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Simultaneous Saccharification and Fermentation of Hydrothermal Pretreated Lignocellulosic Biomass: Evaluation of Process Performance Under Multiple Stress Conditions

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Abstract Industrial lignocellulosic bioethanol processes are exposed to different environmental stresses (such as inhibitor compounds, high temperature, and high solid loadings). In this study, a systematic approach was followed where the liquid and solid fractions were mixed to evaluate the influence of varied solid loadings, and different percentages of liquor were used as liquid fraction to determine inhibitor effect. Ethanol production by simultaneous saccharification and fermentation (SSF) of hydrothermally pretreated *Eucalyptus globulus* wood (EGW) was studied under combined diverse stress operating conditions (30–38 °C, 60–80 g of liquor from hydrothermal treatment or autohydrolysis (containing inhibitor compounds)/100 g of liquid and liquid to solid ratio between 4 and 6.4 g liquid in SSF/g unwashed pretreated EGW) using an industrial *Saccharomyces cerevisiae* strain supplemented with low-cost byproducts derived from agro-food industry. Evaluation of these variables revealed that the combination of temperature and higher solid loadings was the most significant variable affecting final ethanol concentration and cellulose to ethanol conversion, whereas solid and autohydrolysis liquor loadings had the most significant impact on ethanol productivity. After optimization, an ethanol concentration of 54 g/L (corresponding to 85 % of conversion and 0.51 g/Lh of productivity at 96 h) was obtained at 37 °C using 60 % of autohydrolysis liquor and 16 % solid loading (liquid to solid ratio of 6.4 g/g). The selection of a suitable strain along with nutritional supplementation enabled to produce noticeable ethanol titers in quite restrictive SSF operating conditions, which can reduce operating cost and boost the economic feasibility of lignocellulose-to-ethanol processes.

Keywords Inhibitor compounds · High temperature · High solid loading · Fermentation lignocellulosic biomass · Industrial strain · Hydrothermal treatment

Introduction

Nowadays, the use of renewable biomass to supply the increasing energetic needs and to partially replace fossil fuels is recognized as a suitable alternative to attain a sustainable growth based on a bioeconomy. Liquid fuel (as bioethanol) from lignocellulosic biomass is a promising solution since this raw material is renewable, widespread, and with a huge potential for the manufacture of products, without competing with food crops [1]. In order to achieve a cost-effective lignocellulosic bioethanol production process, industrial lignocellulosic fermentations depend on overcoming specific challenges that differ from conventional food fermentations [2]. These limiting conditions are related with the stages involved in the lignocellulosic process to produce ethanol.

Firstly, a pretreatment is necessary to break down the recalcitrant structure of lignocellulosic feedstock. Hydrothermal treatment, as autohydrolysis or liquid hot water, uses water as the only reaction medium and is considered an environmentally friendly pretreatment that improves enzymatic saccharification of lignocellulosic biomass and solubilizes the

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hemicellulosic fraction into oligosaccharides [3, 4]. Nevertheless, the autohydrolysis liquor (liquid phase after pretreatment) from hydrothermal processing also comprises sugar- and lignin-derived compounds (furans, weak acids, and phenolic compounds), considered inhibitors of enzymes and microorganisms used on saccharification and fermentation processes, respectively [5, 6].

Secondly, the whole slurry (liquor and pretreated biomass altogether) can be submitted to simultaneous enzymatic saccharification and fermentation (SSF) process. The saccharification and fermentation carried out in one stage presents more advantages than in separate steps, such as the risk of contamination and sugar inhibition effect are lower and the use of one reactor reduces operational cost [7]. In SSF process, thermotolerance is one of the most desired features of the fermentative microorganism, as high temperature (50 °C) is required for efficient enzymatic saccharification of cellulose to glucose, being the optimal operating temperature for *Saccharomyces cerevisiae* (main yeast used in ethanol fermentation) in the range of 25–30 °C [8]. In addition, the use of high-temperature in industrial fermentation processes can also lead to energy saving by (i) the reduction of cooling costs (mainly in tropical countries where the temperature varies between 30–40 °C throughout the year), (ii) reducing the viscosity leading to lower energy requirements for the homogenization of the fermentation medium, and (iii) facilitating ethanol recovery [2, 9, 10].

Finally, a distillation of the SSF medium, for ethanol purification, is required as the last stage of the process. An industrial process that operates at high solid loading leads to a final ethanol concentration higher than moderate-low loadings, reducing distillation cost and water consumption [11]. For this purpose, ethanol concentration should be >4 % (*w/w*) which corresponds to a lignocellulosic biomass loading of >20 % (*w/w*) for saccharification and fermentation [12]. Nevertheless, the high solid loadings hamper substrate mixing and consequently lead to poor mass transfer.

Together, the combination of these process challenges (inhibitor compounds, high temperature, and solid loadings) can lead to synergistic effects on enzyme and yeast with considerable impact on overall process performance. These synergistic effects could have a higher negative effect than a single factor on ethanol production being a necessary robust microorganism with a stress-tolerant ethanologenic background, which could make the difference to attain feasibility of lignocellulosic bioethanol process [13, 14].

In recent works, *S. cerevisiae* industrial strain PE-2, isolated from Brazilian fuel ethanol industry, has shown noteworthy fermentation efficiency and stress tolerance during industrial fermentation [15–18], showing high ethanol tolerance for very high gravity fermentation [19, 20], inhibitor resistance [16], and high cell viability at temperatures above 35 °C [21]. Moreover, optimized nutritional fermentation media can minimize the toxic effects of inhibitor compounds [22]. The previous work improved the slurry fermentation yield by nutritional supplementation using agro-industrial byproducts (corn steep liquor, cheese whey, and yeast extract supplemented with urea and $K_2O_5S_2$), increasing 2.4 and 7.4-fold ethanol production on separate and simultaneous saccharification and fermentation, respectively [23].

This work aims to investigate and optimize lignocellulosic simultaneous saccharification and fermentation under limiting process conditions (high temperature, inhibitor compounds, and high solid loadings) following the experimental procedure scheme proposed in Fig. 1. For that, the whole slurry from hydrothermal treatment of *Eucalyptus globulus* wood (EGW) was used at different proportions for addressing the solid loading and inhibitor effect. Autohydrolysis liquor (AL) from hydrothermal pretreatment (containing inhibitor compounds) at different percentages (60–80 g of AL/100 g of liquid in SSF) was mixed with unwashed and not dried pretreated EGW at different liquid/solid ratios (4–6.4 g of liquid in SSF (containing different percentage of AL)/g of pretreated EGW) under temperatures in the range 30–38 °C using an industrial robust *S. cerevisiae* strain (PE2) and supplemented with optimized low-cost agro-industrial byproducts in a simultaneous saccharification and fermentation process.

Materials and Methods

Raw Material

EGW was collected in a local paper and pulp factory, milled to pass an 8-mm screen, homogenized and stored in a dark and dry place until use. EGW was assayed for extractives (NREL/TP-510-42618), ashes (NREL/TP-510-42622), and structural carbohydrate and lignin (NRL/TP-510-42618). The composition of raw material (expressed in g/100 g EGW oven-dry basis) was previously analyzed by Pereira et al. [16] and listed in Table 1.

