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Modelling of the Simultaneous Saccharification and Fermentation for a Pine Sawdust Biorefinery

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Abstract: The decline in world oil reserves evidences the need to diversify the sources of equivalent raw materials. The use of biomass is one of the most explored alternatives. This work evaluates the second-generation bioethanol (2G) production from a pine sawdust soda- ethanol pulp by simultaneous hydrolysis and fermentation (SSF) to obtain the best conditions for scaling up the process. Experimental designs have been used to find mathematical models that define the complex situation jointly varying time with other variables (enzyme load and temperature). Time periods in the full model varied from 0 h to 72 h. Given the results (curve shape differences), it was decided to split the design in two, covering periods from 0 h to 24 h (Model I) and from 24 to 72 h (Model II). The pulp chemical composition was 80.2% glucans, 7.2% xylans, 0.3% galactans, 8.4% mannans, 3.7% lignin. Cellic® Ctec2 cellulolytic enzymes were used for saccharification and *Saccharomyces cerevisiae* IMR 1181 (SC 1181) yeast for fermentation. The best conditions found in the two designs were 30 FPU g^{-1} glucans, 39 °C, 24 h for Model I, and 30 FPU g^{-1} glucans, 35 °C, 72 h for Model II. Fermentation optimal values were 63.23 and 81.93 for Models I and II, respectively.

Keywords: pine sawdust; SSF; multilevel factorial design; soda-ethanol pretreatment

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1. Introduction

Second-generation bioethanol, an ecological product whose production process involves simple technologies and renewable resources, could reduce the current demand for oil and the emission of greenhouse gases [1]. The use of bioethanol as a renewable raw material for chemical products manufacture has achieved market development. There have been technological advances in the catalytic conversion of bioethanol into relevant chemical products such as ethylene, propylene, 1,3-butadiene, isobutylene, hydrogen, acetaldehyde, ethylene oxide, n-butanol, acetic acid, ethyl acetate, acetone, and dimethyl ether [2]. Processing could involve dehydration, dehydrogenation, oxidation, reforming, gasification, decomposition, coupling, etc. Many developments are still in the early design stages (laboratory or pilot scale).

Producing and using bioethanol in biorefineries focused on obtaining bioplastics (BioPE) involves two basic steps. First, dehydration to bioethanol is carried out through an endothermic reaction at a suitable temperature and with the help of a catalyst to produce ethylene [3]. Then, a catalyzed polymerization produces biopolyethylene (BioPE).

BioPE is a non-biodegradable plastic of great interest due to its several uses, versatility, and chemical stability [4]. From an economic point of view, its commodity condition is a challenge. That is to say, bioethanol and its derivatives have to be competitive and profitable within a biorefinery. From a technological point of view, there are several items to optimize in the different stages of production on a pilot and pre-commercial scale.

The biorefinery of lignocellulosic biomass does not compete with food. Besides, it favors the integral use of wood through its sustainable conversion to fuels, biomaterials,

and high added value bioproducts [5]. In this context, sawmills byproducts like pine sawdust are valuable raw materials for second-generation bioethanol production [3]. This resource is abundant in the northeast region of Argentina, the most forested area in the country. However, its complex and heterogeneous composition makes it recalcitrant to conversion processes [6].

The processing for bioethanol production involves pretreatment, enzymatic hydrolysis, and sugar fermentation. The pretreatment choice depends on the physical and chemical characteristics of the raw material. Soda-ethanol is a type of pretreatment in which NaOH produces delignification, and ethanol acts as a solvent, helping to extract dissolved lignin from the solid. The soda-ethanol pretreatment is very effective for the following enzymatic hydrolysis process since alkaline medium allows a high delignification, opening the fiber pores and increasing the accessibility of enzymes for the saccharification process [6]. Enzymatic saccharification is a catalytic process in which enzymes act synergistically to produce glucose monomers. Generally, it is performed at relatively high temperatures (40 to 50 °C) and pH between 4.5 and 5.0 [7]. Finally, the fermentation process traditionally uses Saccharomyces cerevisiae yeasts, which efficiently ferments glucose to ethanol [8] at a temperature range between 25 and 35 °C [9]. The most studied yeasts for bioethanol production strain include *Saccharomyces cerevisiae* (commercial baker's yeast), *Kluyveromyces fragilis*, and *Candida utilis*, considered GRAS (Generally Recognized As Safe), suitable for human consumption [3].

