0.45 of xylan, 38.33% $\pm$ 0.88 of Klason lignin and 0.31% $\pm$ 0.01 of arabinan. After

autohydrolysis treatment, 81.5% of cellulose (measured as glucan) and 27.2% of xylan were recovered in the solid phase. On the other hand, composition of hemicellulosic hydrolysate was as follow: 2.25 ± 0.00 g/L of glucose, 27.82 ± 0.38 g/L of xylose, 3.11 ± 0.00 g/L of arabinose, 2.83 ± 0.00 g/L of acetic acid, 1.09 ± 0.27 g/L of furfural and 0.04 ± 0.03 g/L of HMF. Therefore, 68.2% of xylan was solubilized and recovered in the liquid phase (or hemicellulosic hydrolysate) as xylose, after acid hydrolysis of the autohydrolysis liquor. The hemicellulosic hydrolysate was composed mainly by xylose (28 g/L) and acetic acid (2.88 g/L), and its composition was similar to the one reported by Rivas et al. (2002) for sequential stages of autohydrolysis and dilute acid posthydrolysis of corn cob autohydrolysis liquor.

Hemicellulosic hydrolysates obtained from different lignocellulosic biomass (such corn cob, wheat straw, rapeseed straw, brewers spent grain) are the most commonly renewable substrates used for xylitol production (Kumar et al., 2018; López-Linares et al., 2018; Venkateswar Rao et al., 2016). Generally, hemicellulosic hydrolysates for xylitol production are submitted to costly steps of detoxification to improve the fermentation process since most yeast have low tolerance towards inhibitory compounds, such as acetic acid, furfural and HMF (López-Linares et al., 2018; Pereira et al., 2014). In this work, non-detoxified corn cob hydrolysate was used as substrate for xylitol production by the inhibitor-tolerant engineered PE-2-GRE3 strain, under conditions described for the bioreactor assays using synthetic media (Fig. 4a–c). Initial glucose was consumed within 10 h of fermentation and 15 g/L of ethanol was produced from glucose consumed (Fig. 4d). After glucose depletion, hemicellulosic hydrolysate was supplemented with glucose in fed-batch mode. As expected, the xylitol productivity in hemicellulosic hydrolysate (Table 2, fed-batch 4) was lower in comparison with productivities obtained in synthetic media, probably due to the presence of inhibitors such as furfural, HMF and acetic acid that have a negative effect on yeast growth (Perez-Bibbins et al., 2014). As seen in Fig. 4d, 74% of xylose was consumed at 72 h of fermentation with a maximal xylitol production of 17.5 g/L, corresponding to a xylitol yield of 0.89 g/g. These results can be positively compared with the one obtained by a natural xylitol-producing *Candida tropicalis* strain from a non-detoxified hydrolysate, added in fed-batch mode, with a reported yield of 0.7 (Ping et al., 2013). A fed-batch strategy similar to the one used in this work was also proposed by Kogge and Ghosalkar (2017) for xylitol production using a recombinant *S. cerevisiae* XP-RTK strain (overexpressing *GRE3* and a xylose specific transporter from *P. stipitis*), which produced 16 g/L of xylitol from non-detoxified but diluted corn cob hydrolysate with a maximal productivity of 0.21 g/L/h.

3.3. Simultaneous saccharification and fermentation assays of pretreated corn cob for xylitol production

In order to develop an integrated and sustainable process, the solid phase obtained from the autohydrolysis pretreatment and mainly composed by glucan (39.6%) was proposed as co-substrate to supplement glucose for xylose bioconversion into xylitol. Cellulose saccharification of pretreated corn cob for glucose release aims to mimic glucose supplementation in fed-batch mode to keep a basal level of glucose supply. For that, different percentage of solids (5 and 10%) and enzyme loadings (6, 12 and 24 FPU/g) were assayed to evaluate the glucose release by enzymatic hydrolysis. Fig. 5a presents the glucose profile obtained from saccharification of pretreated corn cob biomass. As seen, glucose concentration varied in the range of 6.9–32.9 g/L, corresponding to experiments at 6 FPU/g and 5% of solids and 24 FPU/g and 10% of solids, respectively. Xylose released from the solid by enzymatic saccharification was also quantified (data not shown) achieving concentrations in the range of 3.36–6.25 g/L. Glucose yields of enzymatic saccharification assays are shown in Fig. 5b and varied from 32.4 to

