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Optimization of the enzymatic hydrolysis of cellulose of *triplochiton scleroxylon* sawdust in view of the production of bioethanol

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ABSTRACT

The objective of the work is to optimize the enzymatic hydrolysis step in the production process of bioethanol based on sawdust. For this purpose, sawdust from Ayous (*Triplochiton scleroxylon*) was sampled and characterized. After pre-treatment with the organosovl method, a composite experimental design centred on three factors (substrate loading, enzyme loading and hydrolysis time) was used to study their effects on the release of reducing sugars during hydrolysis. The D-glucose monomers obtained were fermented and the product was distilled. The results obtained reveal that the pretreated Ayous sawdust contains a cellulose content of 53.2 ± 0.3%. At the end of the enzymatic hydrolysis, an increase in the enzyme loading and the hydrolysis time were recorded, followed by a decrease in the substrate loading thus contributing to a better hydrolysis yield of the celluloses. A loading of 9.07% in substrate associated with a loading of 21.36 FPU/gDM of enzyme for 72 hrs constituted the optimal conditions allowing the release of the maximum of reducing sugars and an optimal yield of hydrolysis of celluloses. The optimum values of the reducing sugars and the conversion yield are 21.88 mg/ml and 69%. The yield of Gay - Lussac obtained is 38 g/g. All this shows that it is possible to produce bioethanol from white sawdust.

Introduction

Global demand for energy continues to increase due to global population growth and increasingly energy-consuming consumption patterns. Meanwhile, the main fossil resources (oil, natural gas and coal), which make up most of the current global energy shield, decrease over time. These fossil resources are also partly responsible for large-scale greenhouse gas

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Fig. 1. Pile of sawdust Ayous (*Triplochiton scleroxylon*).

emissions. All these arguments put together militate for the use of renewable energies so most of them are obtained from plant biomass commonly called lignocellulose [1,2].

Cameroon's forest wealth is estimated at over 18 million hectares with the loan of 80 different species sold. Ayous (light white wood) and Sapelli (heavy red wood) account for a third of exports [3]. Ayous is the most abundant and most used gasoline. The industries of first and second processing of these species generate sawdust of about one million cubic meters per year [3]. A significant part is burnt, polluting the atmosphere [3]. Sawdust, however, is part of biomass which is an alternative and renewable energy source. Its transformation into second generation biofuels is based on the conversion of cellulosic biomass into biodiesel by thermochemical gasification [4] or liquefaction by catalytic conversion [5], or into bioethanol by enzymatic hydrolysis [6]. This work is part of the logic of bioethanol production by enzymatic hydrolysis based on sawdust from Ayous (*Triplochiton scleroxylon*).

Several authors have produced bioethanol based on cellulosic biomass [7,8,9,10]. Optimization of the enzymatic hydrolysis step has also been the subject of some work with various substrates. Vasudeo et al., [11], for example, optimized enzymatic hydrolysis using wheat straw by varying the pH, substrate loading, enzyme loading. Pandiyan et al., [12] optimized the enzymatic hydrolysis of the herb (*parthenium sp*) by varying the pH, the temperature, the enzyme loading and the substrate loading. In the same logic, other authors such as Liu and Wang [13], Neifar et al., [14] and Mendes et al., [15] used wood waste, the macro-algae and the sugarcane bagasse as substrates. The general objective in this work is to improve the bioethanol production process based on sawdust from Ayous (***Triplochiton scleroxylon***) considered as waste, by optimizing the enzymatic hydrolysis step. On the one hand, it will be a question of finding the optimal pair (substrate charge-enzyme charge) for obtaining the maximum of reducing sugars. On the other hand, it will be necessary to seek the hydrolysis time necessary to obtain the best saccharification yield under the optimal conditions of this pair (substrate loading-enzyme loading).

Material and methods

Sampling and characterization of the substrate

The substrate used in this work is Ayous sawdust (***Triplochiton scleroxylon***). It was taken from a sawmill located in the town of Ngaoundéré, locatable according to the coordinates 7 ° 19′51,37′′N; 13 ° 35′10.37′′E and 1.27 km altitude. To be sure of having only the Ayous sawdust sample, the sample was taken from a sawdust heap (Fig. 1) obtained during the sawmill from at least 100 boards and bastings made entirely of Ayous wood. It was also essential to participate in the sawmill on the day of the harvest…

Determination of cellulose content

Cellulose was determined in sawdust from Ayous according to the method used by Godin et al., [16]. The principle is based on the delignification of the substrate using ethanol and nitric acid. In practice, 1 g of Ayous sawdust was mixed with a solution consisting of 20 mL of 95% ethanol and 5 mL of concentrated nitric acid. After 1 hrs of boiling in a water bath thermostatically controlled at 95 °C, the solution obtained was decanted and then filtered. The residue from filtration undergoes the same operations of solution mixing (ethanol-nitric acid) three times, heating (boiling), decantation and filtration. The white paste from the fourth heating was first washed with a solution (consisting of a mixture of 40 ml of ethanol and 10 ml of nitric acid), then washed with one litre of hot water before being dried at the oven. The dry residue obtained after