Simultaneous saccharification and fermentation (SSF) is the preferred strategy for bioethanol production since the hydrolysis and fermentation reactions occur in a single reactor. All the produced glucose is immediately consumed by the yeasts, avoiding the inhibition of the enzyme by sugar accumulation, and reducing bacterial contamination. Additionally, using a single reactor reduces processing costs [10].

In bioethanol production, the contribution of the enzymatic hydrolysis process is almost 25% of total production costs. Therefore, it is essential to achieve its optimization to achieve cost-effective levels of sugars [11]. In addition, the possibility of reactivating the used colonies of the *Saccharomyces Cerevisiae* IMR 1181 (SC 1181) yeast strain by the streaking method for their conservation and future use contributes to production cost reduction [12].

Bioethanol production as a platform chemical can be integrated into a biorefinery framework for obtaining bioplastics. Therefore, it is essential to know the optimal production conditions for economically and efficiently obtaining this "raw material" to generate a new and sustainable value chain.

This work evaluates the second-generation bioethanol (2G) production from a pine sawdust soda-ethanol pulp by simultaneous hydrolysis and fermentation (SSF) to obtain the best conditions for scaling up the process. The variables assessed were enzymatic load, temperature, and time at low substrate consistency (2% w/w). Mathematical models that define the complex situation of joint variation of time with other variables (enzyme load and temperature) were obtained using experimental designs. The study is part of a comprehensive study on pine biorefinery, covering different products and materials.

2. Materials and Methods

The raw material was an industrial sawdust mix of *Pinus elliottii* and *Pinus taeda* provided by a local sawmill (Misiones, Argentina), subjected to a soda-ethanol pretreatment [10]. The following operating conditions were chosen to obtain high delignification: liquor-to-wood ratio (L:W) of 5.4:1, a maximum temperature of 170 °C, the time-to-maximum temperature of 60 min, the EtOH:H_2O ratio 35:65% v/v, NaOH 23.3% on dry wood mass, at 140 min. After pretreatment, the pulp was thoroughly washed [13].

Cellic® CTec2 commercial enzymes (Novozymes) were provided by Sigma-Aldrich (Buenos Aires, Argentina) were used for the enzymatic hydrolysis. The *Saccharomyces cerevisiae* IMR 1181 (SC 1181) strain, applied for the fermentation process, was donated by the Institute of Modeling and Technological Innovation IMIT (UTN-CONICET), Resistencia, Argentina.

A Sabouraud glucose agar medium was used for the strain recovery. Agar (2%) with Tween 80 was applied for the micro and macro morphology milk study. Water agar and a Castellani medium were employed for strain conservation.

2.1. Characterization of the Substrate and Liquid Fraction of the Fermentation Process

The chemical composition of the raw material was determined in previous work [14]. Pine sawdust and pulps (substrate) were characterized according to NREL (National Renewable Energy Laboratory) Golden, Colorado, USA standards, including total solid and moisture (NREL/TP-510-42621) [15], and structural carbohydrates and lignin (NREL/TP-510-42618) [16]. HPLC (Waters Corp., Milford, MA, USA), with a SHODEX SP810 column was used with the operational conditions: distilled water as eluent, 0.6 mL/min, 85 °C, and refractive index detector.

Glucans, xylans, mannans, galactans, and arabinans quantification in the solid were calculated by multiplying the sugar concentrations by the stoichiometric factors of hydrolysis. That is to say, 0.88 (132/150) for sugars with five carbons (xylose and arabinose) and 0.90 (162/180) for sugars with six carbons (glucose, mannose, and galactose).

Glucose and ethanol were determined by HPLC liquid chromatography (Waters Corp., Milford, MA, USA), using an AMINEX-HPX97H column (BIO-RAD, Bio-Rad Laboratories, Inc. Argentina). Chromatographic conditions were: 4 mM of H_2SO_4 as eluent, a flow of 0.6 mL/min, 35 °C temperature, and refractive index and diode array detectors.

Yeast concentrations were determined in SSF experiments by direct absorbance measurement at 600 nm (UV-1800 Spectrophotometer, Shimadzu Corporation, Kyoto, Japan). The NREL/TP-510-42630 protocol of the National Renewable Energy Laboratory was followed, including the calibration curve development. The obtained sample is the supernatant after the pulp is decanted. The reference was the absorbance measured at 600 nm of a fermentation broth sample without yeast or pulp.

