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Boosting bioethanol production from Eucalyptus wood by whey incorporation

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ABSTRACT

The mixture of *Eucalyptus globulus* wood (EGW) and cheese whey powder (CWP) was proposed for intensification of simultaneous saccharification and fermentation (SSF) at high temperature and solid loadings using the industrial *Saccharomyces cerevisiae* Ethanol Red® strain. High ethanol concentration (93 g/L), corresponding to 94% ethanol yield, was obtained at 35 °C from 37% of solid mixture using cellulase and β-galactosidase enzymes (24.2 FPU/g and 20.0 U/g, respectively). The use of CWP mixed with pretreated EGW increased the ethanol concentration in 1.5-fold, in comparison with SSF experiments without CWP for both Ethanol Red® and CEN.PK113-7D strains. Moreover, 1.4-fold higher ethanol concentration was obtained with Ethanol Red®, in comparison with CEN.PK113-7D strain. Ethanol Red® strain was genetically engineered for β-galactosidase production in order to advance towards a fully integrated process. This work shows the feasibility of attaining high ethanol concentrations in second generation bioprocesses by a multi-waste valorization approach.

1. Introduction

Bioethanol is the most widely used biofuel worldwide, partially able to replace fossil fuels, reducing the environmental impact of greenhouse gas emissions (Balat, 2011). Currently, bioethanol is produced from sucrose and/or starch from raw materials such as sugarcane and maize (first generation biofuel) (Rodionova et al., 2017). Nevertheless, the use of these raw materials poses sustainability concerns being necessary the search of alternative renewable raw materials for biofuels production (Menon and Rao, 2012).

Lignocellulosic feedstock is considered an interesting alternative since it is available in amounts high enough to sustain large-scale production of second generation bioethanol (Cai et al., 2017). In comparison with starchy raw materials, lignocellulosic biomass shows a complex structure that hinders the polysaccharide fractionation. Pretreatment is mandatory to improve the accessibility of lignocellulosic sugars. Hydrothermal treatment (also known as autohydrolysis or liquid hot water) is an environmentally-friendly treatment that has been successfully used for the bioethanol production by simultaneous saccharification and fermentation (SSF) from lignocellulosic feedstocks (such as hardwoods and agro-industrial residues) (Romaní et al., 2012; Vargas et al., 2015; Dominguez et al., 2017). Hydrothermal treatment solubilizes hemicellulose fraction yielding a solid phase composed mainly of cellulose and lignin, which limits the final ethanol concentration. For instance, the highest ethanol production obtained from hydrothermally pretreated eucalyptus wood at high solid loading was 67 g/L (Romaní et al., 2012). Thus, one of the limitations of lignocellulose-to-ethanol process is the difficulty of using high solid loading to attain elevated ethanol concentration (required to reduce distillation costs) (Kroppam et al., 2014). The production of ethanol by SSF instead of separate hydrolysis and fermentation (SHF) shows several benefits, such as the reduced investment costs and the reduction of end-product inhibition of enzymatic hydrolysis (Olofsson et al., 2008). Nevertheless, the fermentation temperature is another critical condition for productive simultaneous saccharification and fermentation processes as the optimal temperature for enzymatic hydrolysis is higher than the optimal for yeast fermentation. Hence, robust thermotolerant yeast are mandatory for working under the required intensified conditions (high temperature and solid loadings) (Kelbert et al., 2016).

Other raw materials have been used for ethanol production, such as whey residue obtained from the production of cheese and casein (Jin et al., 2016). Cheese whey can be processed by physical and thermal treatments to obtain whey powder, whey protein concentrate, whey protein isolate and whey permeate or by biotechnological processing to produce value-added compounds such as animal feed, bioprotein, single cell protein (SCP), probiotics, organic acids, biofuels, enzymes, carotenoids, biological gums, exopolysaccharides, and bioplastics (Yadav et al., 2015).

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Whey demand is increasing for manufacturing of whey protein due to high functional and nutritional values with application in pharmaceutical and food industry. Separation of whey protein requires additional steps of ultrafiltration or diafiltration which generate high volumes of a lactose-rich stream (whey permeate). The fermentation of lactose in cheese whey or whey permeate into ethanol is an interesting valorization solution for the important environmental problem caused by this high organic matter stream (Guimarães et al., 2010). The use of cheese whey powder (CWP) or concentrated cheese whey permeate improves the economics of process since high ethanol concentration can be obtained reducing distillation costs (Kargi, and Ozmıhcı, 2006). Besides, CWP has also been used as low-cost protein source to nutritionally supplement lignocellulosic to ethanol fermentations (Tomás-Pejó et al., 2012; Kelbert et al., 2015).

Saccharomyces cerevisiae is the most widely used microorganism in industrial ethanol processes due to its remarkable fermentation capacity (Oliveira et al., 2011; Guimarães et al., 2010; Domingues et al., 2005). However, ethanol production from lactose by *S. cerevisiae* requires the addition of β-galactosidase enzyme, which catalyzes the hydrolysis of lactose into glucose and galactose, as wild-type *S. cerevisiae* strains are unable to consume lactose. One of the strategies to avoid the prior hydrolysis of lactose by commercial enzymes would be to develop a recombinant *S. cerevisiae* strain able to secrete β-galactosidase into the culture medium. This approach has already been reported using laboratory strains as host microorganisms (Domingues et al., 2002; Oliveira et al., 2007). Domingues et al. (2002) constructed a flocculent *S. cerevisiae* strain secreting an extracellular *Aspergillus niger* β-galactosidase, encoded by the lacA gene. For that, the auxotrophic strain NCYC869-A3 was transformed with the plasmid pVK1.1, harboring the lacA gene under the regulation of *S. cerevisiae* ADH1 promoter and terminator. On the other hand, Oliveira et al. (2007) integrated the lacA gene into the genome of NCYC869-wt strain, using the delta sequences from retrotransposon Ty1 as target sites for gene integration. A recombinant NCYC869 strain was obtained (NCYC869/pδneo + lacA), harboring multi-copies of the β-galactosidase expression cassette which were stably maintained after 8 sequential batch experiments. Both strains were able to secrete β-galactosidase at interesting titers. However, in biorefineries yeast cells must perform under harsh conditions, including the presence of inhibitors resulting from biomass hydrolysis. In this context, robust industrial yeast strains are preferred over laboratory strains due to their superior tolerance to these compounds (Pereira et al., 2014; Cunha et al., 2015; Romaní et al., 2015; Costa et al., 2017).