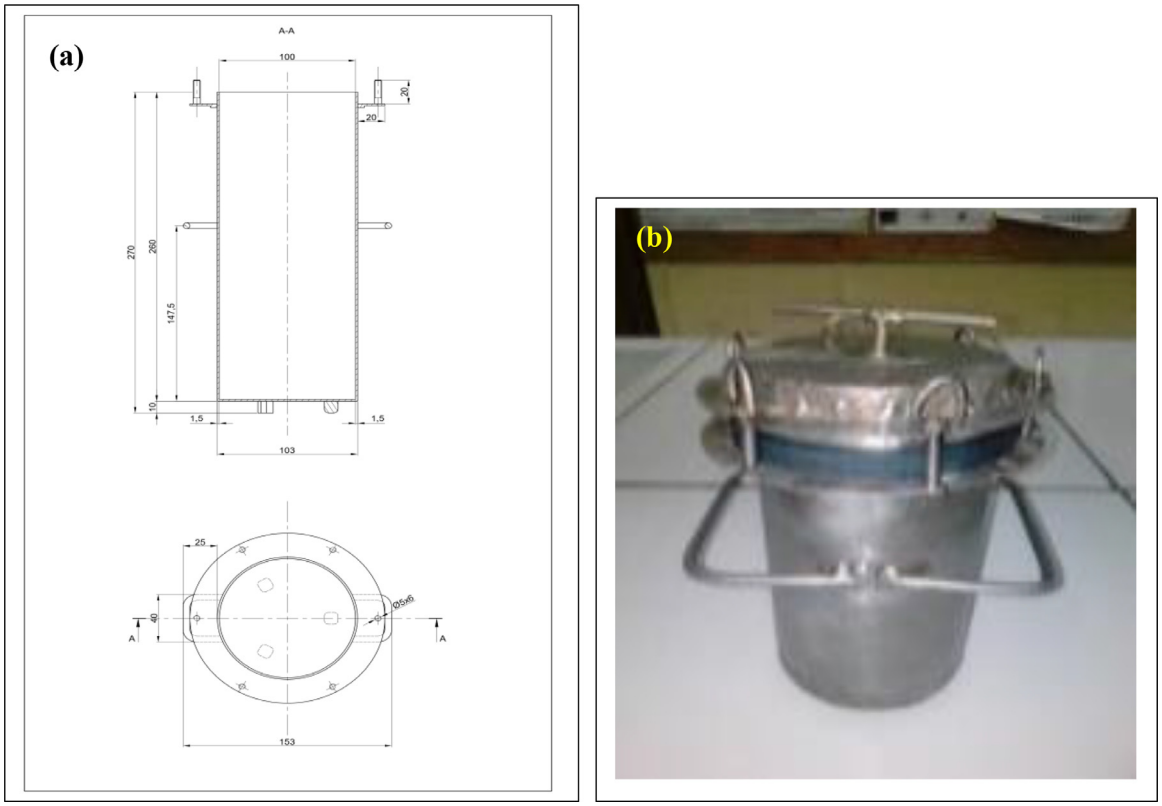

Fig. 2. Technical drawing (a) and image of the 2 L reactor designed (b) for the pre-treatment of sawdust from Ayous by the organosolv process.

drying represents cellulose. Its content was calculated using Eq. (1).

$$\boldsymbol{T} = (\boldsymbol{mi} - \boldsymbol{mf}) * 100 \quad (1)$$

$\boldsymbol{T}$ = cellulose content (%); $\mathbf{m_i}$ = mass of the dry substrate; $\mathbf{m_f}$ = mass of the dry residue.

Bioethanol production process

The process for producing bioethanol from sawdust from Ayous successively followed the stages of pre-treatment, hydrolysis, fermentation and distillation.

Substrate pre-treatment

The substrate was pre-treated according to the organosolv process. Before the pre-treatment operation, however, a reactor was designed by first taking into account the inertness of the material vis-à-vis the suspension to be treated, its high resistance to corrosion and its high conductivity to facilitate thermal transfers. The design also took into account the tightness of the reactor and its ability to withstand temperatures above 250 °C, its thickness and its thread while facilitating heat transfers within the tank. The design ultimately had to take into account the amount of substrate to be pre-treated. The technical drawing produced by AutoCAD-2015 and the image of the designed reactor with a capacity of 2 L are presented in Fig. 2.

The purpose of the pre-treatment is to release the cellulose trapped in the lignin-hemicellulose complex, reduce the cellulosic crystallinity and make the cellulose accessible to enzymes. The pre-processing method used in this work is that of the organosolv process, the optimal points of which were obtained by Tchuidjang et al. [17]. The organosolv process concretely consisted of mixing 50 g of sawdust from Ayous with 1 L of ethanol (23.5%). The mixture was heated at 200 °C for 46 min. The product obtained was cooled and after filtration, the residue was dried in an oven at 105 °C for 24 hrs, until a constant mass was obtained. After the pre-treatment of the substrate, the cellulose content was determined on the pre-treated substrate using the same method used by Godin et al., [16] presented in section "1.2." The pre-treated substrate was then hydrolysed.

Enzymatic hydrolysis

The enzymatic hydrolysis was carried out according to the method used by Bey et al., [18]. The total cellulase activity (filter paper activity) was determined according to the guideline established by the International Union of Pure and Applied

Table 1
Level of each factor.

Factors	Low level (−1)	High level (+1)
Substrate loading X_1)	0.1 (g)/10%	0.2 (g)/20%
Enzyme loading (X_2)	10 FPU/g DM	20 FPU/g DM
Time (X_3)	48 hrs	72 hrs

Table 2
Experience and experimentation matrix.