2.2. Yeast Reactivation

The yeasts were preserved to be recovered and used in this entire works series [13]. They were reactivated in Petri dishes and tubes using a streaking method and were incubated at 28 °C for 48–72 h. The culture medium, 19.6 gL^{-1} of Sabouraud glucose agar suspended in 300 mL of distilled water in a 500 mL Erlenmeyer flask, was heated in a water bath until it reached a gelatinous consistency. The recovered yeast strains were conserved in duplicate on a water agar and Castellani medium and refrigerated at 4–8 °C [17]. The colonies were incubated at room temperature for 24 h for their adaptation to the medium and stored in 1.5 mL Eppendorf tubes with water agar at 4 °C.

The microculture technique (conventional microbiological method), consisting of the macro and micromorphological characteristics systematic studies, was used for the isolated-pure strains phenotype recognition [18].

A support slide was placed in a sterile Petri dish, and another was added perpendicularly to it, on which 3 mL of 2% milk agar medium with Tween 80 were deposited. The yeasts were seeded with a loop in cut lines transverse to the slide, putting previously flamed coverslips on the seed lines. A moistened filter paper at the base of the plate maintained the chamber humidity. Finally, the system was incubated at 28–30 °C for 48 h [18].

2.3. Pre-Inoculum and Inoculum Preparation

100 mL of YPD liquid medium (yeast extract, peptone, and dextrose) were used for the pre-inoculum and inoculum under the following concentrations: 10 gL^{-1} of yeast extract, 20 gL^{-1} of peptone, and 20 gL^{-1} dextroses provided by the standard for SSF strategy (NREL/TP-510-42630) (Dowe and McMillan, 2008). Micronutrients were supplemented: 5 gL^{-1} of potassium phosphate, 1.5 gL^{-1} of ammonium chloride, and 0.65 gL^{-1} of magnesium sulfate heptahydrate, by protocol employed for SSF strategy [13]. First, 2 mL of *Saccharomyces cerevisiae* IMR 1181 (SC 1181) yeast in 10 mL of the YPD liquid medium were incubated until an optical density of 0.8 (pre-inoculum condition). Then, this pre-inoculum

was incorporated into 90 mL of the YPD liquid medium and incubated to an optical density of approximately 0.7 (inoculum condition). Optical density was spectrophotometrically determined at 600 nm, following the standard for SSF strategy (NREL/TP-510-42630) (Dowe and McMillan, 2008). Pre-inoculum and inoculum processes lasted 24 h in a thermal bath at 180 rpm and 37 °C [13]. The precision temperature control of the thermal bath was +/−0.1 °C.

2.4. Simultaneous Saccharification and Fermentation (SSF) Strategy

The solid material was enzymatically hydrolyzed and subsequently fermented with *Saccharomyces cerevisiae* IMR 1181 (SC 1181) yeast according to NREL-LAP 510-42630 [19]. For the SSF process, 200 mL Erlenmeyer flasks containing 2% hydrolyzable cellulose (on a dry matter base) suspended in 50 mL of 0.1 M sodium citrate (pH 5), 40 mL of distilled water, 10 mL of inoculum, 0.028 mL of Tween 80 surfactant, 0.5 gL^{-1} of yeast extract, and 1 gL^{-1} of peptone (a total of 100 mL) were placed in a thermal bath agitated at 130 rpm. Air traps were placed to prevent oxygen from entering the system. They allowed the release of the CO_2 produced by the yeast. Samples were taken every 4 h for the initial 16 h and at 24, 48, and 72 h.

2.5. Experimental Designs

The obtained data were analyzed by factorial experimental designs with central points to evaluate the combined influence of enzymatic load, temperature, and time on bioethanol yields. Factorial designs allow understanding the effects of independent variables on a dependent variable. In a 3^2 factorial design, there are three independent variables, each with two levels of variation. A central point addition incorporates one more level allowing defining surface equation models as surface response designs. These mathematical models based on experimental data represent how changes in variables affect the response of interest. Combining these equations allows for finding the variable levels that optimize the desired response. For model building, it is essential to work with transformed variables. The variables transformation helps fulfill the statistical requirements and avoid correlation problems if they exist.

Enzyme load (FPU g^{-1} glucans), Temperature (°C), and Time (h) were the selected variables. Consistency stayed low to eliminate its influence from the system. Time in the full model varied from 0 h to 72 h. Given the results (differences in the shapes of the curves), it was decided to split the design in two, covering times from 0 h to 24 h (Model I) and from 24 to 72 h (Model II). Table 1 shows the scheme of variables. The Statgraphics Centurion software (The Plains, VA, USA) was used to assess the experimental design results at a 95% confidence level.

Table 1. The variable operating conditions of the SSF strategy.