Autohydrolysis Pretreatment of EGW

The whole slurry (constituted by autohydrolysis liquor and pretreated EGW solid) used in this study was obtained from hydrothermal pretreatment of EGW (Fig. 1). For that, 800 g of water was mixed with 100 g of wood (corresponding to initial solid loading of 12.5 % *w/w*) in a stainless pressurized reactor at maximal temperature (T_{max}) of 210 °C under non-isothermal conditions [24]. The percent of solid loading after treatment was 8.65 g of pretreated EGW solid/100 g of AL. After treatment, pretreated EGW solid was separated from liquid phase (autohydrolysis liquor) by vacuum filtration and washed for characterization of chemical composition and solid yield (SY) determination. The glucan, xylan, and acetyl

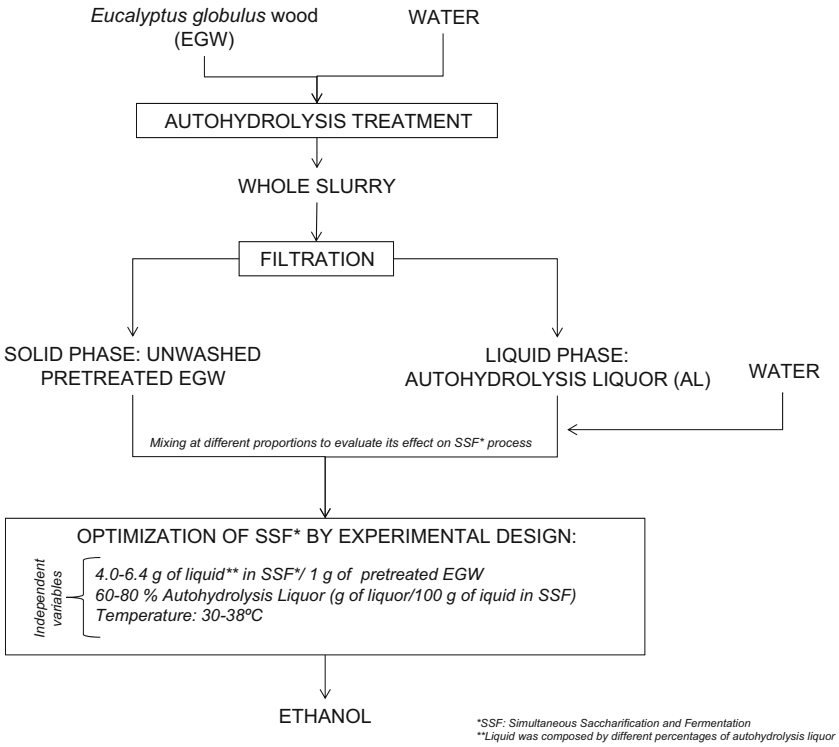

Fig. 1 Schematic representation of the experimental procedure followed in this work

groups and Klason lignin content of solid fraction were quantified following the procedures described above. One aliquot of liquid phase or autohydrolysis liquor was filtered through 0.45-μm membranes to measure the glucose, xylose, acetic acid, furfural, and hydroxymethylfurfural (HMF) by high-performance liquid chromatography (HPLC). Other aliquot was used for oligosaccharide quantification by acid posthydrolysis (4 % of H_2SO_4, 121 °C for 20 min), filtered

Table 1 Composition of *Eucalyptus globulus* wood, EGW (g/100 g of wood in oven-dry basis), hydrothermally pretreated EGW (g/100 g of pretreated wood in oven-dry basis), and autohydrolysis liquor (g/L)

Solid yield, SY (g of pretreated EGW/100 g of EGW)		71.66
Nonvolatile compounds (g/100 g of EGW)		18.63
a. Chemical composition of EGW and pretreated EGW or solid phase after treatment (g/100 g, oven dry basis)		
	EGW	Pretreated EGW (T_{max} 210 °C)
Glucan	44.7	59.26
Xylan	16.01	1.95
Arabinan	1.09	0
Acetyl groups	2.96	0.29
Klason lignin	27.7	33.6
Extractives	2.1	–
Ash	0.2	–
b. Chemical composition of liquid phase or autohydrolysis liquor (g/L)		
Glucooligosaccharides		1.15
Xylooligosaccharides		8.97
Arabinoologosaccharides		0
Acetyl groups		2.55
Glucose		0.64
Xylose		8.85
Arabinose		0.18
Acetic acid		3.11
HMF		0.33
Furfural		1.66

through 0.45 μm and analyzed by HPLC. The oligomer concentration was determined by difference before and after posthydrolysis. A third aliquot was dried at 105 °C to constant weight for non-volatile compound (NVC) determination.

Evaluation of Stress Conditions on Simultaneous Saccharification and Fermentation Assays: Experimental Plan

The evaluation of ethanol production in SSF process was carried using a Box-Behnken design (3 factors and central point with 3 replicates, accounting for 15 total experiments). Three independent variables related with stress conditions were studied: temperature (T, °C), percentage of autohydrolysis liquor (AL, g of liquor/100 g of liquid in SSF or %), and liquid-to-solid ratio (LSR, g of liquid in SSF/g of pretreated EGW solid on dry basis). The variable LSR is inversely proportional to percentage of solids and it can be calculated as follows:

$$\%\ solid\ loading = \frac{1}{LSR} \cdot 100 \quad (1)$$

Table 2 shows the fixed, independent, and dependent variables used in this work, as well as the range studied. The values of LSR and percentage of AL analyzed in this work were chosen in basis of previous experience. Thereby, lower level of LSR was chosen because it was previously shown to be feasible even though in different process conditions (washed pretreated EGW solid was used as substrate by itself without the addition of autohydrolysis liquor as liquid in SSF [25]). On the other hand, 60 % of AL was selected as minimum level of inhibitor loading since this was successfully assayed by Kelbert et al. [23]. Taking into account that the temperature could have synergetic effect on the other two variables, a wide range of temperature between 30 and 38 °C was assayed.

Microorganism and Yeast Cultivation

The strain used in this work was *S. cerevisiae* PE-2, isolated from Brazilian bioethanol industry [16]. The industrial yeast was maintained at 4 °C in agar YPD (2 % of peptone, 2 % of glucose, 2 % of agar, and 1 % of yeast extract) plates. For inoculum preparation, cells were pitched in 1-L Erlenmeyer flasks (containing 400 mL of medium composed by 50 g/L of glucose, 20 g/L of peptone, and 10 g/L of yeast extract) and grown at 30 °C and 150 rpm for 24 h. After that, cells were aseptically collected by centrifugation for 15 min at 8500 g and 4 °C and resuspended in 0.9 % NaCl to achieve a concentration of 200-mg fresh yeast/mL. The SSF experiments were started with an initial inoculum concentration of 8-mg fresh yeast/mL.