Test n°	Coded values X_1	X_2	X_3	Rael values X_1	X_2	X_3
1	−1	−1	−1	10	10	48
2	1	−1	−1	20	10	48
3	−1	1	−1	10	20	48
4	1	1	−1	20	20	48
5	−1	−1	1	10	10	72
6	1	−1	1	20	10	72
7	−1	1	1	10	20	72
8	1	1	1	20	20	72
9	−1.41421	0	0	7,93	15	60
10	1.41421	0	0	22.07	15	60
11	0	−1.41421	0	15	7.93	60
12	0	1.41421	0	15	22.07	60
13	0	0	−1.41421	15	15	43
14	0	0	1.41421	15	15	77
15	0	0	0	15	15	60
16	0	0	0	15	15	60
17	0	0	0	15	15	60
18	0	0	0	15	15	60

X_1: Substrate loading; X_2: Enzyme loading; X_3: hydrolysis Time.

Chemistry (IUPAC), Laboratory and analysis procedure (LAP-006) provided by the National Renewable Energy Laboratory of United State of America (NREL, 2008)). It practically consisted in monitoring the quantity of reducing sugars released at 50 °C by the action of enzymes (appropriate dilution) on Whatman No.1 filter paper. Therefore, the total cellulasic activity used was 71 FPU/mL, with a specific activity of 9088 U/g. To follow the evolution of the conversion over time, the concentration of reducing sugars in the products was measured. It is important to note that enzymatic reactions require reaction media with controlled pH. For this, a 50 mM citrate buffer solution (pH 4.8) was used.

Experimental design

In order to better control the enzymatic hydrolysis, modelling by the methodology of the response surface methodology was used. This is how the centred composite plan was used to determine the optimal levels of the various factors influencing performance. There are many parameters that can vary during the hydrolysis reaction. These are the frequency of rotation of the orbital shaker, the enzymes loading, the substrate loading, the reaction temperature and the reaction time.

It has been established that the frequency of rotation of the orbital agitator would have a very small effect on the efficiency of hydrolysis [19]. The hydrolysis reaction has a significant cost and is subject to time constraints. The variables chosen in this work were the Enzyme loading, the Substrate loading and the time of the hydrolysis reaction. The targeted response is the yield of reducing sugars (RS), which has been maximized in this work. The high and low levels of each factor are inspired by the work of Vasudeo et al., [11] and are defined as shown in Table 1.

The transition from the original variables A to the coded variables X, and vice versa is given by Eq. (2), where A_0 is the central value. The experience and experimentation matrix is presented in Table 2.

$$\boldsymbol{x_i} = \frac{\boldsymbol{A_i} - \boldsymbol{A_0}}{\boldsymbol{Pas}} \tag{2}$$

with

$$\boldsymbol{Pas} = \frac{\boldsymbol{A_{+1}} - \boldsymbol{A_{-1}}}{2} \ \boldsymbol{et} \ \boldsymbol{A_0} = \frac{\boldsymbol{A_{+1}} + \boldsymbol{A_{-1}}}{2} \tag{3}$$

Optimization and modelling of enzymatic hydrolysis

To maximize the reducing sugars and the optimal conversion yield, an optimization to a response was carried out thanks to Minitab version 2018. The optimal points from the experimental design were exploited in the laboratory and kinetics of enzymatic hydrolysis were obtained and materialized by a curve of reducing sugars as a function of time. A fitting of the experimental points was carried out with some algebraic models. This in order to compare the behaviour of our reaction

Table 3
Empirical models for the formation of reducing sugars.

No	Authors	Model Equations
1	Chrastil, [20]	$P = P\infty[1 - \exp(-k' * Eo * t)]^n$
2	Etters, [21]	$P = So[1 - \exp(-k*(t)\hat{}x)]^{\hat{}(\frac{1}{y})}$
3	Väljamäe et al., [22]	$P = So*(1 - \exp(-k * t^{(1-h)})$
4	Ohmine et al., [23]	$P = (\frac{So}{k}) * \ln(1 + \frac{Vokt}{So})$

medium to the enzymatic hydrolysis mechanism described by the model which works best with the experimental points. These models are presented in Table 3.

These models were validated on the basis of the criteria expressed by the equations ranging from 4 to 7.

- R^2 (Rsq) and adjusted R^2 (Rsqaj)
- Chi-square

$$\chi^2 = \left(\frac{\sum_{i=1}^{N} \left(Y_{exp,i} - Y_{calc,i}\right)^2}{N - n} \right)^{1/2} \tag{4}$$

- RMSE (Root Mean Square Error)

$$RMSE = \left(\frac{\sum_{i=1}^{N} \left(Y_{calc,i} - Y_{exp,i}\right)^2}{N} \right)^{1/2} \tag{5}$$

- Average absolute error (AAE)

$$AAE = \sum_{i}^{N} |Y_{cal} - Y_{exp}| \tag{6}$$

- Average relative error (ARE)

$$ARE = \frac{\sum_{i}^{N} |Y_{cal} - Y_{exp}|}{Y_{exp}} \tag{7}$$

Experimentation with enzymatic hydrolysis

The enzymatic hydrolysis of the various solid loading was carried out at 7.93%; 10%; 15%; 20% and 22% in glass bottles containing 10 mL of 0.05 M citrate buffer (pH 4.8). These bottles were sterilized at 100 °C in a thermostatic water bath for 20 min. The sterilized bottles were cooled and the various enzymatic loading announced previously were introduced there. The enzymatic reaction was incubated at 48 °C. The stopping time of the enzymatic reactions is indicated in the experimental matrix. At the end of the enzymatic reactions, the hydrolysates were filtered and the must obtained was sterilized in a water bath at 100 °C for 15 min to inhibit any possible microbial contamination before fermentation. Before fermentation, 0.5 ml of the filtrate was removed to quantify the reducing sugars.

