Lignocellulosic bioethanol production with revalorization of low-cost agroindustrial by-products as nutritional supplements

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ABSTRACT

During the pretreatment of lignocellulosic biomass for second generation bioethanol production, fermentation inhibitors are released. To overcome this, the use of a robust industrial strain together with agro-industrial by-products as nutritional supplementation was proposed to increase ethanol productivity and yields. Two factorial experimental designs were carried out to optimize fermentation of hydrolysate from autohydrolysis of *Eucalyptus globulus*. The most influential variables on ethanol production were cheese whey and $K_2O_5S_2$ (potassium metabisulfite) supplementation. Nutrient addition effect was demonstrated using the whole slurry from autohydrolysis in two process configurations (separate hydrolysis and fermentation, SHF and simultaneous saccharification and fermentation, SSF). Comparing the supplemented SHF and SSF assays with non-supplemented, 2.3 and 7.4 fold higher ethanol concentrations were obtained, respectively. In the case of SSF, 50.4 g L⁻¹ of ethanol concentration and 92.2% of ethanol conversion were attained, demonstrating an improved fermentation performance in industrial lignocellulose fermentations.

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1. Introduction

Bioethanol from lignocellulosic materials (LCMs), also called second generation bioethanol, is considered a promising alternative to first generation bioethanol (obtained from feedstock such as sugar cane, corn, wheat or barley) (García et al., 2014). The LCMs, as *Eucalyptus globulus* (EGW) wood, are renewable and widespread raw materials constituting the world's largest bioethanol renewable resource. An efficient bioethanol production process from lignocellulose involves an effective pretreatment of LCM to improve the accessibility of cellulose and thus enhance the enzymatic saccharification (Buruiana et al., 2014; Singh and Bishnoi, 2013). One interesting approach is to use the whole slurry from treatment, since allows economical and industrial benefits: washing steps are avoided, water consumption is lower and the sugars from liquid phase can be used, increasing ethanol concentration (Tengborg et al., 2001). However, during the pretreatment step some compounds (such as furans, phenolic compounds and weak acids) are produced. Furfural, hydroxymethylfurfural and phenolic compounds result from degradation of pentoses, hexoses and

lignin, respectively and the acetic acid is released in the deacetylation of hemicelluloses (Klasson et al., 2013). These compounds have an inhibitory effect on the microorganisms used for hydrolysate fermentation (reviewed by Palmqvist and Hahn-Hägerdal, 2000a).

In this sense, the success of lignocellulosic biomass fermentations is necessarily dependent on the ability of the yeast strains to cope with the different stresses imposed during these processes. In biomass-based fermentations, yeast cells, besides having to tolerate the presence of the above-referred inhibitors, are also exposed to nutrient starvation and the absence of oxygen (Pereira et al., 2011a). To overcome the problem of toxicity, several strategies have been considered such as a biological or chemical detoxification step prior to fermentation (reviewed by Palmqvist and Hahn-Hägerdal, 2000b) or the improvement of resistance of the organism itself (Heer and Sauer, 2008). Another possible strategy can be the optimization of fermentation environment in order to minimize the toxic effects of inhibitors (Huang et al., 2011). Supplements utilization is an alternative to facilitate the microorganism growth and fermentation in an inhibitory medium (Tomás-Pejó et al., 2012). Taking into account that the nutrient sources in the lignocellulosic hydrolysate are present in low amounts, it is important to optimize the nutritional supplementation for the improvement of fermentation performance. Several authors have reported that the use of supplements

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Fig. 1. (a) Steps for supplementation and fermentation medium optimization using EGW hydrolysate (b) Mass balance and application of the optimized supplementation to whole slurry fermentations.

is able to increase the production yield and productivity of ethanol, with or without stress conditions (Pereira et al., 2010; Jørgensen, 2009; Dragone et al., 2004). In this context, the use of residues or by-products generated in the agro-food industry is an attractive approach since it contributes to re-valorization of these residues and increases the overall economic process feasibility. Different agro-industrial residues, such as corn steep liquor (CSL), raw yeast extract (RYE), cheese whey (CW) and Urea, have been individually studied as supplements for fermentation processes supplying complex nutrients and nitrogen to the medium (Jørgensen, 2009; Salgado et al., 2009; Gullón et al., 2008). On the other hand, nutrients in minor amounts and supplied in salts, as magnesium, calcium and zinc have influence in the rate of sugar fermentation and are necessary as cofactors for several metabolic pathways (Pereira et al., 2010).

In this context, this work aims for the intensification of EGW hydrolysate fermentation processes combined with re-valorization of agro-industrial residues as low-cost nutritional supplements, in order to attain faster and higher ethanol production. For that, a previously selected industrial robust strain of *Saccharomyces cerevisiae* PE-2 (Pereira et al., 2014) was used for efficient fermentation. The addition of nutritional sources was evaluated, optimized and demonstrated in multiple process configurations such as separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) of pretreated *Eucalyptus globulus* wood.