Integration of cheese whey into lignocellulose-to-ethanol processes in a multi-wastes valorization approach could allow the improvement and intensification of fermentation conditions to attain high ethanol concentrations by increasing the carbon source content in the substrate. Recently, this strategy of whey incorporation has been proposed for raw materials from first generation bioethanol such as: wheat flour (Jin et al., 2016), potato tubers and starch (Nakamura et al., 2012). Research works using this approach with lignocellulosic raw materials as substrate are still limited (Oke et al., 2016) and reported final ethanol concentration below 50 g/L (Fischer et al., 2013; Coman et al., 2015; Ferreira et al., 2015). Moreover, integrated bioprocessing, which combines enzyme production, hydrolysis and fermentation in a single process, is recognized as having potential to reduce capital cost involved in lignocellulose-to-ethanol processes (Hasunuma and Kondo, 2012).

In this work, hydrothermally pretreated *Eucalyptus globulus* wood (EGW) was mixed with cheese whey powder (CWP) to produce bioethanol by simultaneous saccharification and fermentation under intensified conditions (high temperature and solid loadings). Operational conditions (cellulase, β-galactosidase and CWP loadings) were optimized for ethanol production using *S. cerevisiae* Ethanol Red® strain and compared with ethanol performance obtained with a laboratory yeast strain (CEN.PK113-7D). In addition, Ethanol Red® strain was genetically engineered for the production of an extracellular β-galactosidase in order to advance towards the development of an integrated and intensified process.

Table 1
Chemical composition of *Eucalyptus globulus* wood (EGW), autohydrolyzed EGW and cheese whey powder (CWP).

a) Chemical composition of EGW and autohydrolyzed EGW, oven-dry basis

	g/100 g of EGW	g/100 g of autohydrolyzed EGW
Glucan	44.7 ± 0.81	63.4 ± 1.49
Xylan	16.0 ± 0.35	0.95 ± 0.25
Arabinan	1.1 ± 0.05	–
Acetyl groups	3.0 ± 0.28	–
Klason Lignin	27.7 ± 0.6	36.0 ± 1.3
Extractives	2.0 ± 0.6	–

b) Chemical composition of cheese whey powder (CWP), (g/100 g oven-dry basis)

Lactose	60 ± 0.58
Protein	12 ± 0.95
Ashes	< 1

2. Material and methods

2.1. Raw materials and analysis of the raw materials

Eucalyptus globulus wood (EGW) was obtained from pulp and paper factory (ENCE, NW Spain), milled to pass an 8 mm screen, air-dried, homogenized and stored in a single lot and dry place until its use. EGW were analyzed for chemical composition (Table 1) following the procedure described by NREL standards (Quantitative acid hydrolysis, NREL-TP-510-42618). Cheese whey powder (CWP) was kindly provided by Lactogal company (Porto, Portugal). Lactose content in CWP was directly analyzed by HPLC with a Aminex HPX-87H column, eluent H_2SO_4 0.005 M at 60 °C and a flow rate of 0.6 mL/min. The crude protein content of CWP was calculated in basis of N determined by Kjeldahl method with appropriated factor 6.25 (Jones, 1941). Ashes was determined by NREL-TP-510-42622 procedure. Chemical composition of CWP was also shown in Table 1.

2.2. Autohydrolysis pretreatment of Eucalyptus globulus wood

EGW was submitted to autohydrolysis treatment. EGW was mixed with water at liquid-to-solid ratio (LSR) of 8 kg water per kg of oven-dry EGW in a pressurized reactor (Parr Instruments Company, Moline, IL). The autohydrolysis treatment was carried out at 150 rpm and heated, following the heating profile (Romaní et al., 2010) up to the desired temperature (T_{MAX} = 230 °C). The operational conditions were chosen on the basis of reported data (Romaní et al., 2012). Once the intended temperature was reached, the experiment was cooled and the liquid and solid phase (autohydrolyzed EGW) were separated by filtration for analysis of chemical composition as described below.

2.3. Strains and cultivation conditions for genetic constructions

Escherichia coli strain NZY5α (Nzytech) used for plasmid construction was grown at 37 °C in Luria-Bertani (LB) medium containing 100 mg/L ampicillin. *Saccharomyces cerevisiae* Ethanol Red® cells were transformed by LiAC/SS carrier DNA/PEG method (Gietz and Schiestl, 2007). After transformation, cells were plated on standard yeast peptone dextrose (YPD) agar plates peptone 2% (w/v), glucose 2% (w/v), yeast extract 1% (w/v) and agar 2% (w/v) supplemented with 200 mg/L geneticin (G418) and X-gal (40 μg/mL) and allowed to grow until colonies appeared. Cells expressing β-galactosidase, i.e., those carrying the plasmid, form blue colonies in X-gal plates.

2.4. Plasmid construction

The pMC1 plasmid was modified from the pVK1.1 plasmid described by Kumar et al. (1992) by inserting a dominant selection marker, the *GEN3* module, allowing the plasmid to be used in prototrophic strains. The *GEN3* module consists of the kanamycin-resistance gene under the control of the *S. cerevisiae* TEF2 promoter and terminator sequences and confers resistance to G418/geneticin. For this, pVK1.1 was digested with *SphI*, removing the *lacA* expression cassette containing the *Aspergillus niger lacA* gene (coding for an extracellular β-galactosidase) flanked by the *S. cerevisiae* ADH1 promoter and terminator. The obtained backbone, YEp24, was then digested with *SalI*. The *GEN3* module was removed from pAGT351 plasmid with *SalI*, digested with *SphI* and ligated to the YEp24 backbone. The *A. niger lacA* cassette was then inserted back into the *SphI* site in YEp24_GEN3. The resulting plasmid was named pMC1 and verified by restriction digestion.