Determination of the quantity of reducing sugars

The reducing sugars were measured according to the method described by Fischer and Stein [24]. This technique is based on the reduction of 2,3-dinitrosalicylic acid (DNS) to 3-amino-5-nitrosalicylic acid by sugars, while hot. The reaction product in basic medium develops an orange-yellow coloration. In practice, the extracted sugar solutions (0.25 ml) previously dispersed in the test tubes were mixed with 1 ml of distilled water. The solution was then mixed with 0.25 ml of the DNS reagent and then incubated in a boiling water bath for 10 min. The whole was cooled, then 4 ml of distilled water was added to it before reading the optical densities at 540 nm. A standard glucose solution of 2 mg/ml was used to make the calibration curve. The quantity of reducing sugars in each test portion was determined by referring to the calibration line. The regression equation of which is expressed in the form of $\mathbf{DO = a\ Q + b}$, where **Q** is the quantity of sugar in the test portion. Furthermore, the quantity of sugars Q (in g/100 g DM) is given by Eq. (11).

$$\mathbf{Q'} = 100 * \left[\frac{\mathbf{Q} * \mathbf{Vt}}{\mathbf{m} * \mathbf{V}(100 - \mathbf{Rw})} * 100 \right] \tag{11}$$

Where **Vt** (50 ml) is the total volume of the extract; **m** is the test sample in g; **V** (0.25 ml) is the volume of sample analysed and **Rw** is the residual water content.

The efficiency of the enzymatic hydrolysis was calculated by comparing the glucose yield after hydrolysis with the initial glucan content of the pre-treated residues. This efficiency is expressed as a percentage of cellulose enzymatically converted

Table 4
Experimental and theoretical responses of the centred composite plan for the production of reducing sugars.

N°	Coded valuers			Reals valuers			Reducing Sugars (mg/mL)	
	X_1	X_2	X_3	X_1	X_2	X_3	Y_{expRS}	Y_{calRS}
1	−1	−1	−1	10	10	48	7.13	6.03
2	1	−1	−1	20	10	48	5.24	5.56
3	−1	1	−1	10	20	48	10.25	11.61
4	1	1	−1	20	20	48	7.20	7.52
5	−1	−1	1	10	10	72	11.75	12.36
6	1	−1	1	20	10	72	10.30	9.87
7	−1	1	1	10	20	72	20.78	21.38
8	1	1	1	20	20	72	13.25	15.28
9	−1.41	0	0	7.93	15	60	16.36	15.71
10	1.41	0	0	22.07	15	60	12.26	11.06
11	0	−1.41	0	15	7.93	60	7.65	8.46
12	0	1.41	0	15	22.07	60	18.89	16.23
13	0	0	−1.41	15	15	43	8.28	8.03
14	0	0	1.41	15	15	77	19.59	17.99
15	0	0	0	15	15	60	15.25	16.34
16	0	0	0	15	15	60	16.50	16.34
17	0	0	0	15	15	60	15.45	16.34
18	0	0	0	15	15	60	16.29	16.34

$\mathbf{X_1}$: Substrate loading; $\mathbf{X_2}$: Enzyme loading; $\mathbf{X_3}$: hydrolysis Time; $\mathbf{Y_{exp}}$: Experimental responses; $\mathbf{Y_{cal}}$: Theoretical responses; **RS**: reducing sugar content;.

(CEC) into glucose using Eq. (8).

$$\%\boldsymbol{CEC} = \frac{\boldsymbol{C} \times \boldsymbol{V} \times \boldsymbol{a}}{\boldsymbol{m} \times \%\boldsymbol{cellulose} \times 1.1} \times \ 100 \tag{8}$$

Where **C** is concentration of reducing sugars in glucose equivalent (g/L); **V** is volume of hydrolysate; **a** is the dilution factor; **m** is the dry matter of the biomass (pre-treated or not pre-treated) before hydrolysis and "**1.1**" is conversion factor from cellulose to glucose.

Fermentation

1.03 g of Saccharomyces cerevisiae was used to ferment 100 ml of hydrolysates from enzymatic hydrolysis. The ethanol yield was calculated by dividing the total amount of ethanol produced (ethanol g) in the fermentation broth by the mass of initial reducing sugars (g) (Eq. (9)). In this equation, 0.511 represents the theoretical yield of ethanol (g) produced per gram of glucose [25]. It can also be expressed as a percentage of ethanol yield compared to the theoretical value using Eq. (10).

$$\boldsymbol{Ethanol\ yield} = \frac{\boldsymbol{Ethanol}\ (\boldsymbol{g})}{0,511\ \times \mathbf{glucose}\ (\mathbf{g})} \tag{9}$$

$$\boldsymbol{Ethanol\ yield}\ \left(\frac{\boldsymbol{g}}{\boldsymbol{g}}\right) = \frac{\mathbf{Masse\ d'\acute{e}thanol}\ (\mathbf{g})}{\mathbf{Masse\ de\ sucres\ r\acute{e}ducteurs}\ (\boldsymbol{g})} \tag{10}$$

Results

Cellulosic potential of sawdust from ayous

Ayous sawdust contains a cellulose content of 49.4 ± 0.5%. With a cellulose content of 53.2 ± 0.3%, pre-treated Ayous sawdust mobilized almost 3% more cellulose than raw sawdust. This shows a fairly good availability of cellulose for the enzymatic hydrolysis step.