2. Materials and methods

2.1. Diagram representation of experimental work

Fig. 1 shows the structure of experimental work carried out in this study. The raw material used was obtained from autohydrolysis

treatment of EGW. To study supplement effect on inhibitory medium, fermentations were performed using only hydrolysate (containing the inhibitory compounds) instead of the whole slurry. Despite the whole slurry being the most close to reality approach, the occurring of enzyme inhibition by sugars, previously analyzed in recent works (Romaní et al., 2014), could interfere with the experimental results, masking inhibitor and/or supplement effects and interfering with the models obtained in the experimental design. The hydrolysate was complemented with 101 g L⁻¹ of glucose and employed for optimization of nutritional supplementation (complex nutrients and salts) in a two-step approach as represented in Fig. 1a. Finally, the optimized nutritional supplement was added in the saccharification and fermentation of whole-slurry fermentations (hydrolysate + autohydrolyzed EGW) for ethanol production, as represented in Fig. 1b.

2.2. Autohydrolysis of *Eucalyptus globulus* wood: hydrolysate and pretreated solid

EGW was previously analyzed by Pereira et al. (2014), containing (expressed in g/100 g of raw material on dry basis, average values of three replicate ± standard deviation): 44.7% ± 0.81 of glucan; 16.0% ± 0.35 of xylan; 1.1% ± 0.05 of arabinan; 3.0% ± 0.28 of acetyl groups; 27.7% ± 0.6 of Klason lignin and 2% ± 0.2 of extractives. EGW was submitted to autohydrolysis treatment. EGW was mixed with water at liquid-to-solid ratio (LSR) of 8 kg water kg⁻¹ of oven-dry raw material in a pressurized reactor (Parr Instruments Company, Moline, IL). The autohydrolysis treatment was carried out at 150 rpm and heated, following the heating profile (Garrote et al., 2008) up to reach the desired temperature ($T_{MAX} = 210^{\circ}\text{C}$). The operational conditions were chosen on the basis of reported

Table 1

Experimental design employed to evaluate the nutritional supplementation with Corn Steep Liquor (CSL), Cheese Whey (CW), Raw Yeast Extract (RYE) and Urea in EGW-hydrolysate and results obtained (ethanol concentration at 24 h, E_{24h}).

Independent variables (g L^{-1})					Dependent variables (g L^{-1})
Run	CSL (x_1)	CW (x_2)	RYE (x_3)	Urea (x_4)	E_{24h} (y_1)
1	0.0	0.0	10	0.75	15.83
2	20	0.0	10	0.75	2.420
3	0.0	20	10	0.75	47.70
4	20	20	10	0.75	28.91
5	10	10	0.0	0.00	38.89
6	10	10	20	0.00	26.93
7	10	10	0.0	1.50	48.83
8	10	10	20	1.50	23.58
9	10	10	10	0.75	37.65
10	0.0	10	10	0.00	49.66
11	20	10	10	0.00	6.380
12	0.0	10	10	1.50	47.89
13	20	10	10	1.50	4.830
14	10	0.0	0.0	0.75	34.14
15	10	20	0.0	0.75	44.30
16	10	0.0	20	0.75	3.640
17	10	20	20	0.75	50.05
18	10	10	10	0.75	34.51
19	0.0	10	0.0	0.75	46.47
20	20	10	0.0	0.75	50.63
21	0.0	10	20	0.75	32.49
22	20	10	20	0.75	4.860
23	10	0.	10	0.00	3.190
24	10	20	10	0.00	51.08
25	10	0.	10	1.50	3.820
26	10	20	10	1.50	51.16
27	10	10	10	0.75	43.32
28	10	10	10	0.75	50.40
29	10	10	10	0.75	48.68
30	10	10	10	0.75	47.96

data (Romaní et al., 2010a). Once the intended temperature was reached, the experiment was cooled and the liquid (hydrolysate) and solid phase (autohydrolyzed EGW) were separated by filtration for analysis of chemical composition (see Section 2.8). Hydrolysate was used as media in fermentation experiments for optimization of nutritional supplementation.

2.3. Microorganism, medium and yeast cultivation

The strain used in this work was *Saccharomyces cerevisiae* PE-2, isolated from Brazilian bioethanol industry and previously shown to withstand EGW-hydrolysate inhibitory conditions (Pereira et al., 2014). Stock cultures were maintained on agar YPD plates [bacteriological peptone 2% (w/v), glucose 2% (w/v), agar 2% (w/v) and yeast extract 1% (w/v)], at 4 °C. Cells were inoculated in 1 L Erlenmeyer flasks filled with 400 mL of YPD medium (50 g L⁻¹ glucose, 20 g L⁻¹ peptone and 10 g L⁻¹ yeast extract) at 30 °C and 150 rpm for 24 h. The cell suspension was aseptically collected by centrifugation for 15 min at 8500 × g and 4 °C, suspended in 0.9% NaCl to a concentration of 200 mg fresh yeast mL⁻¹. The fermentation experiments were inoculated with approximately 5 mg fresh yeast mL⁻¹.