Factor	+1	0	−1
A = Enzyme load (FPU g^{-1} glucans)	30	20	10
B = Temperature (°C)	39	37	35
Full Model = Time (h)	72		4
Model I C = Time (h)	24	16	8
Model II C = Time (h)	72	48	24

The response variable was the SSF yield ($Y_{P/T)}$, calculated using Equation (1) [4].

$$Y_{P/T}\ (\%) = \frac{Ethanol\ produced\ \left(\frac{g}{L}\right)}{0.51\ glucose\ in\ the\ sample\ \left(\frac{g}{L}\right)}\ 100 \quad (1)$$

3. Results and Discussion

3.1. Substrate Characterization

Table 2 shows the sawdust and soda-ethanol pulp chemical composition (% on oven-dry material). Pulp yield was 40.5% and delignification 94.9%, so the pretreatment resulted in the extraction of a high amount of lignin, which increases the enzymes' access to the material. Additionally, enzymes can bind to reactive and non-reactive substances such as lignin, which may irreversibly absorb enzymes, decreasing activity towards a reactive substrate [20]. Results were similar to those obtained by other authors, who reached low-lignin content in an alkaline pulp using similar conditions for Nordic pine (*Pinus sylvestris*) (1.6% to 4.1%) [21].

Table 2. Pine sawdust and soda-ethanol pulp chemical composition.

Chemical Composition	Glucans (% odm *)	Xylans (% odm)	Galactans (% odm)	Mannans (% odm)	Arabinans (% odm)	Lignin (% odm)
Pine sawdust	40.9	7.5	2.6	14.8	0.8	29.2
Soda-ethanol pulp	80.2	7.2	0.3	8.4	-	3.7

* odm: % on oven-dry material.

3.2. Simultaneous Saccharification and Fermentation (SSF) Strategy

3.2.1. *Saccharomyces cerevisiae* Characterization

Macromorphological observation is relevant to identify the strain colony phenotype to predict bioethanol yields. Reis et al. (2013) studied ethanol production via two phenotypes of *Saccharomyces cerevisiae* colonies and observed that smooth colonies produced higher ethanol content than rough ones [22]. The observation of reactivated colonies of *Saccharomyces cerevisiae* IMR 1181 (SC 1181) developed on Sabouraud glucose agar at a temperature of 28 °C showed white, smooth, creamy, and shiny colonies, of oval yeast elements, some monogemated, without pseudomycelio or true mycelium formation, and monogemated blastoconidia. These smooth strains are supposed to be efficient for bioethanol production.

3.2.2. 2G Bioethanol Production

In 2G bioethanol production via the SSF strategy, the glucose released by the enzyme complex during hydrolysis is directly metabolized to ethanol by the Saccharomyces cerevisiae yeasts. Glucose is continuously consumed from the medium, decreasing the end-product inhibition. Therefore, a total process time reduction and higher ethanol yields are obtained [23]. Table 3 provides the 2G bioethanol average yields experimentally obtained by varying the enzymatic load and temperature. In SSF, enzymatic hydrolysis of the solid fraction controls the total ethanol production rate [24].

Results demonstrate that the variation of the enzymatic load plays a fundamental role in bioethanol production since high enzymatic loads generate higher yeast concentrations. For example, with 30 PFU g^{-1} glucans at 16 h, yeast concentrations were between 2.75 to 2.94 gL^{-1}. However, using 10 FPU g^{-1} glucans, it is possible to obtain yeast concentrations between 1.14 to 1.40 gL^{-1} at 16 h. This difference notoriously influences the bioethanol yield.

Comparing yields at 72 h for the three temperatures and enzymatic loads, the increment with the change of load is notorious. Nevertheless, the increment is not even. With low enzymatic loads (10 to 20 FPU g^{-1} glucans), the percentages are 124% (35 °C), 57% (37 °C), and 177% (39 °C), whereas with higher ones, (20 to 30 FPU g^{-1} glucans), they are 57% (35 °C), 52% (37 °C), and 46% (39 °C). Other studies found similar results varying the enzymes load, with a strong positive correlation between enzyme loading and the overall ethanol yield [20,25].

Table 3. Bioethanol yields results obtained through the Simultaneous Saccharification and Fermentation (SSF) of a pine sawdust soda-ethanol pulp (theoretical bioethanol 11.36 gL^{-1}).