Model of the enzymatic hydrolysis of sawdust from ayous

Table 4 presents the values of the responses (reducing sugars) obtained at the end of each experiment in the experimental matrix.

The mathematical model of the second-degree polynomial type generated by this experimental plan, and describing the concentration of reducing sugars released during the enzymatic hydrolysis of sawdust from Ayous is presented by Eq. (12). The validation criteria for this model are presented in Table 5.

$$\begin{aligned}\mathbf{Y_{RS}} = {} & 16,335 - 1,643\,\mathbf{X_1} + 2,746\,\mathbf{X_2} + 3,521\,\mathbf{X_3} - 1,476\,\mathbf{X_1X_1} - 1,996\,\mathbf{X_2X_2} - 1,663\,\mathbf{X_3X_3} - 0,905\,\mathbf{X_1X_2} \\ & - 0,505\,\mathbf{X_1X_3} + 0,863\,\mathbf{X_2X_3}\end{aligned} \tag{12}$$

$\mathbf{Y_{RS=}}$reducing sugars content**;** $\mathbf{X_{1=}}$substrate loading; $\mathbf{X_{2=}}$enzyme loading**;** $\mathbf{X_3}$ = hydrolysis time.

Table 5
Validation indicators for the reducing sugar release model.

Validation indicators	Y_{RS}	Acceptable values	References
R^2	94.08%	> 80%	Joglekar and Mayand Boyaci [27]
Adjusted R^2	87.41%	> 80%	
AADM	0.072	[0–0,3]	Pedersen [28]
Bias factor (Bf)	1	[0,75–1,25]	Pedersen and Jorge-sen [28]
Accuracy factors (Af)	1.075	[0,75–1,25]	

AAMD: Absolute Analysis of Mean Deviation.

Table 6
Meaning of the different effects of the mathematical model for the release of reducing sugars (Eq. (12)).

Factors and their interactions	Reducing sugars	
	Coefficient	P-value
Substrate loading (X_1)	−1.643	0,009
Enzyme loading (X_2)	2.746	0,000
Time (X_3)	3.521	0,002
Substrate loading*Substrate loading (X_1^2)	−1.476	0,038
Enzyme loading*Enzyme loading (X_2^2)	−1.996	0.010
Time*Time (X_3^2)	−1.663	0.023
Substrate loading*Enzyme loading (X_1 X $_2$)	−0.905	0.166
Substrate loading*Time (X_1 X_3)	−0.505	0.420
Enzyme loading*Time (X_2 X_3)	0.863	0.184

Effects with a probability (P-value) <0.05 are considered significant at the 95% confidence level.

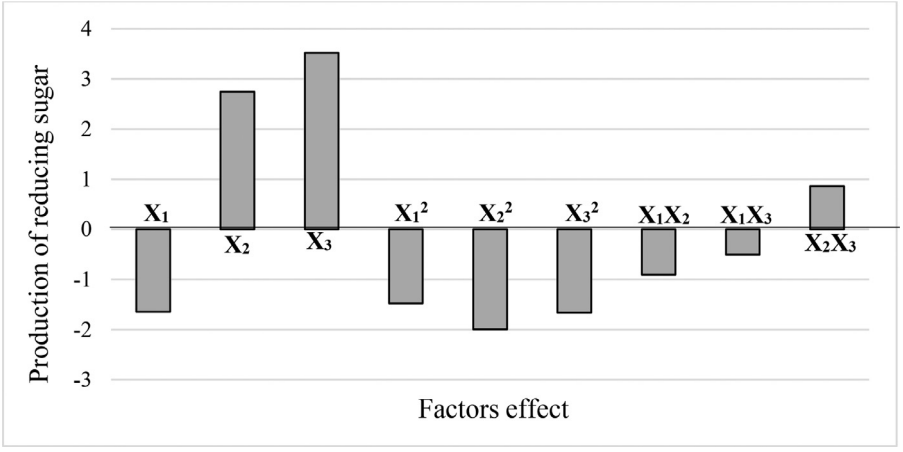

Fig. 3. Contribution of each factor in the model, for the production of reducing sugars.

The validation indicators for the reducing sugar model are in the standard acceptance intervals. This means that the model obtained is valid. This model explains 94.08% of the variability of the response (reducing sugars). Table 6 presents the significance of the direct effects, interactions and quadratic effects of the reducing sugar release model with a probability at the 95% confidence level.