2.5. Strains and cultivation conditions for SSF experiments

The yeast strains used in this work were *Saccharomyces cerevisiae* Ethanol Red®, CEN.PK 113-7D and recombinant Ethanol Red®-pMC1. Wild-type stock cultures were maintained on standard YPD-agar plates at 4 °C and for recombinant Ethanol Red®-pMC1, YPD-agar plates were supplemented with 200 mg/L geneticin (G418). Cells were inoculated in 250 Erlenmeyer flasks with 100 mL of YPD medium at 30 °C and 200 rpm for 15 h. For recombinant Ethanol Red®-pMC1 cells, YPD was supplement with 200 mg/L geneticin (G418). The cell suspension was aseptically collected by centrifugation for 15 min at 8500*g*, 4 °C and resuspended in 0.9% NaCl to a concentration of 200 mg fresh yeast/mL. The fermentation experiments were inoculated with approximately 7.5 mg fresh yeast/ mL.

2.6. Simultaneous saccharification and fermentation: experimental plan and statistical analysis

Simultaneous saccharification and fermentation (SSF) assays of mixture of EGW and CWP were carried out following a Box-Behnken, second-order design based on the three-level incomplete factorial design (3 factors with three replicates of the central point, 15 total experiments). The enzymes used in this work, cellulase (Cellic CTec2) and β-galactosidase (Saphera 2600L), were kindly supplied by Novozymes (Bagsvaerd, Denmark). The Cellic CTec2 was used for saccharification of cellulose (present in pretreated EGW) and Saphera was used for hydrolysis of lactose (present in CWP). The matrix of experimental plan was shown in Table 2. The independent variables studied were:% of cheese whey or x_1, β-galactosidase loading (U/g of lactose in cheese whey) or x_2 and cellulase loading (FPU/g of autohydrolyzed EGW) or x_3. The amount of lignocellulosic substrate (pretreated EGW) was fixed in 25%. The independent variables can be correlated with dependent variables by a second-polynomial equation following the expression:

$$y_i = \beta_{0i} + \beta_{1i}x_1 + \beta_{2i}x_2 + \beta_{3i}x_3 + \beta_{11i}x_1^2 + \beta_{22i}x_2^2 + \beta_{33i}x_3^2 + \beta_{12i}x_1x_2 + \beta_{13i}x_1x_3 + \beta_{23i}x_2x_3 \quad (1)$$

where, yi (i = 1–2) are the dependent variables (corresponding to Ethanol concentration or Ethanol yield); x_1, x_2 and x_3 value of independent variables; β_{0i}, β_{1i}, β_{2i}, β_{3i} are regression coefficients calculated from experimental data by multiple regression using the least-squares method. The experimental data were fitted to the proposed model using commercial software (Statgraphics). The goodness of model fitting was evaluated by the coefficient determination R^2 and the statistical significance by the Fisher's F-test for analysis of variable with a 95% confidence level.

The SSF assays were carried out in a 100 mL Erlenmeyer flask at 35 °C and pH = 5 in an orbital shaker (140 rpm). Pretreated EGW (25% of insoluble solids) and CWP (at soluble solid loading in the range 8.5–25.5%, Table 2) were used as substrates in SSF assays. Autohydrolyzed EGW and water were sterilized at 121 °C for 20 min. CWP was pasteurized at 60 °C for 60 min and added aseptically to autohydrolyzed EGW. Enzymes were added to SSF experiments at loadings shown in Table 2. Samples were withdrawn from experiments at desired times, centrifuged (6000 rpm for 10 min) and analyzed by HPLC for lactose, glucose, galactose and ethanol concentration. Ethanol yield was calculated as follows:

$$EtOH\ yield\ (\%) = \frac{[EtOH]_{max}}{[L]\cdot 0.53 + [fB1.11]0.51}\cdot 100 \quad (2)$$

where, $[EtOH]_{max}$ is the maximal concentration obtained in SSF experiments (g/L), [L] is the concentration of lactose in SSF experiments considering the CWP percentage, 0.53 and 0.51 are conversion factors for lactose and glucose to ethanol based on stoichiometric biochemistry of yeast, respectively. [B] is dry pretreated EGW concentration at the end of SSF assays, f is glucan fraction in pretreated EGW (g/g), 1.111 is the stoichiometric factor that converts glucan to equivalent glucose. Final volume at the end of the SSF experiments was corrected taking into account the solubilized fraction of EGW and CWP during the enzymatic hydrolysis. Considering this, the potential fermentable sugars of SSF assays was in the range of 166–275.5 g/L.

Table 2
Operational conditions (dimensional and dimensionless independent variables): run 1–15 experiments of experimental Box-Behnken design, run 16 and 17 (optimized condition, with and without cheese whey powder) using Ethanol Red® strain and run 18 and 19 (optimized condition, with and without cheese whey powder using CEN.PK113-7D strain).

Run	Dimensional independent variables			Normalized and independent dimensionless variables		
	Cheese whey (%)	β-Galactosidase (U/g)	Cellulase (FPU/g)	x_1	x_2	x_3
1	8.5	5	18.5	−1	−1	0
2	25.5	5	18.5	1	−1	0
3	8.5	20	18.5	−1	1	0
4	25.5	20	18.5	1	1	0
5	8.5	12.5	12	−1	0	−1
6	25.5	12.5	12	1	0	−1
7	8.5	12.5	25	−1	0	1
8	25.5	12.5	25	1	0	1
9	17.0	5	12	0	−1	−1
10	17.0	20	12	0	1	−1
11	17.0	5	25	0	−1	1
12	17.0	20	25	0	1	1
13	17.0	12.5	18.5	0	0	0
14	17.0	12.5	18.5	0	0	0
15	17.0	12.5	18.5	0	0	0
16	10.6	20	24.23	−0.749	1	0.881
17	0	20	24.23	–	1	0.881
18	10.6	20	24.23	−0.749	1	0.881
19	0	20	24.23	–	1	0.881

2.7. Enzymatic activities

The activity of Cellic CTec2 was measured by Filter Paper assay following the procedure described in Ghose (1987), corresponding to 123 FPU/mL. The activity of Saphera was measured as the release of *p*-nitrophenol from *p*-nitrophenyl-*β*-D-galactopyranoside (*p*NPG), as previously described in Domingues et al. (2004). Samples were incubated with 1.7 mmol/dm^3 substrate in 0.075 mol/dm^3 Na acetate buffer, pH 4.5, for 10 min at 65 °C. The pH was raised to 10 with 1 mol/dm^3 Na_2CO_3 and the activity was measured spectrophotometrically at 405 nm on Synergy HT Multi-Mode Microplate Reader (BioTek). The activity of Saphera was 1471.7 U/mL. One unit of activity was defined as the amount of enzyme that hydrolyzed 1 μmol *p*NPG/min at 65 °C. Moreover, activity of β-galactosidase secreted by recombinant Ethanol Red® strain was also measured following the same procedure.