2.4. Fermentations of supplemented EGW-hydrolysate

In order to intensify ethanol production, a two-step approach as represented in Fig. 1a was performed to optimize fermentation media, using inhibitory hydrolysate medium. In a first step, the use of complex nutrients was studied, which allowed the formulation of a complex supplemented medium. In the second step, the effect of salt addition to the previously obtained medium was studied, in order to further enhance yeast performance and ethanol production.

2.4.1. Study of complex nutrients supplementation by Box–Behnken design

The study of complex nutritional supplementation of hydrolysate was carried out using a Box–Behnken design. The complex nutrients used in this work were: corn steep liquor (CSL), raw yeast extract (RYE), cheese whey (CW) and Urea. The experimental design contained three blocks and a central point with three replicates (30 total experiments). The ranges of variables are listed in Table 1 and were selected based on literature (Gomes et al., 2012; Li et al., 2011; Maddipati et al., 2011; Pereira et al., 2010, 2012; Silva et al., 2010; Jørgensen, 2009; Silveira et al., 2001).

The 60% of EGW-hydrolysate, diluted with water in volumetric proportion, was supplemented with 101 g L⁻¹ of glucose and complex nutrients, according to the amounts described in Table 1. Glucose and hydrolysate were autoclaved separately (121 °C, 20 min) and mixed during 30 min. The RYE was kindly provided by a microbrewery (Fermentum, Portugal), dried at 60 °C until no weight variation, crushed and sieved. CW was kindly provided by Quinta dos Ingleses (Agro-Livestock Company, Portugal), and used directly. CW and RYE were pasteurized at 65 °C for 30 min and added aseptically to the hydrolysate. CSL and urea solution were sterilized by autoclaving (121 °C, 20 min) and added aseptically.

Fermentation experiments were performed at 30 °C in an orbital shaker (150 rpm) using 100 mL Erlenmeyer flasks (fixed volume of 30 mL) fitted with perforated rubber stoppers enclosing glycerol-filled air-locks to allow exhaustion of CO₂ while avoiding entrance of air. The pH of fermentation media was adjusted to 4.7 with 1 M NaOH. Fermentations were monitored by measurement of mass loss in the Erlenmeyer flasks through weighing, equivalent to CO₂ production in the fermentation and directly related to fermentation rate (glucose conversion to ethanol and CO₂) considering the process stoichiometry and the fermentation time. Glucose and ethanol concentrations were determined from samples withdrawn at 0 h

Table 2Experimental design employed to evaluate the salts addition in EGW-hydrolysate and results obtained (CO₂ exhaustion at 16 h).

Run	Independent variables (g L ⁻¹)							Dependent variable (g L ⁻¹)
	MgSO ₄	CuSO ₄	CaCl ₂	ZnCl ₂	FeSO ₄	KH ₂ PO ₄	K ₂ O ₅ S ₂	
1	0.0	0.00	0.0	0.01	0.0072	2	0.000	35.06
2	5.0	0.00	0.0	0.0	0.00	2	0.334	42.54
3	0.0	0.075	0.0	0.0	0.0072	0	0.334	12.60
4	5.0	0.075	0.0	0.01	0.00	0	0.000	3.267
5	0.0	0.00	0.8	0.01	0.00	0	0.334	44.52
6	5.0	0.00	0.8	0.0	0.0072	0	0.000	35.33
7	0.0	0.075	0.8	0.0	0.00	2	0.000	5.983
8	5.0	0.075	0.8	0.01	0.0072	2	0.334	2.180
9	2.5	0.038	0.4	0.005	0.0036	1	0.167	17.12
10	0.0	0.00	0.0	0.01	0.0072	2	0.000	39.97
11	5.0	0.00	0.0	0.0	0.00	2	0.334	44.16
12	0.0	0.075	0.0	0.0	0.0072	0	0.334	10.04
13	5.0	0.075	0.0	0.01	0.00	0	0.000	2.660
14	0.0	0.00	0.8	0.01	0.00	0	0.334	45.43
15	5.0	0.00	0.8	0.0	0.0072	0	0.000	35.83
16	0.0	0.075	0.8	0.0	0.00	2	0.000	7.947
17	5.0	0.075	0.8	0.01	0.0072	2	0.334	0.917
18	2.5	0.038	0.4	0.005	0.0036	1	0.167	15.39

and 24 h by HPLC. The experiments were stopped when the fastest fermenting condition of the set entered stationary phase, showing no weight variation.