Simultaneous SSF Process

Pretreated EGW solid (unwashed and not dried) and autohydrolysis liquor were mixed at varying LSRs, the calculations were performed in dry basis (Table 2). Different liquor percentages were applied as liquid fraction (AL 60–80 %; Table 2). Liquor was sterilized by filtration (0.22 μm) to avoid additional sugar degradation, and pretreated EGW was sterilized by autoclave (121 °C, 20 min). pH was adjusted to 4.8, using 0.05 N of sodium citrate buffer. SSF assays were carried out in 100-mL Erlenmeyer flasks in an orbital incubator (150 rpm). The enzymes Cellic Ctec2 and HTec2 (kindly supplied by Novozymes, Bagsvaerd, Denmark) were added to SSF assays at moderate enzyme to substrate ratio of 22.5 FPU/g and 500 UI/g for cellulase and xylanase, respectively [24]. Enzyme activities of cellulase (120 FPU/mL), β-glucosidase (779.8 UI/mL), and xylanase (1690 UI/mL) were measured following standard procedures [26–28].

SSF experiments were supplemented with agro-industrial byproducts (corn steep liquor, cheese whey, yeast extract, urea, and $K_2O_5S_2$) optimized previously [23]. Raw yeast extract (kindly provided by Fermentum Lda. microbrewery, Portugal) was dried at 60 °C until no weight variation, crushed, sieved, and supplemented to SSF experiments with concentration of 4.1 g/L. Cheese whey was kindly provided by Quinta dos Ingleses (Agro-Livestock Company, Portugal) and used directly in a concentration of 16.5 g/L. Cheese whey and yeast extract were pasteurized at 60 °C for 60 min and added aseptically to the SSF experiments. Corn steep liquor and $K_2O_5S_2$ solution were autoclaved (121 °C, 20 min) and added to achieve a final concentration in SSF experiments of 5.8 and 0.33 g/L, respectively. Urea was sterilized by filtration (0.22 μm) and added to the SSF experiment (0.86 g/L).

SSF assays were conducted in Erlenmeyer flasks with perforated rubber stoppers enclosing glycerol-filled air locks to allow exhaustion of CO_2 while avoiding entrance of air. Samples were withdrawn at 0, 7, 23, 31, 47, 71, 96, 122, and 143 h, centrifuged (8000 for 10 min) and analyzed by HPLC for glucose, xylose, acetic acid, and ethanol concentration.

In order to evaluate the pretreatment and SSF process, several parameters were determined to enable data interpretation. Cellulose to ethanol conversion (CEC, g of ethanol/ 100 g of ethanol potential) was calculated as follows (NREL/TP-510-42630):

$$\text{Cellulose to Ethanol Conversion} = \frac{[EtOH]f - [EtOH]0}{0.51 \cdot f\,[Biomass] \cdot 1.111} \cdot 100\% \quad (2)$$

where [EtOH]f is the ethanol concentration at the end of the fermentation (g/L); [EtOH]0 is the ethanol concentration at the beginning of the fermentation (g/L) which should be zero;

Table 2 Operational conditions used on study of simultaneous saccharification and fermentation (SSF) of hydrothermally pretreated EGW

a. Operational conditions of SSF study		
	Abbreviated name	Values or range
Fixed variables		
pH of SSF assays		5
Agitation (rpm)		150
Enzyme loadings		
Cellic Ctec2 (FPU/g)		22.5
Cellic Htec2 (UI/g)		500
Independent variables		
Temperature (°C)	T or x_1	30–38
Percentage of autohydrolysis Liquor (g of liquor/100 g of liquid in SSF or %)	AL or x_2	60–80
Liquid-to-solid ratio (g of liquid in SSF/g of pretreated EGW solid)	LSR or x_3	4–6.4
Dependent variables		
Ethanol concentration at 122 h (g/L)	EC_{122} or y_1	
Cellulose to ethanol conversion at 122 h (g/100 g)	CEC_{122} or y_2	
Ethanol productivity at 96 h (g/Lh)	Qp_{96} or y_3	

[Biomass] is dry biomass or LCM concentration (corrected by the solubilization of glucan and xylan during the enzymatic saccharification) at the beginning of the fermentation (g/L); f is the cellulose fraction of dry biomass (g/g); 0.51 is the factor for glucose to ethanol based on stoichiometric biochemistry of yeast; and 1.111 is the conversion factor of cellulose into equivalent glucose.

Ethanol productivity (Qp_t, g/Lh) was defined as the ratio between ethanol concentration at time t (E_t) and total SSF time, and it was calculated as follows:

$$Qp_t = \frac{E_t}{t} \tag{3}$$

Fitting of Data and Modeling

To obtain the responses of ethanol production, cellulose to ethanol conversion, and ethanol productivity, experimental data were fitted to the proposed models using commercial software (STATISTICA 7 and Statgraphics Plus 5.1). Response surface methodology (RSM) was used for optimization of studied variables.

Analytical Methods

Samples from analytical composition of raw material, pretreated EGW solid, autohydrolysis liquor, and SSF assays were analyzed for glucose, xylose, acetic acid, hydroxymethylfurfural (HMF), furfural and ethanol by high-performance liquid chromatography (HPLC) using a Varian MetaCarb 87H column (300x7.8 mm), eluent H_2SO_4 0.005 M at 60 °C, at a flow rate of 0.7 mL/min with a Jasco 830-IR refractive-index detector (for sugars and acetic acid) and UV detector JASCO set at 210 nm (for furfural and HMF).

Results and Discussion

Hydrothermal Pretreatment of *Eucalyptus globulus* Wood: Chemical Composition

Conditions of hydrothermal treatment were chosen in basis of previous work in which the highest recovery of polysaccharides (measured as glucose from enzymatic hydrolysis of cellulose and as sum of xylose and xylooligosaccharides from liquid phase after pretreatment) was attained [4]. Table 1 shows the chemical composition of raw material, pretreated EGW solid, and autohydrolysis liquor. After treatment, 95 % of cellulose and 87 % of lignin were recovered quantitatively in solid phase and 81.1 % of xylan was solubilized into xylose and xylooligosaccharides achieving a concentration of 8.89 and 8.97 g/L, respectively. As consequence of pretreatment hardness, hexoses and pentoses were dehydrated to HMF and furfural (0.66 and 1.66 g/L), respectively. In addition, the acetyl groups were released to autohydrolysis liquor in the form of acetic acid achieving a concentration of 3.11 g/L. These inhibitor degradation products represented 25 % (*w/w*) of total non-volatile compounds in the autohydrolysis liquor. Chromatogram of autohydrolysis liquor is shown in Fig. S1 of supplementary data. The chemical composition of autohydrolysis liquor and pretreated EGW solid is typically of hardwoods and is comparable with previous reported data [29]. The pretreated EGW was directly

used as substrate in an SSF process without washing and drying steps avoiding additional processing cost which would improve the implementation of a large-scale industrial process.