Temperature (°C)	Time	4	8	12	16	24	48	72
		Enzymatic load = 30 FPU g^{-1} Glucans						
35 °C	Produced ethanol (gL^{-1})	1.27	3.35	4.02	5.37	5.69	n.d.	9.21
	$Y_{P/T}$ (%)	11.11	29.51	35.36	47.28	50.06	n.d.	81.00
	$P_{P/T}$ (gL^{-1} h^{-1})	0.32	0.42	0.34	0.34	0.24	n.d.	0.13
	Yeast concentration (gL^{-1})	n.d.	1.85	1.93	2.94	3.73	3.66	3.80
37 °C	Produced ethanol (gL^{-1})	1.34	1.74	1.85	5.70	4.92	8.28	9.07
	$Y_{P/T}$ (%)	11.82	15.35	16.32	43.27	50.20	72.87	79.82
	$P_{P/T}$ (gL^{-1} h^{-1})	0.34	0.22	0.15	0.36	0.21	0.17	0.13
	Yeast concentration (gL^{-1})	1.54	1.78	1.88	2.75	3.24	3.63	3.73
39 °C	Produced ethanol (gL^{-1})	2.62	4.13	5.15	6.81	7.22	7.93	8.08
	$Y_{P/T}$ (%)	23.07	36.36	45.38	59.92	63.51	69.83	71.1
	$P_{P/T}$ (gL^{-1} h^{-1})	0.66	0.52	0.43	0.43	0.30	0.17	0.11
	Yeast concentration (gL^{-1})	1.21	2.16	2.32	2.93	3.34	3.48	3.48
		Enzymatic load = 20 FPU g^{-1} glucans						
35 °C	Produced ethanol (gL^{-1})	1.75	2.37	3.05	3.66	4.12	n.d.	5.87
	$Y_{P/T}$ (%)	12.7	20.86	26.82	32.20	36.26	n.d.	51.63
	$P_{P/T}$ (gL^{-1} h^{-1})	0.44	0.30	0.25	0.23	0.17	n.d.	0.08
	Yeast concentration (gL^{-1})	n.d.	1.77	2.03	2.09	3.26	3.94	3.99
37 °C	Produced ethanol (gL^{-1})	0.61	1.72	3.20	3.98	4.56	4.83	5.98
	$Y_{P/T}$ (%)	5.37	15.13	28.19	35.06	40.13	42.53	52.61
	$P_{P/T}$ (gL^{-1} h^{-1})	0.15	0.22	0.27	0.25	0.19	0.10	0.08
	Yeast concentration (gL^{-1})	1.45	1.63	1.88	2.07	3.10	3.69	3.95
39 °C	Produced ethanol (gL^{-1})	1.75	3.01	3.91	5.15	4.96	5.57	5.55
	$Y_{P/T}$ (%)	15.41	26.46	34.38	45.34	43.63	48.99	48.84
	$P_{P/T}$ (gL^{-1} h^{-1})	0.44	0.38	0.33	0.32	0.21	0.12	0.08
	Yeast concentration (gL^{-1})	1.15	2.11	2.13	2.78	3.03	3.67	3.81
		Enzymatic load = 10 FPU g^{-1} glucans						
35 °C	Produced ethanol (gL^{-1})	0.57	1.19	1.25	1.87	2.41	n.d.	2.62
	$Y_{P/T}$ (%)	5.04	9.50	11.89	16.41	21.18	n.d.	23.07
	$P_{P/T}$ (gL^{-1} h^{-1})	0.14	0.15	0.10	0.12	0.10	n.d.	0.04
	Yeast concentration (gL^{-1})	n.d.	0.88	1.06	1.40	2.89	3.43	3.77
37 °C	Produced ethanol (gL^{-1})	0.00	0.46	1.48	1.74	2.09	3.03	3.82
	$Y_{P/T}$ (%)	0.00	4.06	13.03	15.31	18.41	26.69	33.59
	$P_{P/T}$ (gL^{-1} h^{-1})	0.00	0.06	0.12	0.11	0.09	0.06	0.05
	Yeast concentration (gL^{-1})	0.65	1.61	0.99	1.39	2.45	3.21	3.85
39 °C	Produced ethanol (gL^{-1})	1.02	1.61	1.91	2.45	2.36	2.22	2.00
	$Y_{P/T}$ (%)	9.02	14.11	16.77	21.56	20.79	19.52	17.62
	$P_{P/T}$ (gL^{-1} h^{-1})	0.26	0.20	0.16	0.15	0.10	0.05	0.03
	Yeast concentration (gL^{-1})	0.62	1.88	1.37	1.14	2.71	3.31	3.74

n.d.: not determined.

