

Sodium borohydride treatment to improve the fermentation of acid hydrolysates from pruning olive-tree biomass. Reduction of phenolic and furans compounds

A.J. Moya, S. Peinado, S. Mateo, S. Sánchez

Department of Chemical, Environmental and Materials Engineering, University of Jaén, 23071 Jaén (Spain)
Presenting author email: ajmoya@ujaen.es

Abstract

The application of an effective removal treatment to minimise inhibitory compounds, present after hydrolysis treatment, might mean a great advance on renewable biofuel generation strategy since inhibitors substances, such as phenolic compounds and furans, are one of the major problems in the development of the fermentative step. Detoxification of olive-tree pruning hydrolysates using NaBH₄, as reducing agent, has had a positive effect on fermentative parameters using *Pichia stipitis* CBS 6054. Oxalic acid treatment conditions have been optimised, considering the combined severity factor (CSF). Sodium borohydride detoxification process has been studied using a response surface methodology, 2³ central composite design matrix, conducting both optimisation and verification of the model by comparing theoretical and predicted values. The use of NaBH₄ has been demonstrated to be a successful method for almost entirely furans elimination as well as 40% of phenolic compounds reduction. As result of this inhibitors reduction, it has been possible to ferment the hydrolysate which did not ferment without such treatment, obtaining a maximum ethanol yield of 27% in treated hydrolysates under optimum conditions.

Keywords: Olive–tree biomass, Detoxification, Sodium borohydride, Fermentation, Inhibitors

1. Introduction

Olive-tree pruning biomass is the most abundant source of lignocellulosic biomass in Andalusia (Spain) and other Mediterranean countries. Annually, large volumes of this waste are produced but there is no profitable use of them. There are, in essence, two primary problems associated with this lignocellulosic biomass: the fact that, currently, there is no application considered as technologically and economically viable and, on the other hand, the tradition of burning this renewable material on lands, generating serious problems related to air pollution, ground mineralisation or fire hazards.

Nowadays, bioethanol production is basically centered in fermentation of D-glucose from lignocellulosic biomass. This fact has the drawback of the difficulty of polymers decomposition into simple sugars (mainly D-glucose and D-xylose), and the reduction of inhibitory compounds present after hydrolysis treatments. The amount and type of toxic or inhibitors compounds generated depend on both the hydrolysis severity and the biomass source [1]. The development of effective strategies to minimise the effect of inhibitory compounds might mean a great advance on renewable biofuel production. This work presents the possibility of using a reducing agent (NaBH₄) under mild reaction conditions as a novel way to reduce the content of phenolic and furanic compounds from olive-tree pruning hydrolysates to make fermentation easier and thus improving ethanol yield.

2. Material and methods

2.1 Biomass

Olive-tree (*Olea europaea L.*) pruning biomass was collected from 15 to 20 years old trees (located between 411730-411740 m EW and 4 196 882-4 196 893 m NS relative to UTM coordinates) after the fruit harvest; the samples were taken from fresh branches located around 1.5 m above the ground. Subsequently, the material was air-dried at room temperature to equilibrium moisture content (~8%) and milled using a laboratory hammer mill (Retsch). The fraction graded to a particle size between 0.425 and 0.600 mm, according to ASTM guidelines, was stored into glass jars sealed until used.

2.2 Oxalic acid pretreatment

Acid hydrolysis of olive pruning biomass was conducted in a stainless steel discontinuous pressure reactor, stirred tank, Parr model 4842 (Moline, IL, USA) with a stirring speed of 15.71 rad/s. Oxalic acid was used as acid catalyst. The solid and liquid ratio was 1–10. Nine different conditions were selected for pretreatment: 130, 150 and 170 °C with 50, 75 and 100 mM oxalic acid. The reaction time was maintained 30 min after the targeted temperature was achieved. Remaining lignocellulosic material was separated by filtration. Both solid substrates and hydrolysates were stored at 4 °C. In order to compare results in different hydrolysis conditions and to determine a quantitative measure of the acid treatment intensity, a semi-empirical combined severity factor, CSF, was employed.

The severity factor for hydrolytic treatments carried out with the olive tree pruning, has been evaluated according to the expression of Overend et al. [2], who defined R_0 as Eq. (1).

$$R_0 = \int_0^t e^{\frac{T(t)-100}{w}} dt \quad (1)$$

Where: $T(t)$, is the function concerning the temperature variation of the reaction system with the treatment time, °C.; t , is the treatment time, min; w , is an empirical parameter related to the activation energy, equal to 14.75 for materials of similar characteristics to olive-tree pruning biomass.

Calculating R_0 from Eq. (1) will therefore allow us to measure the combined effect of both variables (temperature and time) in a given treatment. Regarding hydrolysis experiments, three different phases can be distinguished: heating, holding and cooling.