Simultaneous Saccharification and Fermentation Assays of Pretreated EGW Solid Using Autohydrolysis Liquor as Liquid Medium

In order to evaluate operational conditions of SSF process, pretreated EGW solid (unwashed and not dried) was mixed with autohydrolysis liquor under conditions described in Tables 2 and 3. This substrate while having carbon source is nutritionally deprived compromising the good performance of the yeast strain that has to deal simultaneously with different stress factors. Currently, byproducts from agro-food industries are generated in considerable quantities worldwide [30], being their use as nutritional source an attractive alternative to attain more cost-effective process. Corn steep liquor (byproduct of corn wet milling) is a clear example of this, used commercially as a supplement to growth media. In this context, low-cost nutritional supplementation (composed by corn steep liquor, cheese whey, raw yeast extract, urea, and $K_2O_5S_2$), previously optimized [23], was added to improve fermentation rates. Industrial *S. cerevisiae* PE2 strain was chosen on the basis of previous screening using the same autohydrolysis liquor at 30 °C [16], since it showed higher fermentation performance comparing to other strains isolated from industrial environments (cachaça, first-generation bioethanol and cacao industries) and to laboratorial strains.

Glucose concentration (data not shown) was consumed within the first 8 h for experiments carried out at 30 °C independently of inhibitor loading (autohydrolysis liquor) and at 34 °C for the 60 % of AL. On the other hand, the glucose was consumed within 48 h in the experiments performed at 34 °C for the other percentages of AL at LSR >4 g/g and at 38 °C for 60 and 70 % of AL. It is important to highlight that the glucose was not totally consumed in SSF assays at 38 °C, for the 70 and 80 % AL and LSR of 4 and 5.2 g/g (intermediate and extreme conditions of the experimental design) in which the glucose was accumulated to 58 and 80 g/L in the late fermentation phase. This low fermentation performance was also observed by Zhu et al. [31] using diluted acid pretreated corn stover at 38 °C probably due to low viability of the yeast strain. On the other hand, in this work, glucose was consumed before 96 h of saccharification and fermentation when the temperature was 34 °C and the autohydrolysis liquor was 80 %. These results show a clear influence of temperature on glucose consumption by the industrial strain in presence of high percentage of autohydrolysis liquor. More inhibitory effect of ethanol concentration with an increase of temperature has been reported by different authors [32, 33]. The same synergistic effect pattern of autohydrolysis liquor percentage and temperature has been previously reported in which an increase of the inhibitor liquor effect could be observed with the rise of temperature [34]. High temperature and presence of inhibitor compounds induce common stress responses to protect yeast cell from damage associated with them [35].

Xylose concentration (data not shown) varied in the range of 10.3–23.1 g/L, achieving a complete hydrolysis of xylooligosaccharides into xylose. The highest values of xylose were obtained in experiments 4, 8, and 12 where the temperature (34 and 38 °C) and autohydrolysis liquor percentage (70 and 80 %) were higher. This fact can be related with

Table 3 Box-Behnken experimental design employed to assess the simultaneous saccharification and fermentation of hydrothermally pretreated EGW under stress conditions of temperature (T), presence of inhibitors (AL), and high solid loadings (LSR)

Run	T (°C), x_1	AL[a] (%), x_2	LSR[b] (g/g), x_3	EC_{122} (g/L), y_1	CEC_{122} (g/100g), y_2	Qp_{96} (g/Lh), y_3
1	30 (−1)	60 (−1)	5.2 (0)	46.0	79.8	0.427
2	38 (1)	60 (−1)	5.2 (0)	47.3	82.0	0.478
3	30 (−1)	80 (1)	5.2 (0)	44.5	77.2	0.413
4	38 (1)	80 (1)	5.2 (0)	12.4	21.5	0.111
5	30 (−1)	70 (0)	6.4 (1)	38.2	79.8	0.332
6	38 (1)	70 (0)	6.4 (1)	41.0	85.6	0.373
7	30 (−1)	70 (0)	4 (−1)	53.6	74.1	0.519
8	38 (1)	70 (0)	4 (−1)	10.3	14.2	0.092
9	34 (0)	60 (−1)	6.4 (1)	46.2	96.5	0.440
10	34 (0)	80 (1)	6.4 (1)	42.9	89.7	0.398
11	34 (0)	60 (−1)	4 (−1)	59.2	81.9	0.614
12	34 (0)	80 (1)	4 (−1)	48.5	67.0	0.072
13	34 (0)	70 (0)	5.2 (0)	47.8	82.9	0.464
14	34 (0)	70 (0)	5.2 (0)	46.5	80.6	0.467
15	34 (0)	70 (0)	5.2 (0)	48.1	83.5	0.492

[a] Percentage of autohydrolysis liquor: g of liquor/100 g of liquid in SSF

[b] Ratio between g of liquid (composed by different percentages of AL)/g of pretreated EGW

higher autohydrolysis liquor percentage (containing xylose and xylooligosaccharides) and favorable conditions for enzyme activity (temperature > 30 °C), contributing to the hydrolysis of the enduring xylan in the pretreated EGW biomass by cellulases and hemicellulases. Regarding acetic acid, the concentration was almost constant during SSF process, with concentrations in the range of 2.97–3.95 g/L.

Figure 2 displays the time course of ethanol production for all conditions studied in this work, in which different lag phases of SSF assays can be observed, directly related with the glucose uptake discussed previously. Furan compounds (furfural and HMF) are reduced to less inhibitor alcohols by yeast, being the main cause for different lag phase times in ethanol fermentations at different percentages of autohydrolysis liquor (containing inhibitor compounds) [36]. Variation in the lag phases between 1.5 and 25 h were also reported for fermentation of spruce hydrolysate at 0, 25, and 50 % and 35 °C, as well as absence of growth with 80 % of hydrolysate after 140 h [36].

In this work, most experiences entered stationary phase at 96 h of saccharification and fermentation (Fig. 2), in which the ethanol productivity (Qp_{96}) was calculated and listed in Table 3. Results show that the highest productivity value (0.614 g/Lh) was obtained at intermediate temperatures (34 °C), low inhibitory loading (60 % of AL) and the highest solid loading (LSR = 4 g/g). Low liquid-to-solid ratio (corresponding to high solid loading) and low percentage of autohydrolysis liquor allowed good yeast performance due to higher substrate availability and reduced furan compound inhibition, alongside with intermediate temperature which allows good enzymatic performance without compromising yeast viability. As a general trend, ethanol productivities >0.4 g/Lh were achieved at 30 and 34 °C using LSR of 4 and 5.2 g/g.