2.4.2. Study of salts supplementation by Plackett–Burman

The effect of salts addition to the supplemented hydrolysate was evaluated using a Plackett–Burman design with central point and two replicates (total number of 18 fermentation trials). The salt concentrations were selected based on literature (Cao and Liu, 2013; Geddes et al., 2013; Hashem and Darwish, 2010; Nieves et al., 2011; Niladevi et al., 2009; Pereira et al., 2011b; Jørgensen, 2009; Silveira et al., 2001). For this, the optimized fermentation medium with complex nutrients (obtained in Section 2.4.1) was used as substrate and the addition of different salts was carried out under conditions listed in Table 2. The salts solutions (except K₂O₅S₂) were sterilized by autoclaving (121 °C, 20 min) and added aseptically to the medium. The K₂O₅S₂ was sterilized by filtration (0.22 μm).

The fermentation experiments were carried as described in Section 2.4.1. The fermentation profile was measured by weight loss equivalent to CO₂ production during time-course of fermentations, allowing evaluation of fermentation rates. Samples were collected and analyzed by HPLC for ethanol and glucose concentration.

2.5. Statistical analysis

The STATISTICA 7 software was used for generation, analysis and optimization of the experimental design, the ethanol and CO₂ production were studied as response variables.

2.6. Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) supplemented with optimized nutrients

In order to evaluate the impact of nutritional supplementation strategy on overall process yields, two strategies of saccharification and fermentation (SHF and SSF) were conducted.

For SHF experiment, the saccharification of autohydrolyzed solid was carried out in an orbital shaker at 150 rpm, 48.5 °C and pH 4.8, using commercial enzymes (“Cellic Ctec2” cellulases and “Cellic HTec2” hemicellulases), which were kindly provided by Novozymes (Bagsvaerd, Denmark). The cellulase activity of Ctec2 was 120 FPU mL⁻¹, measured as described by Ghose, (1987). β-Glucosidase and hemicellulase activity for Ctec2 and HTec2 were 779.8 UI mL⁻¹ and 1690 UI mL⁻¹, respectively (Paquot and Thonart,

1982; Bailey et al., 1992). In the SHF experiment, the enzyme loadings were 22.5 FPU g⁻¹ and 500 U g⁻¹ of cellulase and hemicellulase, respectively. The enzymatic saccharification was performed mixing the pretreated solid and hydrolysate at liquid to solid ratio (LSR) of 6.4 g g⁻¹. The liquid was composed by 60% of hydrolysate to maintain the same conditions of experimental design. The operational conditions of enzymatic saccharification were chosen on basis of previous results (Romaní et al., 2014). The hydrolysate and pretreated solid were sterilized separately and mixed aseptically. After 96 h of saccharification, the medium (containing glucose from cellulose and hydrolysate) was separated from solid phase by centrifugation (15 min at 8500 × g) and used as fermentation media. The fermentation conditions were the same as described in Section 2.4.

The SSF was carried out at 35 °C and pH = 4.8 (using 0.05 N of sodium citrate buffer) in 100 mL Erlenmeyer flasks with orbital agitation (150 rpm). For comparative reasons, the loadings of enzymes and solids employed in SSF and SHF were the same. The SSF assay was inoculated with 8 mg fresh yeast mL⁻¹ of final medium. The reaction time of SSF was between 0 and 120 h. At the desired times, samples were withdrawn from the media, centrifuged, filtered and analyzed by HPLC for ethanol and glucose concentration. The glucan conversion (GC) into glucose by enzymatic hydrolysis was calculated as determined by the National Renewable Energy Laboratory (NREL) protocols, 2004 (NREL/TP-510-42630):

$$\% \text{Glucan Conversion} = \frac{[\text{Glucose}] + 1.053 [\text{Cellobiose}]}{1.111 \times f [\text{Biomass}]} \times 100\% \quad (1)$$

where [Glucose] is the glucose concentration at time t (g L⁻¹); [Cellobiose] is the concentration of cellobiose (g L⁻¹) at time t , in the experiments cellobiose was not detected; f is the glucan fraction of dry biomass (g g⁻¹) [Biomass] is dry biomass or LCM concentration at the beginning of the experiment.

2.7. Determination of fermentation parameters

Ethanol conversion (EC, %) was calculated as it is described the NREL protocols, 2004 (NREL/TP-510-42630):

$$\% \text{Ethanol Conversion} = \frac{[\text{EtOH}]_f - [\text{EtOH}]_0}{0.51 \cdot f [\text{Biomass}] \times 1.111} \times 100\% \quad (2)$$

where [EtOH]_f is the ethanol concentration at the end of the fermentation (g L⁻¹) minus any ethanol produced from the enzyme and medium; [EtOH]₀ is the ethanol concentration at the beginning of the fermentation (g L⁻¹) which should be zero; [Biomass]

is dry biomass or LCM concentration at the beginning of the fermentation (g L^{-1}); f is the glucan fraction of dry biomass (g g^{-1}); 0.51 is the factor for glucose to ethanol based on stoichiometric biochemistry of yeast and 1.111 is the conversion factor of glucan into equivalent glucose

The volumetric productivity (Q_p , $\text{g L}^{-1} \text{h}^{-1}$) was also determined and was calculated as:

$$Q_p = \frac{E_t}{t} \quad (3)$$

where E_t is the ethanol concentration (g L^{-1}) at time t , t is the necessary time (h) to reach the stationary phase.