The variance analysis of the coefficients of the model reveals that the direct effect of the substrate loading (X_1), the quadratic effects of the substrate loading (X_1^2), of enzyme loading (X_2^2) and of time (X_3^2) are significant, but negatively influence the yield of reducing sugar. These coefficients also contribute 10.7%, 9.6%, 13.03% and 10.08% respectively to the decrease in the yield of reducing sugars. On the other hand, the direct effects of the enzyme loading (X_2) and time (X_3) are significant at the 95% confidence level and positively influences the enzymatic hydrolysis of celluloses from sawdust. These two coefficients respectively contribute 17.92% and 22.9% to the increase in the yield of reducing sugars. These observations are more noticeable on the representation in the form of a histogram of the coefficients of the different effects of the factors for the production of reducing sugars, presented in Fig. 3.

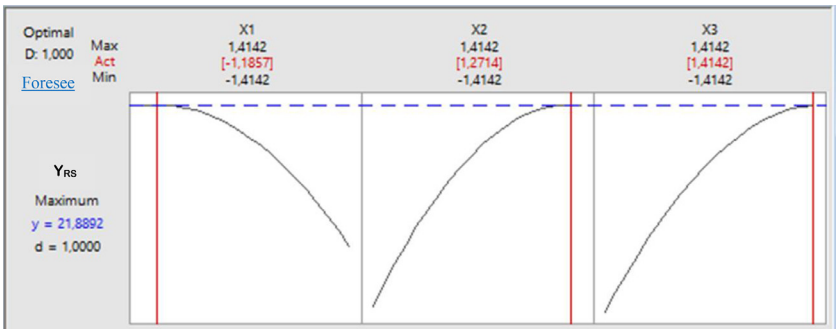

Fig. 4. Optimization diagram of the reducing sugar model.

Table 7
Combination of factors for optimizing a response from the reducing sugar release model.

Factors	Optimum	Real values
Substrate loading	−1.1857	9.07%
Enzyme loading	1.2714	21.36 FPU/g DM
Hydrolysis Time	1.4142	72 hrs

Optimized data

Fig. 4 shows the optimal points for the three factors (substrate loading, enzyme loading and hydrolysis time) obtained after optimization to a response performed using Minitab 2018 version.

It appears from this Fig. 4 that the reduction in the substrate loading favors both the hydrolysis of the cellulose and the increase in the enzyme loading in the reactor and the time of hydrolysis. The points for each factor (from this Fig. 4) to be exploited during the enzymatic hydrolysis to obtain a maximum of reducing sugar are moreover presented in table 7.

Optimizing a response gives a predictive value of 21.88 mg/mL for reducing sugars. The conversion yield of celluloses into reducing sugar obtained is 69%.

Enzymatic kinetics

Fig. 5 represents the kinetic curve of enzymatic hydrolysis of Ayous sawdust celluloses from the optimal points.

It appears from this Fig. 5 that at an optimal time of 72 hrs of hydrolysis (Table 7), the content of reducing sugars is 21.35 mg/mL. This is close to the prediction value (21.88 mg/mL) obtained above. However, the kinetics of enzymatic hydrolysis integrating the optimal values of the factors monitored, shows that the enzymatic reaction continues for an optimal time around 93 hrs. The additional 21 hrs of hydrolysis reaction imposed by the kinetics can increase the reducing sugar content by a little more than 2 mg/mL. The kinetics of enzymatic hydrolysis of sawdust cellulose from Ayous was compared with certain models, the characteristics of which are presented in Table 8.

It appears from Table 8 that only the model of Väljamäe et al., [22] fits best with experimental data, because it presents the smallest sum of the minima of 0.640. This means that the kinetics of enzymatic hydrolysis of sawdust from Ayous is a fractal kinetic model. Under these optimal hydrolysis conditions, the optimal (Gay - Lussac) production yield is 0.38 g of ethanol /g of reducing sugars. However, there is a visible difference between the theoretical optimal hydrolysis time (72 hrs) obtained from the experimental design and the actual optimal hydrolysis time (93 hrs) obtained after implementation of the optimal points.

Discussion

Effect of factors on the production of reducing sugars

The second-degree polynomial equation generated by the experimental design revealed that the quadratic effects of the substrate loading (X_1^2), enzyme loading (X_2^2) and hydrolysis time (X_3^2) contribute to a significant reduction in production yield reducing sugars. This negative influence can be explained by a slow diffusion of the enzyme in the cellulosic structure of the substrate which is dense due to its overload. Indeed, the direct effect of the substrate loading (X_1) already having a negative influence on the enzymatic hydrolysis (Table 6). it is obvious that its quadratic effect (X_1^2) goes in the same direction, because the high concentration or density of the substrate in the reactor contributes to slow down the diffusion of the enzyme within the cellulosic structure, even if the concentration of enzyme is high. These experimental conditions therefore lead to a slowing down of the formation of the enzyme-substrate complex. In addition, the high density of the

Table 8
Statistical coefficients of the comparative models for the formation of reducing sugars.