In this work, the maximal concentration of ethanol for each SSF assay was achieved at 143 h and varied in the range of 13.7–61.1 g/L (CEC of 18.9 and 84.4 %) corresponding to experiments 8 and 11, respectively. Maximal ethanol concentration was obtained for the highest solid loading (LSR = 4 g/

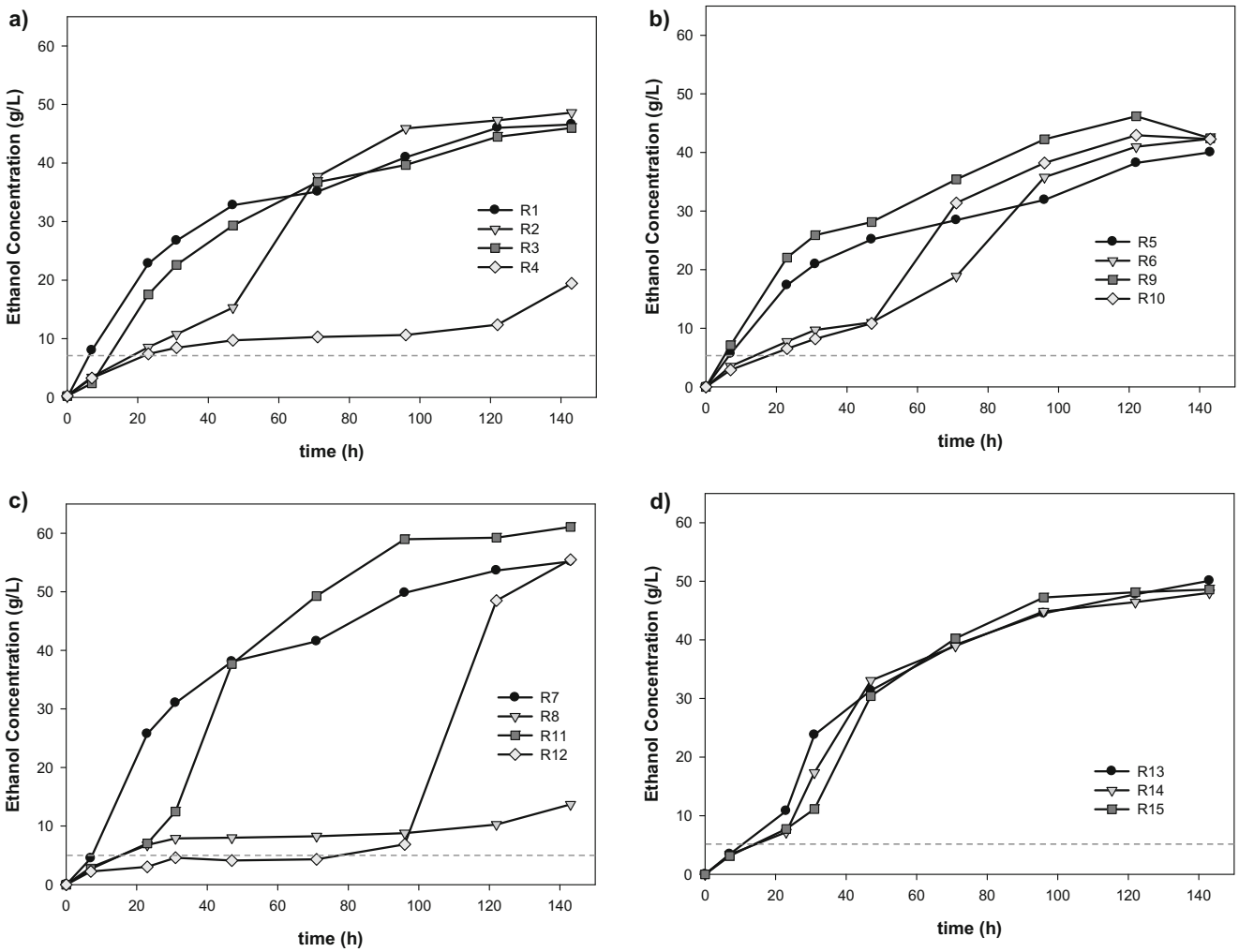

Fig. 2 Time course of ethanol concentration of simultaneous saccharification and fermentation (SSF) assays (runs 1–15 listed in Table 3), *dotted line* is indicating lag phases

g), intermediate temperature (34 °C) and lowest percentage of autohydrolysis liquor, corresponding to better performing conditions previously discussed regarding productivity. In comparison with results obtained at 30 °C, the difference in the ethanol concentration could be due to a deficient enzymatic hydrolysis taking into account the low temperature. Ethanol concentration higher than 55 g/L was obtained in experiments 7, 11, and 12 in which the LSR was 4 g/g or 25 % of solids. Moreover, ethanol concentration >40 g/L was obtained at 143 h in all studied conditions except for experiments 4 and 8, carried out at 38 °C with 70 and 80 % of AL. Chromatogram of the sample from the SSF experiment (run 9 at 47 h) is shown in S2 of supplementary data.

Regarding the cellulose to ethanol conversion, the highest values were achieved for the lowest solid loading (LSR = 6.4 g/g) independently of temperature and percentage of autohydrolysis liquor. This effect of the solid loading on cellulose to ethanol conversion can be linked to a reduction on enzymatic efficiency. Higher solid loadings can influence negatively the mixing properties in the fermentation medium, reducing enzymatic access to the cellulose and conversion efficiency.

Response Surface Methodology Assessment

Screening studies for tolerant *S. cerevisiae* are generally aimed at individual stresses, while the resistance to multiple stresses has received less attention [37–39]. This integrated approach should be considered since the combined effect of stresses is the great challenge of saccharification and fermentation process for industrial lignocellulosic ethanol production. Moreover, when taking into account SSF processes, the imposed stresses can affect simultaneously the enzyme and yeast performance, amplifying the need for an integrated study taking into account multiple inhibitions during the whole cellulose to ethanol conversion process. The influence of SSF operational conditions on ethanol production, ethanol conversion, and ethanol productivity presents an elevate grade of complexity due to the variable interaction, as it can be seen in Tables 3 and 4. In this sense, the RSM is a useful tool for the easy visualization of independent variable effects on dependent variables as well as for the prediction of results within the studied range [40]. Table 3 recollected the dependent variables studied in this work: ethanol concentration at 122 h (EC_{122} in g/L), cellulose to ethanol conversion at 122 h (CEC_{122} in g/100 g), and ethanol productivity at 96 h (Qp_{96} in g/Lh). Ethanol concentration and conversion were chosen at 122 h of saccharification and fermentation as in the next time point, the ethanol concentration started to decrease in some experiments. These experimental variables were correlated with independent variables (T, AL, and LSR) by a second-order polynomial equation, as follows:

$$y_j = b_{0j} + \sum_{i=1}^{3} b_{ij}x_i + \sum_{i=1}^{3}\sum_{k\geq i}^{3} b_{ikj}x_i x_k$$

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

Table 4 recollects the regression coefficients ($b_{0j} \ldots b_{23j}$), the statistical significance (based in the Student's t test), and the statistical significance of the model (based on Fischer's F parameter). The parameters summarized in Table 4 verified the good fitting of dependent and independent variables by the empirical models. The average coefficient (R^2) of the models was >0.96 for studied dependent variables, which shows that the model is suitable to represent the correlation among selected variables.