The productivity analysis also shows that 37 °C produces the best results. Productivity increases by 25% when changing from 35 °C to 37 °C for 10 FPU g^{-1} glucans, but there is no change for 20 and 30 FPU g^{-1} glucans. However, when rising the temperature from 37 °C to 39 °C, there is a productivity decrease for 10 and 30 FPU g^{-1} (extreme values) and no changes for 20 FPU g^{-1} glucans.

The main interest of this series of works is the use of pine sawdust to obtain bioproducts, including ethanol. The evaluation involved the effect of temperature, enzymatic load, and time on this particular substrate at a low concentration of hydrolyzable cellulose (HC). In the previous work with the same substrate, at 37 °C with 30 FPU and 1% HC, an ethanol concentration of 5.68 g/L (100% yield) was achieved [13]. In this work, the maximum theoretical concentration (2% HC) was 11.36 g/L. At 72 h, 81% was the maximum obtained yield in optimal conditions (that is, 9.21 g/L of ethanol). The yield difference is due to the higher concentration, which, although still low, already shows stirring difficulties with the laboratory system used.

Temperature produces much smaller increments, but 37 °C seems the best option. When using *Saccharomyces cerevisiae*, the usual SSF process range is 35–37 °C, a compromise between this yeast operating temperature (30–32 °C) and the higher temperatures required by enzymatic hydrolysis [26].

Given the recalcitrance of softwoods, obtaining high ethanol yields implies a high enzyme load requirement [27]. The drawback is that enzymes compose the utmost hydrolysis cost, so it is necessary to reduce it to make enzymatic hydrolysis economically feasible [20]. Some alternatives are the recycling of the enzyme [20] and the use of cellobiose-fermentation yeast (for example, *Brettanomyces clausenii*) [24], among others.

3.3. Experimental Designs

3.3.1. Full Model

The analysis of the complete design gave, as a result, the model represented by Equation (2) (in transformed variables, $R^2 = 93.5$).

$$Y_{P/T\ Full\ Model} = 43.5 + 18.3\ z + 17.5\ t + 5.90\ T^2 - 3.46\ T\ t + 11.5\ Ez\ t - 18.34\ t^2 \quad (2)$$

where T: temperature; t: time; Ez: enzymatic load.

The fit of the equation for fermentation yields (observed vs. predicted by the model) is shown in Figure 1.

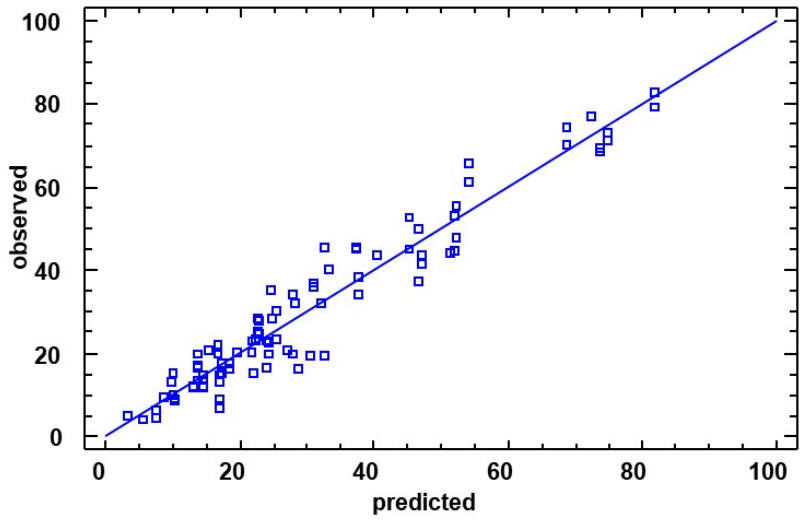

Figure 1. Bioethanol yield observed versus predicted by Equation (2).

The model indicates that temperature has a minor influence. Additionally, the primary interaction involves time. This interaction shows that the effect of temperature on the system is much stronger at high times, evidencing that the process would require a temperature adjustment somewhere in between.

Time shows a quadratic trend, meaning that production stops before 72 h. Nevertheless, the experimental data show maximum yields at the end of the reaction, indicating that the model fails at high times. To further understand the process, it was decided to split the full design into two separate models (short and long times).

3.3.2. Model I

For low times, the equation of the fitted model for the response variable $Y_{P/T}$ (%) in transformed variables ($R^2 = 96.3$) involves the three factors (enzyme load, temperature, and time) and their interactions, showing a quadratic shape (Equation (3)).

$$Y_{P/T\ Model\ I}\ (\%) = 32.3 + 14.8\ z + 3.80\ T + 8.90\ t + 1.87\ Ez\ T + 2.93\ Ez\ t + 4.65\ T^2 - 5.97\ t^2 \quad (3)$$

Figure 2 represents the yield model obtained with the SSF strategy, with variations of the enzymatic load of 10–30 FPU g^{-1} glucans, 8–24 h for 39 °C.