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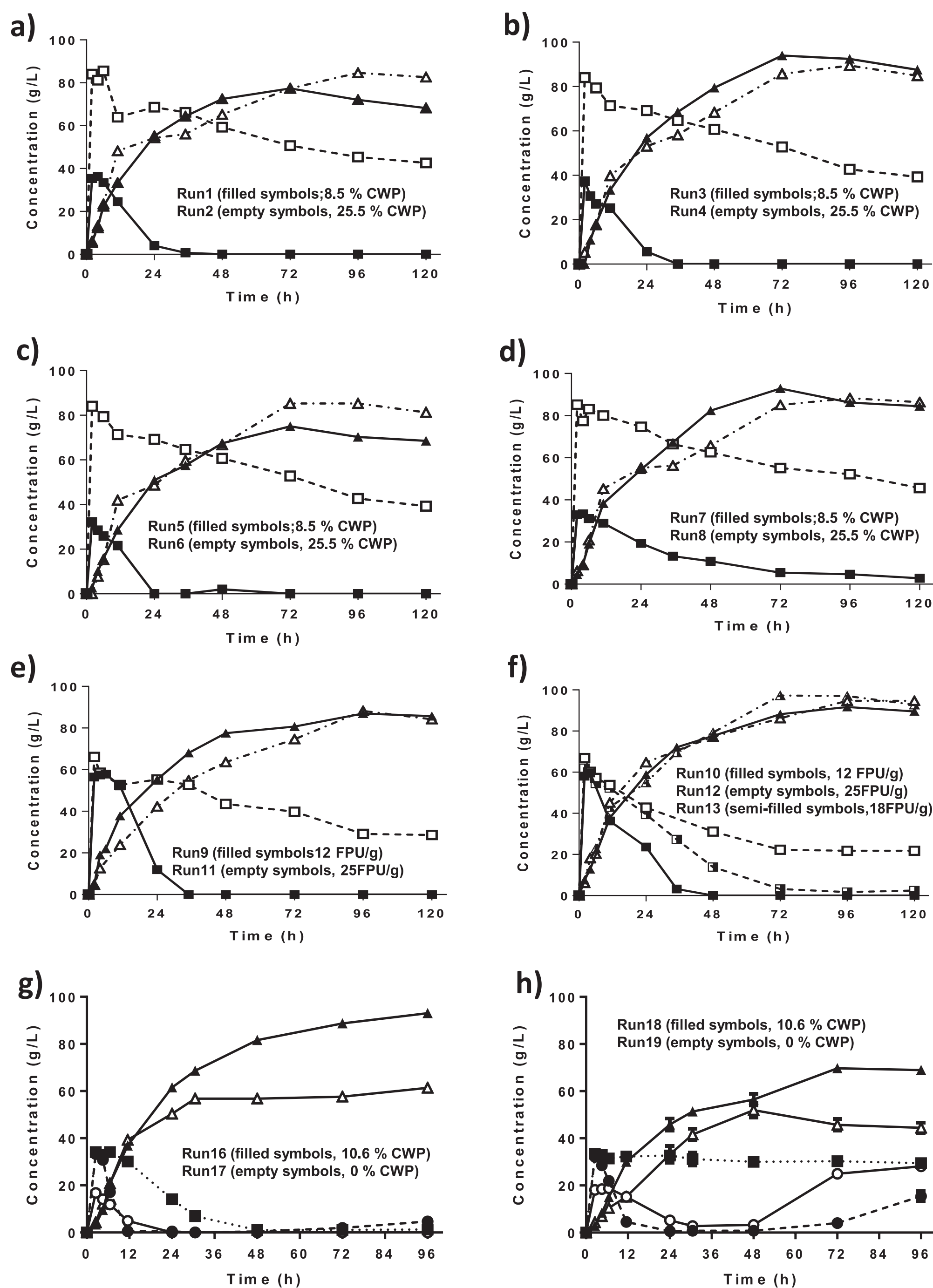

Fig. 1. Time course of galactose (square), glucose (circle) consumptions and ethanol (triangle) production from simultaneous saccharification and fermentation (SSF) assays of pretreated *Eucalyptus globulus* wood and cheese whey powder mixture under operational conditions listed in Table 2: a–f) SSF assays of experimental Box-Behnken design using Ethanol Red® strain; g) SSF assays under optimized conditions with and without cheese whey powder using Ethanol Red® strain, and h) SSF assays under optimized conditions with and without cheese whey powder using CEN.PK113-7D strain.

3. Results and discussion

3.1. *Hydrothermally pretreated Eucalyptus globulus and selection of operational conditions for simultaneous saccharification and fermentation*

EGW was subjected to hydrothermal treatment (or autohydrolysis) at 230 °C (conditions optimized by Romaní et al., 2012). Autohydrolysis treatment solubilizes the hemicellulose fraction and alters the recalcitrant structure of EGW, improving the enzymatic saccharification of cellulose (Romaní et al., 2010). The chemical composition of pretreated EGW was shown in Table 1. The autohydrolysis treatment allowed the glucan enriched of EGW from 44.7 to 63.4%. After treatment, 69.9 g of hydrothermally pretreated EGW per 100 g of raw material were recovered. The glucan recovery was 99.2% considering the glucan present in the raw material. Pretreated EGW was previously used as substrate in SSF optimization at high solid loadings for ethanol production in which 67.4 g/L of ethanol was achieved at liquid to solid ratio of 4 g of liquid per g of pretreated EGW (corresponding to 25% of solids) and Celluclast 1.5 L loading of 16 FPU per g of substrate (Romaní et al., 2012).