Figure 3 shows a graphic representation of the interaction of temperature, percentage of autohydrolysis liquor, and liquid solid loading on ethanol concentration, fixing three levels (−1, 0, and 1). The linear coefficient was significant at $p \leq 0.05$ for the temperature and percentage of autohydrolysis liquor (Table 4). On the other hand, the quadratic coefficient was significant for temperature and LSR and interaction coefficient for the combination of temperature and LSR (Table 4). Figure 3a shows the different behaviors obtained at 30, 34, and 38 °C for LSR and percentage of autohydrolysis liquor on ethanol production. The reported results at 38 °C showed the clear influence of temperature on ethanol concentration, leading to higher concentration between 30 and 50 g/L comparing to results obtained at 30 °C, where concentration of ethanol was in the range of 30–38 g/L at high solid loadings (LSR = 4 g/g) and percentage of autohydrolysis liquor <76 %. This performance is probably due to a lower enzyme activity at 30 °C, showing that the temperature was a more limiting variable than low LSR (or high solid loading) and percentage of autohydrolysis liquor on ethanol concentration. In fact, Mutturi and Lidén [34] reported a decrease of glucose release (18 %) of saccharification of pretreated arundo 32 °C comparing to 39 °C, indicating that yield of ethanol at 32 °C on SSF process could be significantly lower than at 39 °C. At 34 °C, an ethanol concentration higher than 40 g/L was obtained under all conditions. This pattern is in agreement with reported works in which the selected temperature for SSF process is usually 35 °C [36, 41]. Results indicate that the inhibitor effect is more expressive at higher temperatures. High temperature stress has great influence on cellular processes: inhibition of cell division, imbalance of protein homeostasis, and difficulty on coupling of oxidative

Table 4 Regression coefficients and statistical parameters measuring the correlation and significance of models for independent variables: liquid/solid ratio (LSR), temperature (T), and percentage of autohydrolysis liquor (AL)

Model parameters	E_{122h} or y_1 (g/L)	CEC_{122h} or y_2 (g/100 g)	Qp_{96h} or y_3 (g/Lh)
b_0 (Intercept)	47.447	82.335	0.474
Linear			
T (b_1)	−8.926[b]	−13.453[a]	−0.080[a]
AL (b_2)	0.4118[b]	−10.599[b]	−0.121[a]
LSR (b_3)	1.769	−14.282[a]	−0.031[c]
Quadratic			
T (b_{11})	−6.298[b]	−18.766[b]	−0.085[b]
AL (b_{22})	−11.675[c]	1.575	−0.033
LSR (b_{33})	−0.003	−0.150	−0.061[b]
Interaction			
T × AL (b_{12})	−8.342	−14.476[b]	−0.088[a]
T × LSR (b_{13})	−11.540[b]	−16.436[b]	−0.117[a]
AL × LSR (b_{23})	−1.872	−2.015	−0.125[a]
F	5.846	9.628	24.075
R^2	0.913	0.945	0.977
Significance level	>96	>98	>99

[a] Coefficient significant at ≥ 99 % confidence level

[b] Coefficient significant at ≥ 95 % confidence level

[c] Coefficient significant at ≥ 90 % confidence level

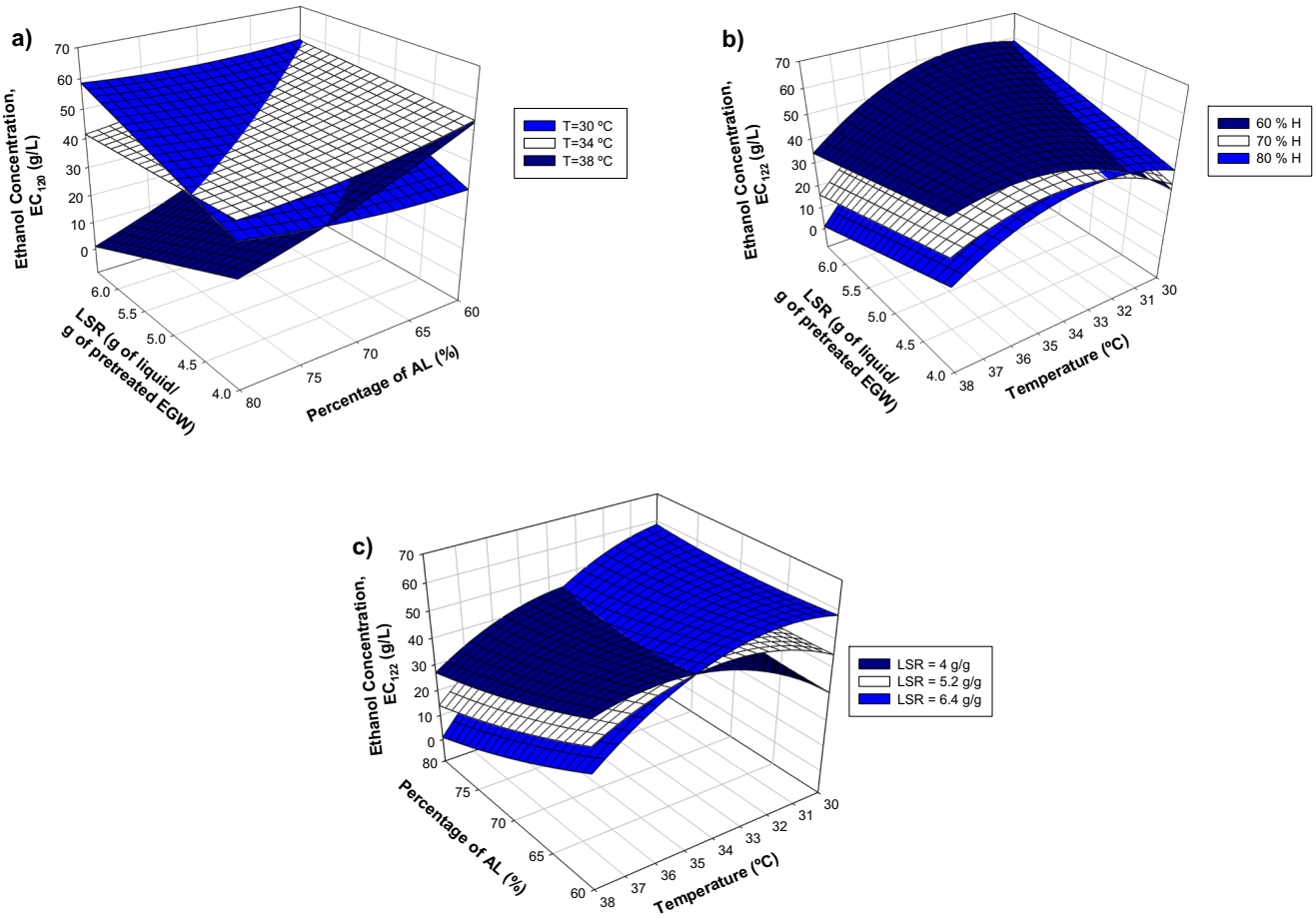

Fig. 3 Response surface of ethanol concentration at 122 h (EC_{122}) of simultaneous saccharification and fermentation process on **a** percentage of autohydrolysis liquor (AL) and LSR (fixed temperature at 30, 34, and 38 °C); **b** autohydrolysis liquor and temperature (fixed percentage of AL at 60, 70, and 80 %); and **c** temperature and autohydrolysis liquor (fixed LSR at 4, 5.2, and 6.4 g/g)

phosphorylation [41] which could hinder the biological detoxification of inhibitor compounds. Studies related with temperature tolerance of *S. cerevisiae* have demonstrated that differences of less than 1 °C have great influence on growth, non-growth, and death of yeast at temperature range of 37–43 °C [42]. Figure 3b, c show ethanol concentration as function of temperature and LSR (fixing the percentage of autohydrolysis liquor at 60, 70, and 80 %) and temperature and percentage of autohydrolysis liquor (fixing LSR at 4, 5.2, and 6.4 g/g), respectively. At 38 °C, ethanol concentration increased slightly with a decrease of LSR. On the other hand, ethanol concentration decreased for increasing of autohydrolysis liquor percentage. At fixed percentage of autohydrolysis liquor of 60 %, ethanol concentration was 1.7-fold higher at 38 °C compared to 30 °C. On the other hand, ethanol concentration was 1.4-fold higher at 30 °C than 38 °C with 80 % of autohydrolysis liquor. In addition, differences in the autohydrolysis liquor loading of 5 % (60 to 65 % of AL) implied a decrease of ethanol concentration of 39.2 % (34 to 24.4 g/L). Favaro et al. [38] also reported a decrease of ethanol concentration (43.4 to 18.6 g/L) with 50 and 75 % of sugarcane hydrolysate, respectively, using a thermotolerant *S. cerevisiae* Fm17 strain isolated from grape marc.