Authors	Equations	**MinSu**	**k**	$\mathbf{V_0}$	$\mathbf{E_0}$	$\mathbf{P_\infty}$	$\mathbf{S_0}$	**h**	**x**	**y**	**n**
Väljamäe et al., [22]	$P = S_0\ [1\text{-}\exp(-k*t^{(1-h)})]$	0.640	0.0028				31.401	−0.3738			
Chrastil's, [20]	$P = P_\infty\ [1\text{-}\exp(-k'*E_0{}^*t)]^n$	0.742	0.126		0.126	10.729					1.499
Etters, [21]	$P = S_0[1\text{-}exp(-k^*t^{(x)})]^{(\frac{1}{y})}$	0,805	0.046				38.2931		0.7827	0.4718	
Ohmine et al., [23]	$P= (\frac{So}{k})^*(\ln(1+\frac{Vokt}{So})$	2.828	0.006	0.297			67.5				

MinSu = minimum sum; ***k*** = speed constant; $\mathbf{V_0}$ = initial speed at Ohmine; $\mathbf{E_0}$ = initial enzyme loading; $\mathbf{P_\infty}$ = Products that diffuse; $\mathbf{S_0}$ = Initial cellulose substrate; ***h*** = the fractal dimension; **-k, x** and **y** are the specific parameters for each bit rate; ***n*** = order of the apparent reaction (**n** tends to **1** if the diffusion resistance is small. If the diffusion resistance is high, then **n** is small.).

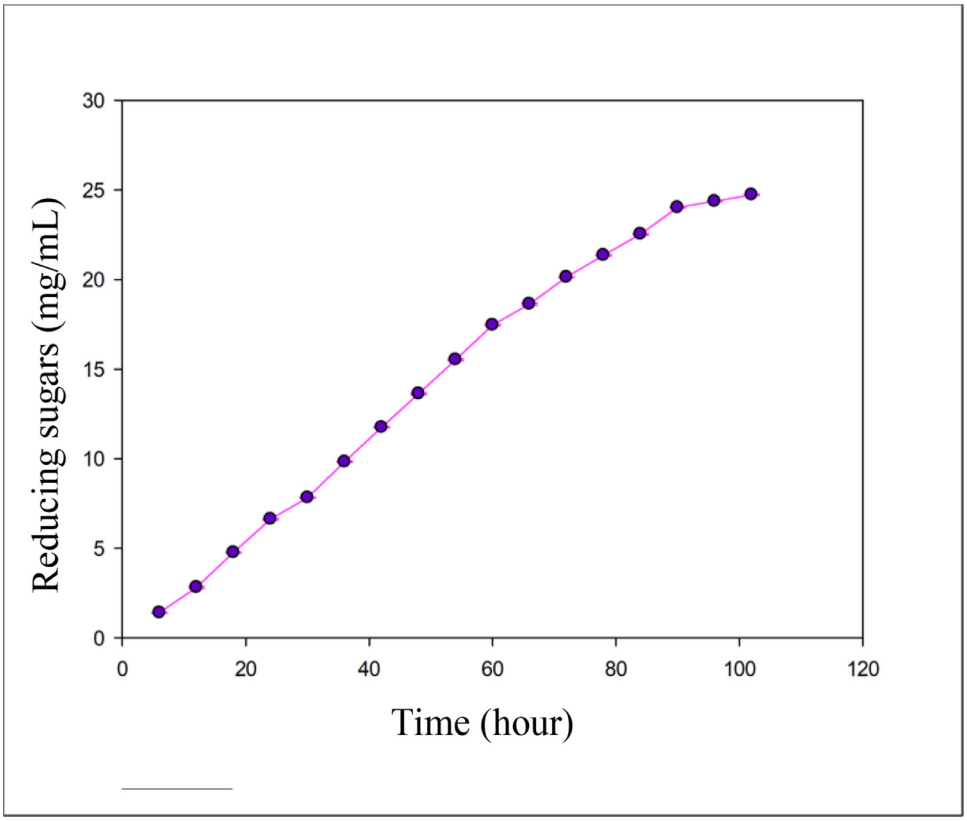

Fig. 5. Kinetics of enzymatic hydrolysis of Ayous sawdust with the optimal values of the substrate loading and the enzyme loading.

substrate can sometimes even inhibit the enzymatic action during the evolution of hydrolysis [29,30]. This is moreover verified by the negative effect of the substrate loading - enzyme loading ($X_1 \times_2$) interaction visible in Table 6.

The negative effects of the substrate loading (X_1 and X_1^2) inevitably require more hydrolysis time (X_3^2), with unfortunately a low yield of reducing sugars if necessary. Especially since the negative effect of the interaction between substrate loading and hydrolysis time ($X_1 \times_3$) demonstrates that the good progress of the enzymatic hydrolysis of Ayous cellulose does not depend on the simultaneous variation of these two factors. The scenario described above is even more striking when the cellulosic structure of the exploited substrate is rigid as is often the case for wood. Indeed, the rigidity of the cellulosic structure does not favour an easy enzymatic activity within it [31,32]. So, the density of Ayous sawdust linked to its high loading in the reactor, associated with its rigid structure clearly justify the significant but negative influence of its quadratic effect, as well as that of the enzyme loading on the production of sugars reducers.

The polynomial equation (Eq. (12)) shows, on the other hand, that the direct effects of the enzyme loading (X_2) and the hydrolysis time (X_3) significantly favour the hydrolysis of the cellulose of Ayous sawdust. The positive influence of these two factors on the production of reducing sugars can be explained by the fact that the increase in hydrolysis time promotes better formation of the cellulase-cellulose complex. In fact, the catalytic site of the cellulase is effectively fixed on the Ayous cellulose when the hydrolysis lasts over time, thus favouring the release of the monomers [33]. This observation is also verified through the positive effect of the enzyme loading - hydrolysis time ($X_2 \times_3$) interaction, appreciable in Table 6.