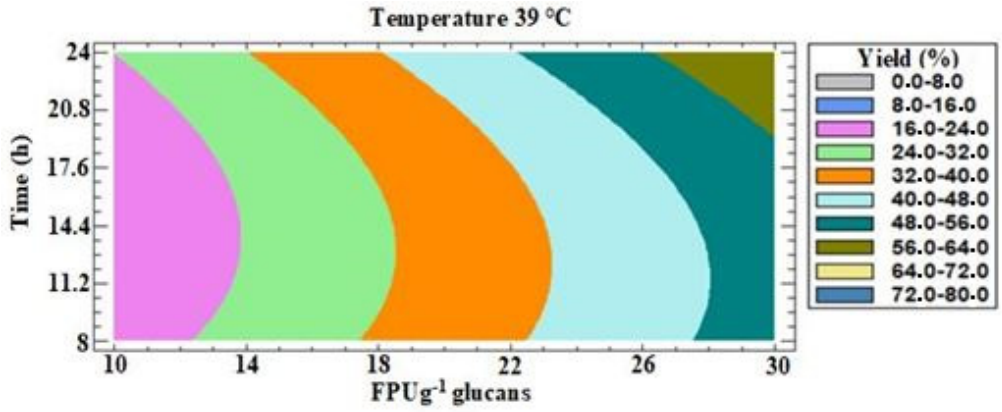

Figure 2. Contours of the estimated response surface of Model I (39 °C).

To perform their function efficiently in a relatively short time, both the enzyme and the yeast require that the process parameters be in their optimal conditions. As the enzyme works at T = 50 °C, and the yeast supports a maximum of T = 39 °C, the maximum yield found by the experimental design corresponds to the upper limit (+1) for the three factors, that is, with an enzymatic load of 30 FPU g^{-1} glucans, 39 °C temperature for 24 h.

In the first hours of the process (before 24 h), the enzyme requires its optimal conditions to depolymerize the cellulose into glucose monomers as fast as possible so that the yeast can convert it into ethanol molecules.

The fermentation yield values calculated by the model adjust to the experimental values (Figure 3). That is, fermentation yields obtained by the model and experimentally in 24 h are 63.2 and 63.5%, respectively, with an enzymatic load of 30 FPU g^{-1} glucans and at 39 °C.

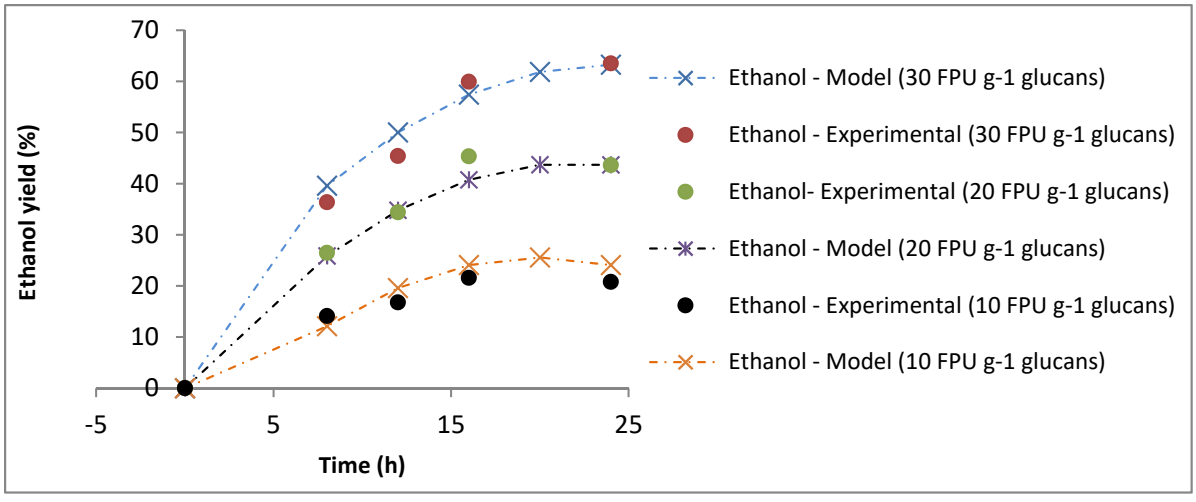

Figure 3. Experimental and theoretical (calculated using Model I) fermentation yields at 39 °C.