78.8%. The glucose concentration and glucose yield were significantly influenced by the increase of enzyme loading. Considering that glucose at high concentration limits xylose uptake by yeast (Oh et al., 2013), the use of 5% of solid (Fig. 5a) seemed more suitable to maintain a low level of glucose thus to and improve xylitol yield and productivity. Moreover, a previous study using pretreated corn cob (only the solid fraction resultant from alkali pretreatment) for xylitol production by SSF revealed higher yields when using a 5% solid loading (Latif and Rajoka, 2001). Fig. 6a–b shows the SSF assays carried out for xylose bioconversion into xylitol using 6 and 12 FPU/g. As seen, SSF carried out with 6 FPU/g showed an incomplete xylose consumption (54.19%) and a xylitol production of 14.13 g/L (Table 3). Under this condition, the glucose released during enzymatic hydrolysis was not sufficient to allow the complete conversion of xylose into xylitol. On the other hand, the SSF assay at 12 FPU/g and 5% of pretreated corn cob (Fig. 6b) showed a xylose consumption of 89.9% with a xylitol production of 23.2 g/L which corresponded to 0.91 g/g of xylitol yield (Table 3). The increase of enzyme loading allowed a higher cellulose saccharification which improved the xylitol production (1.64 fold higher than xylitol produced by SSF at 6 FPU/g). Xylitol yield in this condition was also considerably higher than others reported for an SSF of the solid fraction of an alkali pretreated corn cob using *S. cerevisiae* and *C. tropicalis* strains (0.71 g/g by *C. tropicalis*, 0.52 g/g by *S. cerevisiae* and 0.69 g/g in co-culture) (Latif and Rajoka, 2001).

A saccharification step before the SSF process, also known as Pre-saccharification and simultaneous saccharification and fermentation (PSSF), is proposed as an alternative strategy for xylitol production from whole slurry of corn cob. This strategy aims to mimic the conditions used for xylitol production from synthetic media (Section 3.2), where the initial glucose concentration is used for yeast biomass growth aiming an increase in xylitol productivity. Fig. 6c shows glucose production during the pre-saccharification step (9 g/L). After this stage, yeast cells were added and glucose was rapidly consumed (< 3 h) resulting in the maximal ethanol production of 4.8 g/L. At 30 h and 54 h, PSSF assay was supplemented with 5% of solid loading (pretreated corn cob) in order to maintain a glucose feed during the bioconversion process of xylose into xylitol. In this experiment, 79.9% of xylose was consumed at 96 h of PSSF process and maximal xylitol concentration of 29.6 g/L with a xylitol yield of 0.93 g/g was achieved (Table 3). Xylitol productivity (not considering the 24 h of pre-saccharification) achieved a maximal value of 0.54 g/L/h, being slightly lower than the maximal productivity obtained in SSF-2 (0.74 g/L/h). Nevertheless, xylitol production was increased compared to SSF-2 experiment (Table 3), reaching 27% higher xylitol concentration. This increase is caused by the addition of xylan-containing solid at different times of PSSF, resulting in a proportional increase of available xylose for conversion.