Table 3
Sugars (lactose, glucose and galactose) concentration and dependent variables (ethanol concentration and ethanol yield) obtained from simultaneous saccharification and fermentation assays of Eucalyptus wood and cheese whey mixture using Ethanol Red® strain (run 1–17) and CEN.PK113-7D strain (run 18 and 19).

Run	Lactose 0 h (g/L)	Glucose 11 h (g/L)	Galactose 120 h (g/L)	Ethanol concentration(g/L) or *y1*	Ethanol yield (%) or *y2*
1	51.08	0.00	0.00	77.36	82.75
2	154.08	0.00	42.63	82.59	60.96
3	53.66	0.00	0.00	93.98	100.53
4	152.74	0.00	0.00	89.46	66.03
5	51.97	0.24	0.00	75.02	80.24
6	152.07	1.32	12.81	85.32	62.98
7	52.02	0.00	2.79	92.91	99.38
8	151.23	1.91	45.64	88.33	65.19
9	107.24	1.03	0.00	86.95	76.10
10	101.05	0.00	0.00	91.74	80.30
11	102.08	1.30	0.00	88.17	77.17
12	0.00	2.53	21.76	94.69	82.88
13	100.97	0.00	3.00	99.98	87.51
14	101.84	0.00	2.47	97.31	85.17
15	101.23	0.00	1.87	95.35	83.46
16	62.30	0.51	1.29*	93.03	94.30
17	0	0.00	–	61.38	83.72
18	61.86	4.20	29.17*	69.16	70.10
19	0	15.55	–	45.95	62.68

* Galactose concentration (g/L) at 96 h.

In this work, cheese whey powder was mixed with hydrothermally pretreated EGW to provide an additional source of carbon (lactose) and protein in order to intensify the lignocellulose-to-ethanol process. Therefore, the percentage of hydrothermally pretreated EGW was fixed based on the above described work (Romaní et al., 2012). The percentage of CWP and the enzymes loadings (cellulase and β-galactosidase) were evaluated and optimized using an experimental Box-Behnken design. Levels of independent variables evaluated (CWP, cellulase and β-galactosidase) were listed in Table 2. The percentage of CWP varied in the range of 8.5–25.5% corresponding to 5–15% of lactose, these concentrations were chosen based on literature (Guimarães et al., 2008). The range of β-galactosidase (5–20 U/g lactose) and cellulase (12–25 FPU/g of pretreated EGW) loadings were also selected based on published works (Romaní et al., 2014; Jin et al., 2016).

3.2. Co-fermentation of substrates for ethanol production by simultaneous saccharification and fermentation

SSF assays were carried out under conditions listed in Table 2. Time course of galactose consumption and ethanol production are displayed in Fig. 1. The operational conditions evaluated have a clear effect on sugars consumption and ethanol production using the mixture of substrates (EGW and CWP). In all of the evaluated conditions, lactose was hydrolyzed into glucose and galactose within 3 h of SSF process (data not shown). Glucose from lactose hydrolysis was preferably fermented within 11 h of fermentation in all assays (Table 3). This fact is probably due to the catabolite repression phenomenon (Belinchón and Gancedo, 2003; Trigueros et al., 2016). Similar behavior was observed in the co-fermentation of 25% of wheat and 16.7% of cheese permeate in which glucose was totally consumed and galactose uptake was lower (Jin et al., 2016).

In fact, SSF profiles showed clear differences in the galactose consumption depending on lactose concentration. Galactose was fermented by *S. cerevisiae* Ethanol Red® within 24 h of fermentation at the lowest amount of CWP (8.5%) evaluated (Fig. 1a–c, filled symbols). In these SSF assays, co-fermentation of glucose and galactose was clearly observed, being the glucose consumption rate higher than galactose

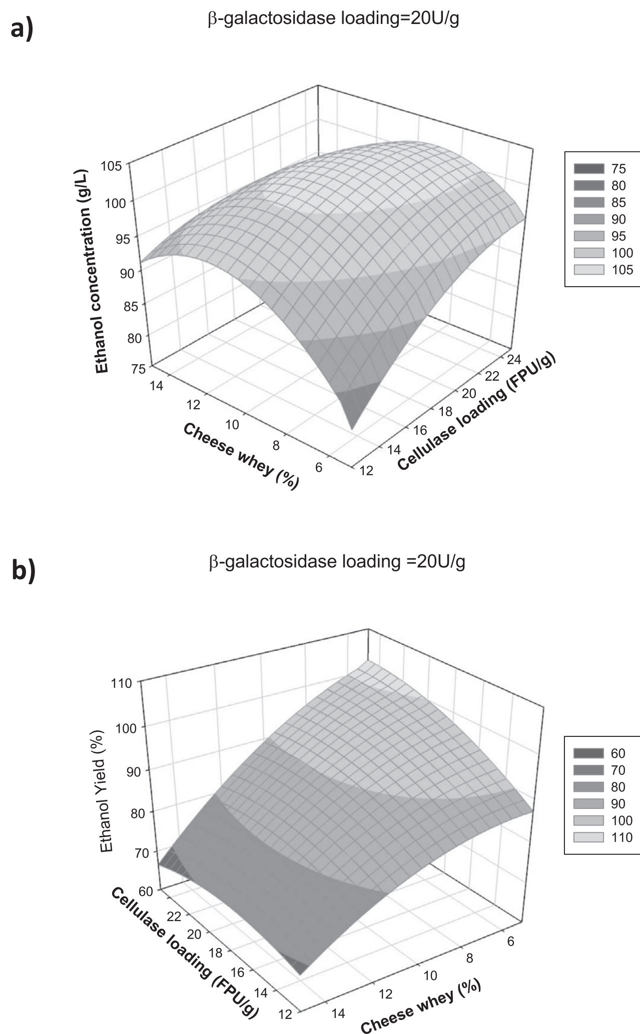

Fig. 2. Response surface as function of cellulase loading and cheese whey percentage (fixed β-galactosidase at 20 U/g of: a) ethanol production in g/L) and b) ethanol yield in %.

uptake rate (Table 3 and Fig. 1). On the other hand, the galactose consumption was lower than 30 g/L when the percentage of CWP was the highest (25.5%, see Fig. 1a–c, empty symbols). For SSF experiments with intermediate CWP percentage (17.0%), galactose was consumed within 120 h of fermentation showing differences depending on the quantity of added cellulase enzyme (12–25 FPU/g). Galactose was totally consumed within 35 h of fermentation in runs 9 and 10 in which the cellulase loading was the lowest (12 FPU/g). In SSF assays (Fig. 1d, runs 11 and 12) where the cellulase loading was the highest (25 FPU/g), galactose was consumed more slowly in comparison with runs 9 and 10 (Fig. 1c). This fact is probably related to a lower saccharification of cellulose when using 12 FPU/g (Romaní et al., 2010), which reduces the glucose concentration in the medium and could improve the galactose consumption by alleviating catabolite repression.