Figure 3c shows that ethanol concentration for LSR of 6.4 g/g had an almost linear behavior regarding variation of temperature and percentage of autohydrolysis liquor. Nevertheless, LSR of 4 and 5.2 g/g show a maximum at 34 °C. The optimal conditions for maximal concentration of ethanol were calculated and predicted a concentration of 58.6 g/L under the following conditions: 30 °C, 60 % of AL, and LSR = 6.4 g/g.

Cellulose to ethanol conversion correlation with independent variables was represented in Fig. 4a in which LSR was fixed at 4 g/g (higher solid loadings). As it can be seen, cellulose to ethanol conversion higher than 90 % was obtained in a wide range of operating conditions at temperatures above 31.5 °C. In addition, cellulose to ethanol conversion of 100 % was also achieved for a percentage of autohydrolysis liquor lower than 70 %. These results show an interesting range of limiting process conditions (high solid and autohydrolysis liquor loadings) in which ethanol conversion achieves competitive values. This aspect is important from the industrial point of view since the reduction of additional washing steps (using the autohydrolysis liquor) and water consumption (with low liquid-to-solid ratios) without losses on ethanol yield has a direct impact in the economic efficiency of lignocellulosic ethanol processes. A remarkable decrease on ethanol yield was reported by Liu et al. [43] when increasing the solid loading from 20 to 25 % of whole slurry from steam-exploded corn cob at 39 °C. For the results hereby presented, the empirical model predicted a maximal ethanol conversion of 100 % using 60 % of autohydrolysis liquor, liquid/solid ratio of 4 g/g (or 25 % solids) and temperature of 35.9 °C. The presence of inhibitors seems more harming at high LSR. This result can be due to lignocellulosic biomass ability to absorb or react with compounds present in the autohydrolysis liquor. Considering that SSF has a solid (lignocellulosic biomass) and a liquid (autohydrolysis liquor) phase, interaction between phases can occur. Compounds present in the autohydrolysis liquor can be absorbed or react with the lignocellulosic biomass, making them less available in the liquid phase and therefore less harmful for the yeast. Liu et al. [43] also observed this behavior at high glucan loading for enzymatic saccharification. However such relation cannot be directly established given that the coefficient for interaction between percentage of autohydrolysis liquor and LSR was not considered statistically significant considering the empirical model for cellulose to ethanol conversion. According to Table 4, linear, quadratic, and interaction coefficients were significant at $p \leq 0.05$ for temperature. Linear coefficients were also significant for variables: percentage of AL and LSR. Moreover, Fig. 4b represents the predicted values of ethanol conversion (CEC) as function of temperature and LSR at fixed 80 % of autohydrolysis liquor (the highest inhibitor loading). Eighty percent of ethanol conversion was achieved at temperature <36 °C and LSR of 4 g/g. These data reveal the influence of autohydrolysis liquor percentage on SSF process since, with washed pretreated EGW (without autohydrolysis liquor) under same conditions of pretreatment (T_{max} of 210 °C), the ethanol conversion increases with rise of LSR [25], showing a different behavior.

Figure 5a, b represents the ethanol productivity fixing the percentage of autohydrolysis liquor in 60 % (in which the ethanol productivity was more elevated) and LSR = 4 g/g (the highest solid loading). The interaction of all studied variables had a significant effect ($p \leq 0.01$) (see Table 4). Maximal ethanol productivity ($Qp_{96} = 0.631$ g/Lh) was predicted by the empirical models for the following operational conditions: 31.4 °C, 60 % of AL, and LSR = 6.4 g/g.

Currently, literature collects few studies of lignocellulosic ethanol production with the evaluation of more than one stress factor on simultaneous saccharification and fermentation process [13, 14, 38, 43, 44]. Most of them follow strategies as screening of industrial strains and/or improvement of strain robustness tools such as evolutionary engineering and/or genome shuffling [35, 36]. Results obtained in this work with high solid and autohydrolysis liquor loadings can be favorably compared to previously reported data [38, 43].

Optimization of Ethanol Production: Operational Conditions Selection and Model Validation

Ethanol concentration and conversion were maximized by multiple response optimization models. The predicted condition (55.8 g/L and 100 % of conversion) was obtained under the following conditions: temperature of 37 °C, 60 % of AL,