2.8. Analytical methods

An aliquot of hydrolysate was analyzed by HPLC for acetic acid, ethanol, furfural, glucose and hydroxymethylfurfural (HMF) determination with a Varian MetaCarb 87H column, eluent H_2SO_4 0.005 M at 60°C at a flow rate of 0.7 mL min^{-1} using a refractive-index detector and UV detector. A second aliquot was analyzed for oligosaccharides determination by quantitative post-hydrolysis procedure (with 4% H_2SO_4 at 121°C for 20 min). The chemical composition of autohydrolyzed EGW was analyzed as described in Romani et al. (2010b) for glucan, xylan and Klason lignin quantification. Total phenolic compounds of hydrolysate were determined as described in Conde et al. (2011) by absorbance measurement at 720 nm of the complex formed with the Folin–Ciocalteu reagent. A standard curve with caffeic acid was used for quantification of phenolic compounds, expressed as caffeic equivalents.

3. Results and discussion

3.1. Autohydrolysis pretreatment: Autohydrolyzed Eucalyptus globulus wood (EGW) and hydrolysate

The autohydrolysis treatment allows the solubilization of hemicellulose fraction of EGW and the alteration of its recalcitrant structure. The chemical composition of autohydrolyzed EGW was 59.26, 1.95 and 33.60% of glucan, xylan and Klason lignin, respectively. The glucan and Klason lignin were almost recovered quantitatively in the solid phase (95 and 86%, respectively). On the other hand, the xylan was solubilized (9% of xylan remained in the pretreated solid). The composition of liquid phase after the pretreatment (hydrolysate) was 0.64 g L^{-1} of glucose; 8.85 g L^{-1} of xylose; 0.18 g L^{-1} of arabinose; 3.11 g L^{-1} of acetic acid; 0.33 g L^{-1} of hydroxymethylfurfural; 1.66 g L^{-1} of furfural; 1.15 g L^{-1} of glucosyloligosaccharides; 8.97 g L^{-1} of xylooligosaccharides; 2.55 g L^{-1} of acetyl groups and 2.01 g L^{-1} of phenolic compounds. The main components of hydrolysate were xylose and xylooligosaccharides that represented 60.5% of total identified compounds in the liquid phase. These results are in agreement with previously reported data (Romani et al., 2010a,b). Taking into account the percentage of these sugars in the liquid phase, their fermentation would be of upmost interest to increase process yields, and several efforts are being made currently to obtain robust yeast strains capable of fermenting all sugars present in the hydrolysate (Demekke et al., 2013). However, as observed for glucose, xylose fermentations can only be feasible if yeasts are able to cope with the inhibitors present in the hydrolysate, which can be facilitated by proper nutritional balance of the fermentation medium.

3.2. Study of hydrolysate fermentation supplemented with complex nutrients

In order to provide optimal nutritional supplementation to enhance inhibitor resistance, the effect of complex nutrients (CSL,

CW, RYE and Urea) on the hydrolysate fermentation was evaluated using a Box–Behnken experimental design. The operational conditions can be seen in Table 1.

Time course of fermentations was followed by measurement of mass losses in culture flasks, corresponding to CO_2 production and exhaustion in the glycerol lock system. Mass loss data allowed the fermentation profiles construction, which showed the differences in CO_2 production among the experiments during time (represented in Fig. S1, Supplementary data) related to the subsequent differences in fermentation rate. Fermentations assays finished at 24 h, when the stationary phase was achieved in the fastest fermenting cultures. Samples were collected at the chosen time (24 h) for ethanol quantification (included in Table 1). The concentration of ethanol varied in the range $2.4\text{--}51.2 \text{ g L}^{-1}$ (experiments 2 and 26, respectively).

The experimental design allowed mathematical modelling of ethanol production depending on supplement concentrations used, with a second degree polynomial regression expressed (as it is expressed in the following equation):

$$y_j = b_{0j} + \sum_{i=1}^2 b_{ij}x_i + \sum_{i=1}^2 \sum_{k \geq i}^2 b_{ijk}x_i x_k \quad (4)$$

where y_j ($j=1$ to 2) is the dependent variable; x_i or x_k (i or k : 1 to 3, $k \geq i$) are the normalized, independent variables (defined in Tables 1 and 2), and $b_{0j} \dots b_{ijk}$ are regression coefficients calculated from experimental data by multiple regression using the least-squares method.