As promising results were obtained from this strategy, the laboratory scale-up to a 3.7 L bioreactor was evaluated (PSSF-2, Fig. 6d). The initial percentage of pretreated corn cob was increased to 10% (Table 3), to achieve a higher concentration of initial glucose and consequently increase biomass growth, which resulted in the production of 25 g/L of glucose in the 24 h of pre-saccharification step. After yeast addition, glucose was fermented into ethanol achieving a maximal concentration of 16.4 g/L. This unexpected high ethanol yield, which limits glucose use for yeast growth, was probably caused by the bioreactor design that may not be appropriate to work with a moderate-high solid loading (15%), hindering the oxygen mass transfer. Nevertheless, bioconversion of xylose into xylitol started at 28 h. It should be noted that while xylitol concentration progressively increases after 28 h, there seems to be a stabilization of xylose concentration in the medium up until 96 h, which is explained by the gradual release of xylose (ca. 9.39 g/L in total) from the xylan-containing solid loads. Even with the non-optimal conditions of solid load and aeration, more than 76% of available xylose was consumed in 96 h, resulting in the production of 24.3 g/L of xylitol with a yield of 0.88 g/g. Despite the need of additional optimization regarding bioreactor design, air flow

Fig. 5. Time course of: (a) glucose concentration (g/L) and (b) glucose yield (%) in enzymatic saccharification experiments.

Fig. 6. Strategies for xylitol production from corn cob whole slurry by simultaneous saccharification and fermentation (SSF) process: (a) using 5% of solid and 6 FPU/g and (b) 5% of solid and 12 FPU/g and by presaccharification and simultaneous saccharification and fermentation (PSSF) process (added solid in fed-batch): (c) using 15% of solids and 12 FPU/g and (d) 15% and 20 FPU/g in bioreactor. Xylose and glucose consumption as well as xylitol and ethanol production. The arrows indicate the addition of solid.

and solid loading these results pave the way for the possibility of scaling-up xylitol production from lignocellulosic whole slurry envisioning an industrial scale.

4. Conclusions

This work shows that overexpressing the *GRE3* endogenous gene in the robust and innate xylitol accumulation *S. cerevisiae* PE-2 strain enhances xylitol productivity when compared with expression of different

xylose reductases in the same or different yeast chassis. In fed-batch fermentations, with limited-glucose feeding, the PE-2-GRE3 strain efficiently produce xylitol from remarkable high xylose concentrations. In addition, high yields of xylitol from non-detoxified corn cob hydrolysates were attained, in spite of the presence of inhibitory compounds. Furthermore, this work shows, for the first time, the feasibility of using whole slurry corn cob for xylitol production in a simultaneous saccharification process.

Table 3

Operational conditions and main results obtained from simultaneous saccharification and fermentation (SSF) and pre-saccharification and saccharification and fermentation (PSSF) of corn cob whole slurry.

Fermentation (PSSF) of corn cob whole slurry											
Experiment	Operational conditions						Results				
	Solid loading (%)			Enzyme load (FPU/g)			Xpot* (g/L)	Xf (g/L)	Xylitol max (g/L)	Yp/s (g/g)	Qpmax (g/L.h)
	0 h	30 h	54 h	0 h	30 h	54 h					
<i>Batch</i>											
SSF-1	5	–	–	6	–	–	30.58 ± 0.87	14.01 ± 1.21	14.13 ± 0.87	0.85 ± 0.08	0.30 ± 0.01
SSF-2	5	–	–	12	–	–	28.63 ± 0.83	3.14 ± 0.67	23.24 ± 2.14	0.91 ± 0.11	0.74 ± 0.01
<i>Fed-batch</i>											
PSSF-1	5	5	5	12	12	12	39.81 ± 0.97	8.07 ± 0.44	29.61 ± 1.43	0.93 ± 0.04	0.54 ± 0.01
PSSF-2	10	5	–	24	12	–	36.41 ± 0.90	8.63 ± 0.98	24.32 ± 2.37	0.88 ± 0.12	0.30 ± 0.02

* Xpot was calculated considering the sum of xylose concentration in the t₀ of SSF or PSSF with the xylose produced from xylan saccharification.

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