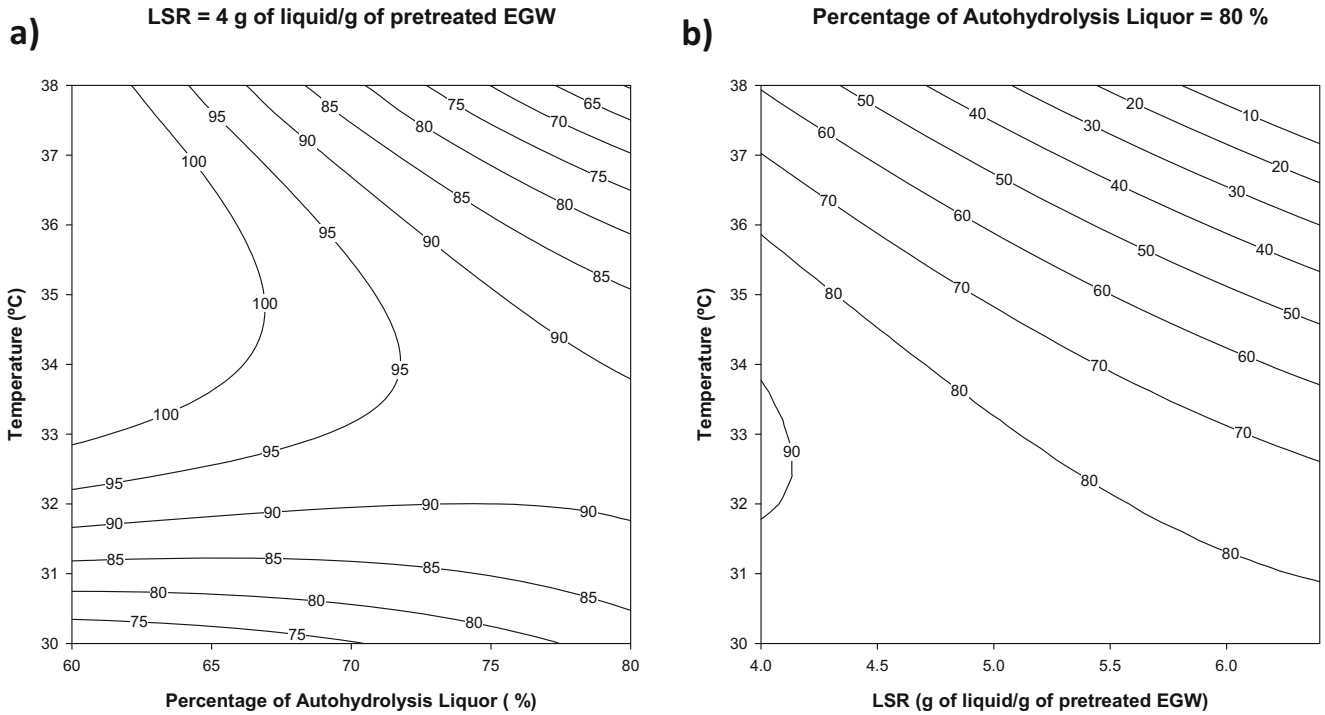

Fig. 4 Response surface of cellulose to ethanol conversion at 122 h (CEC_{122}) of simultaneous saccharification and fermentation process on **a** temperature and autohydrolysis liquor, AL (fixed LSR at 4 g/g); **b** temperature and LSR (fixed percentage of AL at 80 %)

and LSR of 6.4 g/g. In order to validate this optimal condition, an additional experiment was carried out. The experimental validation results were as follows: 53.8 g/L, 85 %, and 0.51 g/Lh, respectively (relative error ≤10 %). In order to compare with separate hydrolysis and fermentation (SHF) process, an additional assay was carried out under optimal conditions. First step of hydrolysis was performed at 50 °C to favor the enzyme action and the second step of fermentation at 37 °C.

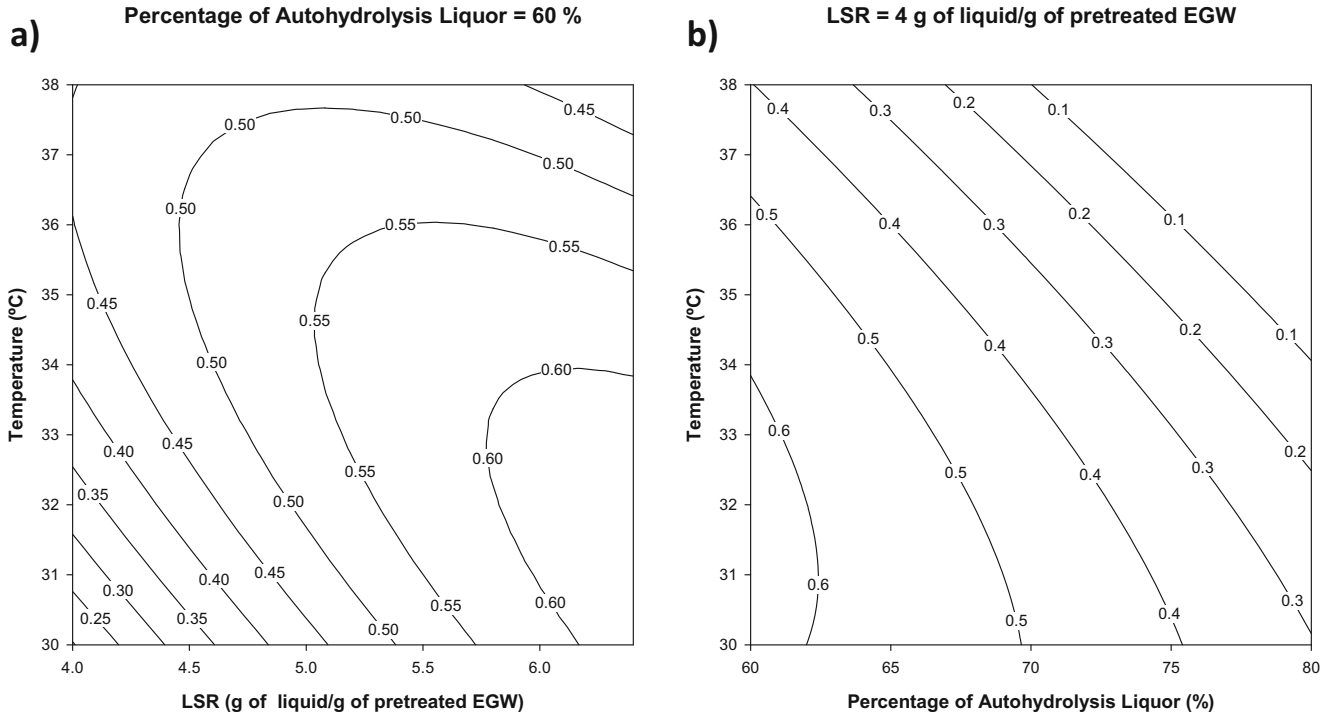

Fig. 5 Response surface of ethanol productivity at 96 h (Qp_{96}) of simultaneous saccharification and fermentation process on **a** temperature and LSR (fixed 60 % of AL); **b** temperature and percentage of autohydrolysis liquor (fixed LSR at 4 g/g)

After 96 h of enzymatic hydrolysis, 86 g/L of glucose was obtained which was used for fermentation. Ethanol concentration was 39.7 g/L (corresponding to 74 % of cellulose to ethanol conversion). Data were displayed in supplementary data Fig. S3.

Besides this optimization, in a wide range of restraining conditions of SSF process, suitable results of cellulose to ethanol conversion (60–80 %) were obtained for a temperature range between 36 and38 °C using 80 % of AL at LSR = 4 g/g (or 25 % solid loading). In this study, the complete separation of the liquid and solid phases of the whole slurry was carried out to study the effect of the different stress factors. In a process perspective, partial separation by a simple operation unit like for instance decantation could be conducted to adjust the whole slurry to reach the optimal LSR and percentage of autohydrolysis liquor hereby determined.

Conclusion

The integrated approach followed in this work addresses the real requirements of lignocellulosic ethanol industry. Synergistic effects between temperature, percentage of autohydrolysis liquor, and liquid/solid ratio and their effect on the responses studied were shown, by the empirical models, as having negative impact on ethanol production, taking into account the whole SSF process. These results indicate a significant influence of temperature on yeast tolerance to inhibitor compounds present in the autohydrolysis liquor. Interestingly, at high solid loadings (LSR of 4 g/g), ethanol conversion is enhanced at high percentage of autohydrolysis liquor compared to results at lower solid loadings (LSR of 6.4 g/g). Overall, the strategy followed in this work (robust industrial *S. cerevisiae* and low-cost nutritional supplementation) to tackle the challenges identified in lignocellulose-to ethanol processes allowed noticeable results of cellulose to ethanol conversion (>90 %) under quite restrictive SSF process conditions (80 % of AL, LSR = 4 g/g, and temperatures between 32 and 33.6 °C), representing a step forward for the realization of a cost-effective lignocellulose-to-ethanol process.

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