Regression coefficients are listed in Table 3 and it can be observed that the relation between supplement concentration and ethanol production was well described by the empirical model ($R^2 = 0.8763$ and predicted- $R^2 = 0.761$). The significance of regression coefficients was determined by p -value. Data of p -value < 0.10 , < 0.05 and < 0.01 indicate a significant level of 90%, 95% and 99%, respectively. These results showed the influence of each factor and the interaction among factors. On basis of results observed in Table 3, the most influent variable on ethanol production was CW with $p = 0.00$ (representing $> 99.99\%$ of the significance level). CSL and RYE also had a significant effect ($> 99\%$) on ethanol concentration, nevertheless their addition had a negative influence on the studied variable.

For a better results interpretation, a response surface was represented (Fig. 2). Fig. 2a shows the variation of ethanol concentration at 24 h with independent variable CW and CSL fixed the variables Urea and RYE in 0.86 and 4.1 g L^{-1} , respectively. The maximal concentration of ethanol (54.97 g L^{-1}) was achieved for values of $\text{CW} > 16 \text{ g L}^{-1}$ and concentration of $\text{CSL} < 6 \text{ g L}^{-1}$. CW showed high influence on ethanol concentration in all of the experiments.

Table 3
Effects of nutrient sources on the ethanol concentration after 24 h of fermentation.

	Coefficient	Standard error	t-Value	p-Value
b_{0j}	43.75	3.660	11.960	0.000
b_{1j}	−11.84	2.590	−4.570	0.000
b_{11j}	−9.520	3.420	−2.780	0.014
b_{2j}	17.51	2.590	6.770	0.000
b_{22j}	−9.750	3.420	−2.850	0.012
b_{3j}	−10.14	2.590	−3.920	0.001
b_{33j}	−1.180	3.420	−0.340	0.735
b_{4j}	0.330	2.590	0.130	0.900
b_{44j}	−7.250	3.420	−2.120	0.051
b_{12j}	−1.340	4.480	−0.300	0.768
b_{13j}	−7.950	4.480	−1.770	0.096
b_{14j}	0.060	4.480	0.010	0.990
b_{23j}	9.060	4.480	2.020	0.061
b_{24j}	−0.140	4.480	−0.030	0.976
b_{34j}	−3.320	4.480	−0.740	0.470

Fig. 2. Response surfaces of calculated ethanol concentration: (a) calculated dependence of ethanol concentration at 24 h (E_{24h}) on Cheese Whey (CW) and Corn Steep Liquor (CSL); (b) calculated dependence of ethanol concentration at 24 h (E_{24h}) on Urea and Raw Yeast Extract (RYE).

Ethanol concentration decreased with increasing of CSL, as can be observed in Fig. 2a. Moreover, Fig. 2b represents the effect of Urea and RYE on the ethanol production fixing the variables CW and CSL in 16.5 and 5.79 g L⁻¹, respectively. As can be seen in Fig. 2b, it was not observed a significant effect of Urea on ethanol concentration at 24 h of fermentation. The maximal concentration of ethanol was obtained for intermediate values of Urea and RYE. This influence can be related to the supplement complex composition and a possible synergetic effect between all supplements during fermentation trial. Besides being a source of protein and nitrogen, the complex supplements used in this study also have salts, organic acids and vitamins in their constitution (Guimarães et al., 2010). Walker (2004), described the effect of metal ions in yeasts, identifying K and Mg as key minerals for growth and ethanol production, accompanied by Zn, Ca, Cu, and Fe among other microelements, all of them present in the complex supplements used in this study. Furthermore, as reported by Chandrasena et al. (1997), the synergetic effect attained between these metal ions in culture media is of upmost importance for ion interaction in yeast performance

Fig. 3. Profiles of CO₂ production obtained in data from the complex supplemented EGW-hydrolysate medium with K₂S₅O₂ (■), without K₂S₅O₂ (◆) and control without any supplementation (▲).

reporting an optimal ion combination to maximize ethanol production, which can justify the difference in the effects and impacts observed between all supplements. Taking this into account, it can be considered that effects obtained in the model reflect a nutritional balance that goes beyond nitrogen addition, including synergies between supplements and their composition in minerals and other compounds, where CW could have the most advantageous constitution for yeast fermentation performance.

The predicted supplement concentrations for maximal ethanol production at 24 h (54.97 g L⁻¹) included low amount of CSL and RYE (5.79 and 4.1 g L⁻¹, respectively), higher concentrations of CW (16.5 g L⁻¹) and intermediate concentration of urea (0.86 g L⁻¹). To validate the model and the values obtained, an assay using optimal concentrations was conducted. Fermentation with supplemented hydrolysate was conducted in parallel with non-supplemented hydrolysate to illustrate the experimental supplementation effect, presented in Fig. 3. As it can be seen in the results, the use of supplementation increased significantly yeast performance in comparison with the non-supplemented control. Supplemented culture was able to reach stationary phase in less than 20 h, while non-supplemented culture was still in exponential phase. Ethanol concentration in the supplemented assay at 24 h was 49.06 ± 2.31 g L⁻¹, exceeding the value of 33.13 g L⁻¹ obtained in the non-supplemented hydrolysate for the given time. The calculated relative error for the optimized assay was 10.67% (or 5.87 g L⁻¹, absolute error). These data confirmed the sustainability of the model for reproducing and predicting the experimental results. A percentage of 30% more ethanol concentration was obtained in the optimized supplemented medium in comparison with the fermentation without addition of nutrients. In this sense, for the fermentation of hydrolysate with inhibitors, the composition of medium has a great effect on the fermentation performance in conformity with previous data (Tomás-Pejó et al., 2012), representing an efficient and low cost solution for industrial application.