All of this information reveals that to optimize the hydrolysis of Ayous sawdust pre-treatment cellulose, it is necessary to reduce the substrate loading and increase the enzyme loading as much as the hydrolysis time (Fig. 4). The optimal enzymatic hydrolysis yield of Ayous cellulose obtained in this work (69%) is lower than the 72.5% and 79.6% respectively obtained by Lu et al., [34] and Fang et al., [35] on the hydrolysis of cellulose from corn stalks pre-treated with vapour explosion and organosolv, respectively. These differences can be explained on the one hand by the nature of the substrates used (sawdust from Ayous and corn stalks), which can be observed not only through the difference in the cellulose contents existing between the two substrates, but also through the difference in the cellulosic structures constituting the two substrates. Indeed, it is logical that the substrate richest in cellulose (corn Stover 58% [35]) can produce after enzymatic hydrolysis more reducing sugar than the substrate less rich in cellulose (Sawdust Ayous (49%)). It is also logical that the substrate having a more flexible cellulosic structure, favours more the enzymatic diffusion and therefore the formation of the cellulase-cellulose complex for a higher enzymatic hydrolysis yield (corn stalk) than, the substrate having a structure rigid and more crystalline cellulosic (sawdust from Ayous) which slows down the enzymatic diffusion within it. This is also the reason why the results

of this optimization reveal that to optimize the enzymatic hydrolysis of the cellulose (rigid structure) of sawdust from Ayous, it is necessary to decrease the substrate loading and increase the enzyme loading.

The optimal enzymatic hydrolysis yield of Ayous cellulose obtained in this work (69%) is also lower than the 74.65% obtained by Romaní et al., [36] who worked on the pre-treated *Eucalyptus globulus* wood. by hydrothermal. This other difference can be explained by the pre-treatment methods used (organosolv and hydrothermal).

Enzymatic hydrolysis model of ayous cellulose

The kinetics of cellulose hydrolysis of sawdust from Ayous follows the model of Väljamäe et al., [22], characteristic of a fractal kinetic model. This means that there can be various phenomena at different fractions of the curve during the enzymatic kinetics carried out. Moreover, the 21 hrs difference between the theoretical optimal hydrolysis time (at 72 hrs) and the real or experimental hydrolysis time (at 9rs3 h) observable in Fig. 5, give the impression that the fraction of the kinetic curve between the 72nd hour and the 93rd hour of enzymatic hydrolysis was not taken into account by the values of the optimization of the enzymatic hydrolysis. However, this difference is more or less predicted by the polynomial equation for optimizing the production of reducing sugars (Eq. (12)) through the value of its constant ($\varepsilon = 16{,}335$), which expresses the error or the difference between the values following the experiment. This difference is justified by the difficulty that cellulase had in diffusing within the cellulose of sawdust from Ayous, due to its rigid structure [37]. Indeed, at the end of the 72 hrs of enzymatic hydrolysis (Fig. 5) predicted by the optimization of the operation, a significant number of catalytic sites of the cellulose was not reached by the cellulase because of the slow diffusion of the latter, caused by the rigid cellulosic structure of the substrate. The consequence was an extension of the enzymatic activity until 93 hrs when a plateau is initiated, characterizing the drop in enzymatic hydrolysis. This kinetics of the enzymatic hydrolysis made it possible to appreciate the additional hydrolysis time necessary which should be added to the theoretical optimal time, if it is essential to exhaust the cellulose polymer in monomer. It is important to insist on the choice of whether or not to add this additional hydrolysis time, since extending the time for enzymatic hydrolysis after the 72 hrs of prediction involves additional consumption of energy. Knowing that the 21 hrs of additional hydrolysis will produce a little more than 2 mg/mL of reducing sugars representing almost 9.7% of the amount of reducing sugars produced in 72 hrs.

As part of this work, an optimal yield of 0.38 g of ethanol / g of reducing sugars was obtained. This result is close to that of Millati et al., [38] who obtained a yield of 0.35 g of ethanol /g of reducing sugars at the end of the fermentation of fir hydrolysates pre-treated with sulfuric acid.

Conclusion

The objective of this work was the optimization of the enzymatic hydrolysis of Ayous wood sawdust celluloses (***Triplochiton scleroxylon***) through organosovl pre-treatment for the production of bioethanol. It emerges from this study that the substrate loading negatively influences the enzymatic hydrolysis reaction of Ayous sawdust cellulose and therefore rather contributes to lowering the production yield of reducing sugars. However, the increase in the enzyme loading and the hydrolysis time favour the enzymatic hydrolysis and the yield of reducing sugars. The kinetics of enzymatic hydrolysis of sawdust Ayous follows a fractal model. The rigid cellulosic structure of this species of wood does not favours the diffusion of cellulase within it, which leads to an increase in hydrolysis time and therefore an overconsumption of energy. In view of the optimal ethanol yield per gram of reducing sugars obtained in this work, we believe that this method of producing bioethanol would be more favourable from both a yield point of view and an energy cost, using a substrate having a softer cellulosic structure.

Declaration of Competing Interest

We are the authors of the manuscript entitled “Optimization of the enzymatic hydrolysis of cellulose of ***Triplochiton scleroxylon*** sawdust in view of the production of bioethanol”, and we declare that no conflict of interest exited amongst us.

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