3.3.3. Model II

This model was obtained using the SSF strategy for prolonged periods (24 h, 48 h, and 72 h). All studied factors and interactions significantly influence the response variable $Y_{P/T}$ (%). The fitted model with transformed variables (R^2 = 95.8) is presented in Equation (4).

$$Y_{P/T\ Model\ II}\ (\%) = 45.2 + 22.7\ Ez + 5.56\ t + 5.15\ Ez\ t - 3.41\ T\ t \quad (4)$$

Figure 4 shows the representation of the fermentation yield model in the SSF strategy with variations of the enzymatic load of 10–30 FPU g^{-1} glucans and temperatures of 35–39 °C at 72 h.

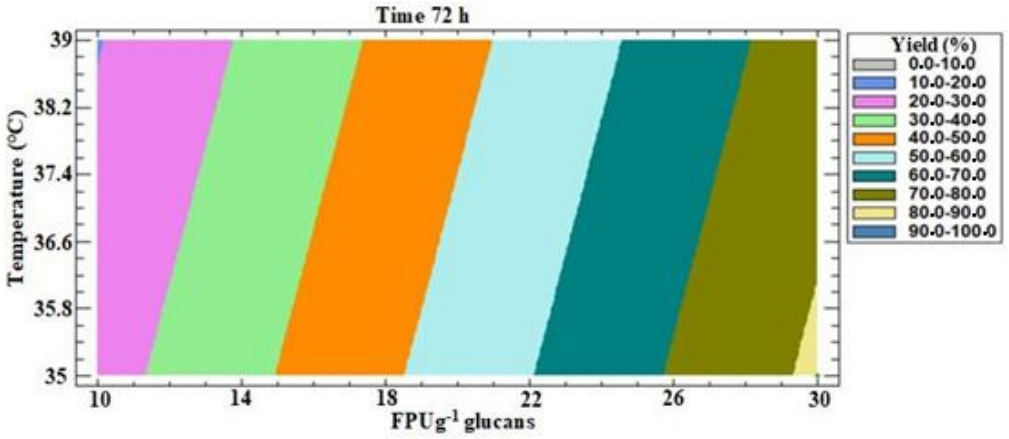

Figure 4. Contours of the estimated response surface of Model II (72 h).

As seen in Equation (4), after the initial 24 h, the temperature has no significant effect on the yield of the fermentation process (it only acts through a mild interaction with time), so both the enzyme and the yeast do not require optimal temperature conditions to carry out their function. However, the results are slightly better at the lower temperature limit (−1 = 35 °C).

The maximum enzymatic load is necessary to maximize cellulose depolymerization into glucose monomers, with the best performance at 72 h reaction (+1 = 30 FPUg^{-1} glucans).

For 72 h, the theoretical fermentation yield obtained from Model II is 81.9, whereas the experimental value is 81.0, with an enzymatic load of 30 FPU g^{-1} glucans at 35 °C.

4. Conclusions

Pine soda-ethanol pulp is a suitable substrate for 2G bioethanol production. Given the low pulp consistency, good bioethanol yields were obtained for this highly recalcitrant pine species using the SSF strategy.

Enzyme load and temperature define fermentation level at the end of the reaction. The enzyme complex showed good performance in the SSF process despite using lower temperatures than the optimal (35–39 °C) for the saccharification process.

The calculated fermentation yield values fit the experimental values, establishing the validity of the obtained models. Fermentation yields optimal values were 63.2% and 81.9% for Models I (short times) and II (long times), respectively. The best conditions found by the experimental designs were 30 FPU g^{-1} glucans, 39 °C and 24 h for Model I, and 30 FPU g^{-1} glucans, 35 °C and 72 h for Model II. This result could serve to adjust the experimental process.

Pine sawdust is an abundant resource. It is bulky, humid, and generally geographically dispersed, all of which complicate its use as an energy resource. Soda-ethanol processing was demonstrated to be an adequate pretreatment of this recalcitrant biomass. The main interest of this series of works is the use of soda-ethanol pretreated pine sawdust to obtain ethanol. This study allowed us to define adequate conditions for each SSF processing lapse, which can be adjusted to achieve maximum bioethanol production.

The obtained results allow defining the best working conditions for the scale change study, which will be carried out at about 12.5% pulp consistency (10% hydrolyzable cellulose). On the other hand, several perspectives need evaluating in future research to reduce costs of 2G bioethanol production, such as enzyme recycling.

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