3.3. Evaluation of salt addition to the optimized medium with complex nutrients

To further intensify ethanol production from the EGW hydrolysate, the effect of mineral salts addition was studied using a Plackett–Burman design, using the previously optimized supplemented medium as basis for the individual screening. Salts screening was carried out using the experimental plan described in

Table 4

Values of significance (based on a *t*-test) of the regression coefficients and statistical parameters measuring the correlation and significance of the model developed for mineral salts screening on ethanol fermentation.

Term	Coefficient	Standard error	<i>t</i> -Value	<i>p</i> -Value
b_{0j}	22.27	0.75–7	29.42	0.000
b_{1j}	–2.166	0.803	–2.697	0.022
b_{2j}	–17.33	0.803	–21.58	0.000
b_{3j}	–0.760	0.803	–0.946	0.366
b_{4j}	–1.277	0.803	–1.590	0.142
b_{5j}	–1.536	0.803	–1.913	0.085
b_{6j}	–0.682	0.803	–0.849	0.416
b_{7j}	2.271	0.803	2.828	0.018

Section 2.4.2 under the conditions listed in Table 2. For the analysis of results, the dependent variable studied was the CO₂ production at 16 h, given that it reflects directly the variations observed in the fermentation rates (time courses of fermentation are shown in Fig. S2, Supplementary data). As described in Section 3.2, the dependent variable (CO₂ at 16 h) was correlated with independent variables (mineral salts) for the evaluation of their effect on the fermentation performance using Eq. (5). An adequate adjustment of model was obtained considering that the measuring of correlation (*R*²) was 0.989 and predicted-*R*² of 0.966. Moreover, Table 4 shows regression coefficients and statistical significance (based in the *p*-value). The coefficients of salts (MgSO₄, CuSO₄, and K₂O₅S₂) were significant at the 95% (*p*-values < 0.05). On the other hand, FeSO₄ had a significant effect >90%, the other variables (CaCl₂, ZnCl₂ and KH₂PO₄) did not show a significant effect (*p*-value > 0.1) on the dependent variable. Only the presence of K₂O₅S₂ salt had a positive effect on CO₂ production at 16 h, as shown by the coefficients obtained in the model. The absence of positive effects and even the negative impact of most salts is coherent with the previously discussed for the complex nutrients. Salt addition effects obtained in the model suggest that further addition of salts is not necessary and even disturbs the nutritional balance optimized previously. Nevertheless, the favorable influence of K₂O₅S₂ can be due to the capacity to reduce or minimize the hydrolysate toxicity to the yeast cells. Geddes et al. (2013) reported that the combination of pH 9 ammonia treatment of hydrolysate and addition of K₂O₅S₂ 1.5 mM reduced toxicity sufficiently to allow the improvement of fermentation performance with media prepared using 100% of hydrolysate. Moreover, according to Nieves et al. (2011), the addition of K₂O₅S₂ (0.5 mM or 1 mM) in fermentation with 30% of the hydrolysate using *E. coli* mutants, generated a substantial improvement in hydrolysate tolerance, minimizing inhibition of growth and fermentation. Additionally, these authors suggested that other toxins than furfural are being neutralized.

Considering the results obtained for K₂O₅S₂ in the experimental design, a validation assay was carried out adding this salt to the optimized medium with complex nutrients. Simultaneously, a non-supplemented fermentation was carried out for comparative reasons. Time course of fermentations is shown in Fig. 3 in which the effect of salt addition can be seen. The addition of K₂O₅S₂ to the previously optimized supplemented medium allowed further improvement of the process, leading to a significant decrease on fermentation time (of about 5 h) for the same ethanol production obtained with only complex nutrients, having a subsequent impact on process productivity. Overall, the results obtained demonstrate that the use of low cost complex nutrients along with K₂O₅S₂ addition improved significantly the fermentation performance of PE-2 strain in the fermentation of EGW hydrolysate under high stress conditions. Ethanol concentration and productivity were improved and process efficiency was pushed towards industrial feasibility.