Differences in the galactose consumption were directly related with ethanol production (Fig. 1 and Table 3). The highest ethanol production (99.98 g/L, corresponding to 87.51% of ethanol yield) was achieved at intermediate conditions (run 13 in Table 3). Ethanol concentration higher than 85 g/L was obtained with 17% of CWP, independent of enzyme loadings used. Interestingly, high ethanol concentration (> 90 g/L) was produced with the lowest CWP percentage (8.5%) using cellulase loading of 25 FPU/g (run 3 and 7). Recent studies have shown the importance of the utilization of low cost materials as co-substrate for bioethanol production (Jin et al., 2016; Oke et al., 2016; Parashar

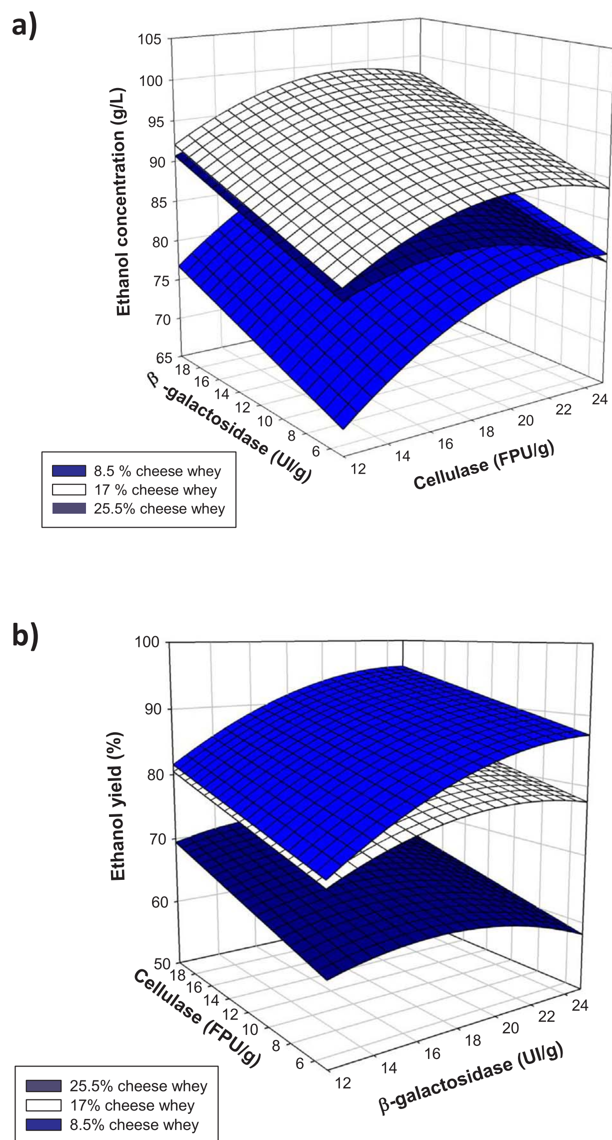

Fig. 3. Response surface as function of cellulase and β-galactosidase loadings (fixed cheese whey powder at 25.5, 17 and 8.5%) of: a) ethanol production in g/L and b) ethanol yield in %.

et al., 2016). However, just a few studies referred this approach in fermentations using lignocellulosic materials (Fischer et al., 2013, Coman et al., 2015; Ferreira et al., 2015). High ethanol concentration (> 90 g/L) obtained in this work can be positively compared with literature using pretreated lignocellulosic biomass mixed with cheese whey. Ferreira et al. (2015) reported a maximal ethanol production of 49.65 g/L, obtained using 80 g/L of pretreated sugarcane bagasse by sequential stages of sulfuric acid and NaOH pretreatments mixed with ricotta whey using *Kluyveromyces marxianus* CCT 7735. In another study, steam exploded sugarcane bagasse (30 g/L) was mixed with milk whey to produce 17.9 g/L of ethanol (Fischer et al., 2013).

3.3. Generalization of models and response surface methodology assessment

Table 3 collected the studied dependent variables in this work (maximal ethanol concentration in g/L (or y_1) and ethanol yield in % (or y_2)), obtained from SSF assays. Experimental variables were correlated following the second-order polynomial Eq. (1) (shown in Section 2.6). The proposed mathematical models describing the CWP percentage (x_1), beta-galactosidase (x_2) and cellulase loadings (x_3) as function and using normalized values are described by Eqs (3) and (4) for ethanol concentration (y_1) and ethanol yield (y_2), respectively.

$$y_1 = 97.548 + 4.351x_2 + 3.133x_3 - 3.723x_1x_3 \quad (R^2 = 0.904\ ;\ F = 5.211) \tag{3}$$

$$y_2 = 85.354 - 13.465x_1 + 4.094x_2 + 3.126x_3 - 4.991x_1^2 - 4.231x_1x_3 \quad (R^2 = 0.964\ ;\ F = 14.959) \tag{4}$$

The statistical significance of the model (based on Fischer's F parameter) showed the good fitting of evaluated variables ($R^2 > 0.9$).