3.4. Application of optimized complex and salt nutrients on SHF and SSF configurations

For a more close-to-reality approach, the autohydrolyzed EGW was used as source of glucose by the saccharification of cellulose present in pretreated EGW. In order to evaluate the efficiency of the optimized nutritional supplementation (containing CSL, CW, RYE, Urea and K₂O₅S₂), in configurations suitable for industrial application, its nutritional effect was tested on two second generation ethanol productions using distinct process configurations. The strategies employed were: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The saccharification and fermentation experiments were carried out under conditions mentioned in Section 2.6, in which enzymatic hydrolysis was evaluated for saccharification of whole slurry from autohydrolyzed EGW and the results obtained of glucose concentration saccharification yield were satisfactory. Additionally, a fermentation assay without supplementation was carried out for comparison and validation of supplementation effect.

3.4.1. Separate hydrolysis and fermentation (SHF)

The autohydrolyzed EGW was mixed with 60% of hydrolysate. This slurry (hydrolysate + solid) was saccharified by enzymes obtaining a fermentable medium composed by 88.42 g L^{–1} of glucose, 18.60 g L^{–1} of xylose, 2.6 g L^{–1} of acetic acid, 0.2 g L^{–1} of HMF and 0.57 g L^{–1} of furfural after 96 h of saccharification. These results show a high yield of saccharification (GC = 94.4%) with an elevated concentration of glucose. Fig. 4a shows the results obtained during SHF experiments. As it can be observed, the CO₂ production was much faster in supplemented medium, reaching the stationary phase within 14 h of fermentation corresponding to a high volumetric productivity (*Q_p*) of 2.7 g L^{–1} h^{–1}. The experiment without nutrient supplementation achieved the stationary phase at 27 h (see Fig. 4a) with a *Q_p* of 1.58 g L^{–1} h^{–1}. As it can be seen in Fig. 4a, the fermentation with nutrient supplementation showed an improvement on fermentation rate, as observed for CO₂ production at 14 h being 2.3 fold more when compared with the non-supplemented fermentation. The ethanol production after 14 h was calculated in basis of CO₂ concentration in the experiments. In the supplemented experiment with complex and salt nutrients, 39.6 g L^{–1} of ethanol was obtained while in the non-supplemented assays 17.1 g L^{–1} of ethanol was achieved. Thus, the utilization of the optimized supplement in the SHF assay leads to a more efficient fermentative performance. The supplemented assay obtained a higher Ethanol Conversion (EC) than the non-supplemented experiment, of 88 and 38%, respectively. Similar concentration of ethanol was obtained from steam exploded corn stover supplemented with CSL (40 mL L^{–1}) using an engineered strain for resistance, nevertheless 1.45 lower productivity was achieved (Li et al., 2011).

3.4.2. Simultaneous saccharification and fermentation (SSF)

The fermentative production of ethanol from LCM can be carried out in a single step (SSF) which presents more advantages than SHF such as: (i) decreased product inhibition, (ii) limited operational costs and (iii) decreased contamination risks (Sassner et al., 2008). Fig. 4b shows ethanol and glucose concentrations of SSF experiments (with and without complex nutrients). In the non-supplemented experiment 65.6 g L^{–1} of glucose and 6.8 g L^{–1} of ethanol were obtained at 120 h. These data indicate that strain was not able to ferment all glucose present in the medium without nutritional supplementation and therefore, EC was low (13.0%). On the other hand in experiment with supplementation, the glucose was practically consumed after 6 h, since it was not observed glucose accumulation. The ethanol obtained in the supplemented SSF assay was 50.4 g L^{–1}. Comparing the maximum ethanol production in both experiments, 7.41 fold more ethanol was produced in

Fig. 4. (a) Profiles of CO₂ production assay with (■) and without (●) supplementation in Separate Hydrolysis Fermentation (SHF); (b) Profiles of glucose (□) and ethanol (■) concentrations with supplementation and glucose (○) and ethanol (●) concentrations without supplementation in Simultaneous Saccharification and Fermentation (SSF).

experiment with nutrient supplementation. EC of nutritional supplemented SSF assay was 96.1% showing a good ethanol production performance. Remarkably, the supplementation strategy followed rendered the SSF process feasible with satisfactory ethanol yields and productivity. Without supplement utilization, SSF fermentation was compromised, as shown by glucose accumulation during the time course of fermentation.

Finally, comparing the results obtained in SHF and SSF, the supplemented assays showed overall higher ethanol concentration. Overall, supplement effect was highlighted for multiple process configurations demonstrating the importance of the described supplementation in the feasibility of 2nd generation ethanol production from EGW.

4. Conclusions

An intensified process of 2nd generation bioethanol was attained by addition of agro-industrial by-products and salts as nutritional supplements. The optimized supplementation in SSF and SHF lead to high fermentation performances with high ethanol yields (96 and 88%, respectively). Ethanol production in the

supplemented SSF and SHF processes was 7.41 and 2.3 fold higher comparing with non-supplemented assays. Overall results demonstrate that low-cost nutritional strategy allowed the intensification (in case of SSF) and even the unblocking (in case of SHF) of second generation ethanol production from EGW-hydrolysate, representing a technology for further application to other raw-materials and fermentation processes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.indcrop.2014.10.056>.

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