The effect of these independent variables (CWP percentage, cellulase and β-galactosidase loadings) were evaluated by response surface methodology assessment (RSM). RSM is a useful tool for the easy interpretation of independent variables effect on dependent variables (Kelbert et al., 2016). Fig. 2a and b show the effect of cheese whey powder percentage and cellulase enzyme loading on ethanol concentration and yield, with a fixed loading of β-galactosidase at 20 U/g. As seen in Fig. 2a, ethanol concentration higher than 90 g/L was obtained when CWP was > 12.75%, independent of cellulase loading. CWP had a remarkable positive effect on ethanol concentration, while cellulase loading effect was lower. On the other hand, a cellulase loading > 17.9 FPU/g and CWP < 16.2% was necessary to obtain ethanol yields higher than 90%. The increase of cheese whey concentration influenced positively on ethanol concentration (with concentration higher than 100 g/L of ethanol in the range of 12.75–21.25% of cheese whey or 7.5–12.5% of lactose) and negatively on ethanol yield (Table 3). Negative effect of lactose on ethanol yield was also observed by Sansonetti et al. (2010), who optimized the operational conditions of ricotta cheese whey fermentation for ethanol production by *K. marxianus* CBS 397.

Fig. 3 shows the effect of β-galactosidase and cellulase at different cheese whey percentages (25.5, 17 and 8.5%) on ethanol concentration and ethanol yield. Interestingly, lower concentrations of ethanol were achieved operating at 25.5% of cheese whey in comparison with 17% of cheese whey (Fig. 3a). The inhibitory effect of concentrated whey media (lactose concentration in the range of 100–150 g/L) has been reported by several authors (Guimarães et al., 2010). The β-galactosidase and cellulase showed higher effect on ethanol production operating at low CWP (8.5%). This fact might suggest an enzyme inhibition effect due to the high substrate loading (33.3–50.0%) employed in this work. Fig. 3b shows similar ethanol yields (in the range of 70–80%) obtained with 8.5 and 17% of cheese whey with low β-galactosidase loading (12 U/g), independently of cellulase loading.

3.4. Optimization and model validation

In order to optimize the SSF process of EGW and CWP mixture, an optimal condition was calculated to obtain the highest ethanol yield and final titer. For that, dependent variables (y_1 and y_2) were used as response variable for the multiple response optimization of model. The model predicted a maximum ethanol concentration and ethanol yield using the following conditions of operation: 10.6% of cheese whey, 20.0 U of β-galactosidase/g and 24.23 FPU of cellulase/g. The predicted ethanol concentration and ethanol yield were 99.96 g/L and 100%, respectively. To validate these results, additional SSF assay was carried out (Fig. 1g, run 16). Experimental data obtained from validation assay were 93.04 g/L of ethanol concentration corresponding to 94.3 of ethanol yield (Table 2, run 16). These results showed a relative error of 7.44% for ethanol concentration and 6.04% for ethanol yield. These data verify the sustainability of the model for reproducing and predicting the experimental results.

The integration of CWP in lignocellulose-to-ethanol processes increases the source of carbon and provides a source of nitrogen, required

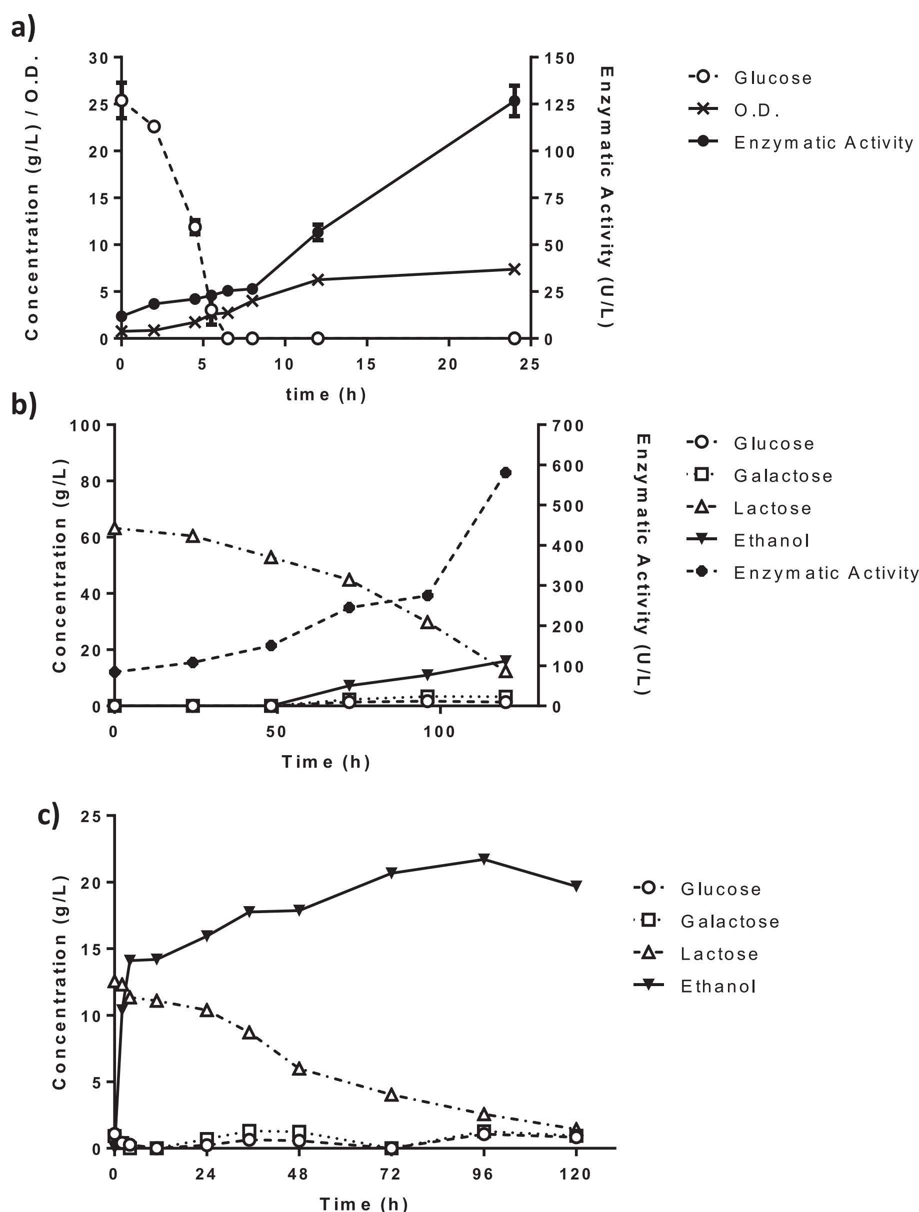

Fig. 4. Fermentation profiles of recombinant Ethanol Red®-pMC1 strain in different culture conditions: a) β-galactosidase production in standard YPD medium (data are average values for two independent experiments); b) β-galactosidase production and lactose consumption in a cheese whey based-medium; c) Simultaneous saccharification and fermentation of 5% of pretreated Eucalyptus wood and 2% of cheese whey at cellulase loading of 24.23 FPU/g.

for a nutritionally balanced fermentation (Kargi, and Ozmıhcı, 2006). In this sense, an additional SSF experiment without CWP was carried out to quantify the improvement of the process with the CWP supplementation (Fig. 1g, run 17). Taking into account the superiority of industrial strains in lignocellulose-to-ethanol fermentation (Pereira et al., 2014; Cunha et al., 2015; Costa et al., 2017), a laboratory *S. cerevisiae* (CEN.PK113-7D) strain was used for comparison under the same conditions with and without CWP supplementation (Fig. 1f, run 18). Main results from these SSF assays under optimized conditions are listed in Table 3. Ethanol Red® strain clearly shows a superior fermentation performance, practically consuming all sugars (Fig. 1g), while 17.0 g/L of glucose and 29.2 g/L of galactose remained to be consumed by CEN.PK 113–7D at the end of the experiment (Fig. 1f). These results show the importance of selecting robust yeast strains for intensified fermentation conditions (such as high temperature and elevated solid loading). On the other hand, higher ethanol concentrations (1.5-fold) were obtained by the two *S. cerevisiae* strains when the cheese whey was mixed with Eucalyptus wood due to the increment of total sugars. Moreover, the addition of cheese whey improved the ethanol yield obtained by laboratory and industrial strains (11.84 and 12.64%, respectively). The mixing of CWP with pretreated EGW also improved the ethanol productivity of SSF. The ethanol productivity at 48 h (Qp_{48h}) for Ethanol Red® was 1.7 g/L h with cheese whey and 1.18 g/L h without cheese whey. On the other hand, the ethanol productivity of CEN.PK 113–7D at 48 h was 1.21 g/Lh with cheese whey and 1.13 g/Lh without cheese whey. Similar ethanol productivity (1.8 g/L h) using cheese whey as sole carbon source and a *K. fragilis* (Kf1), isolated from cocoa fermentation, was reported by Dragone et al. (2011). Ethanol Red® showed better fermentation capacity, consuming glucose and galactose simultaneously, while the laboratory strain was unable to ferment galactose in the presence of glucose, probably due to catabolite repression effect.

3.5. Recombinant Ethanol Red® strain for β-galactosidase secretion: integration in the SSF of EGW and CWP mixture for ethanol production

Considering the obtained results for the SSF process of substrate mixture at 35 °C and high solids loading with Ethanol Red® strain, we selected this industrial strain as host for recombinant β-galactosidase secretion in order to advance towards an integrated process. Ethanol Red® was transformed with the pMC1 plasmid, a multi-copy expression vector based on pVK1.1, containing the β-galactosidase expression

cassette and a dominant selection marker (*GEN3*), allowing its use in prototrophic strains. β-Galactosidase expression by Ethanol Red®-pMC1 was firstly assessed on shake flask experiments in standard YPD medium (Fig. 4a). As expected, glucose was rapidly exhausted (24 g/L under 7 h) and β-galactosidase activity levels reached approximately 130 U/L at the end of the experiments. The recombinant strain was then assayed in a cheese whey powder based-medium (30 °C and 200 rpm) in order to quantify the enzyme production and its capacity to hydrolyze lactose and produce ethanol, as depicted in Fig. 4b. 60 g/L of lactose were hydrolyzed in 125 h, being glucose immediately consumed, while galactose consumption was slower. β-Galactosidase activity levels reached almost 600 U/L at the end of the experiment, while ethanol concentration was just below 20 g/L (Fig. 4b). Domingues et al. (2002) and Oliveira et al. (2007) obtained higher β-galactosidase expression levels in Semi Synthetic lactose 5% (SSLactose) with recombinant strains NCYC869-A3/pVK1.1 and NCYC869/pδneo + lacA, around 2000 U/L and 25 g/L ethanol. However, as already mentioned, in order to develop an intensified process, robust industrial strains are required.

The β-galactosidase activity levels obtained with recombinant Ethanol Red®-pMC1 strain were very low in comparison with those used in the experimental design. In this sense, the use of recombinant strain in SSF process for fermentation of EGW and CWP mixture was carried out with 5% of EGW and 2% of cheese whey (corresponding to 1.2% of lactose) in order to increase the enzyme to substrate ratio. Fig. 4c shows time course of SSF assays with CWP and EGW mixture. Lactose was consumed within 120 h of fermentation. No galactose accumulation was observed in the medium (< 1.3 g/L), which shows a good performance of recombinant strain for both sugars (glucose and galactose) uptake. Ethanol concentration achieved 21.7 g/L (corresponding to 96% of ethanol yield). Under these operational conditions, results obtained from simultaneous saccharification and fermentation of renewable sources (CWP and EGW) mixture were quite satisfactory proving the feasibility of this consolidated approach. The level of β-galactosidase expression should now be fine-tuned in the recombinant yeast. In addition, β-galactosidase secretion can also be optimized by process conditions (Castro et al., 2012). To the best of our knowledge, this is the first time that an integrated strategy using recombinant industrial strain for multi-waste valorization into bioethanol was carried out.

4. Conclusions

SSF process was optimized to obtain high ethanol concentration (93 g/L corresponding to 94% of ethanol yield) using a mixture of pretreated EGW and CWP under a multi-wastes valorization approach. The integration of cheese whey in EGW fermentation together with the use of a robust thermotolerant yeast strain boosted the ethanol concentration in 1.5-fold comparing to results obtained without CWP. Furthermore, and for the first time, an industrial strain was engineered for β-galactosidase production to evolve towards an integrated process. Recombinant strain successfully fermented lactose from cheese whey, as well as, the mixture of eucalyptus wood and cheese whey.

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