In order to calculate the severity factor, the functions $T(t)$ have been adjusted to second-order polynomial, constant and linear for heating, maintenance and cooling, respectively. The overall severity factor is determined as the sum of those corresponding to the three phases. Finally, CSF was calculated attending to Eq. (2) [3].

$$CSF = \log R_0 - pH \quad (2)$$

Several studies described the CSF as a more representative factor than severity one ($\log R_0$), since it includes, in addition, the effect of acid catalysts [4].

2.3 Sodium borohydride treatment

To determine the best conditions of the three variables studied: time from 3.2 to 36.8 min, NaBH_4 concentration from 9.6 to 110.4 mM and pH from 3.32 to 6.68, a response surface methodology has been applied using Statistica 6.0 software (Statsoft, USA). The treatment of the lignocellulosic hydrolysates was performed in glass vessels equipped with magnetic stirred bars and placed on a magnetic plate at room temperature adjusting pH with NaOH and adding powdered NaBH_4 directly to each vessel at different concentrations, enabling this chemical compound to react during each programmed time. All experiments were performed in duplicates.

2.4. Fermentation of hydrolysates

The microorganism *P. stipitis* CBS 6054 was stored at 15 °C in 100 cm³ test tubes on a sterilised solid culture medium composed of (in g/dm³): yeast extract 3; malt extract 3; peptone 5; D-xylose 10; agar 20. Before the start of each experiment, the microorganism was inoculated under sterile conditions into glass test tubes containing the solid culture medium described above. These tubes were then kept in an incubator at 30 °C for 48 h to obtain cells at the same growth stage for every experiment. The concentration of the inoculum at the beginning of each experiment was approximately 0.3 g/dm³.

Fermentation assays were conducted in 100 cm³ Erlenmeyer flasks containing 40 cm³ of hydrolysate, supplemented with half of Lindegren medium components [5], at 30 °C, initial pH of 5.5 and 15.71 rad/s. Flasks were closed with hydrophobic cotton.

2.5 Sugars and inhibitors analysis

Sugars (D-glucose, D-xylose and L-arabinose), acetic acid as well as ethanol concentrations in the hydrolysate were quantified using HPLC (Waters 2695 system, Alliance, USA), outfitted with an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, USA) and a refractive index detector (Waters 2414 system, Alliance, USA). The mobile phase was 5 mM H₂SO₄ at a flow rate 0.6 cm³/min for 1 h. Total furans were determined by the spectrophotometric method described by Martinez et al. [6]. Total content of phenolic compounds was estimated by the Folin–Ciocalteu modified method using caffeic acid as standard [7].

3. Results and discussion

3.1 Oxalic acid pretreatment

Fig. 1 shows the corresponding adjustments of total sugars (sum of D-xylose, D-glucose and L-arabinose), the main monomers obtained, and the acetic acid concentration versus the combined severity factor. These equations have made it possible to determine the optimum value of CSF in order to maximise sugars concentrations. So, the best values of the combined severity factor would be in the range 1.5–1.6.

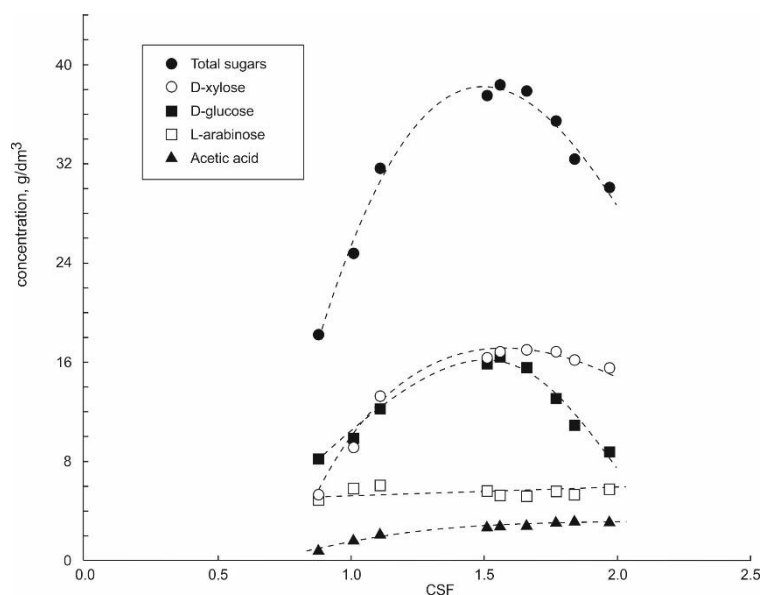


Fig. 1 Variations of total sugars, D-xylose, D-glucose, L-arabinose and acetic acid concentrations with the combined severity factor.

As a result of the experimental design, the optimal hydrolysis conditions were determined, being 150 °C and 75 mM of oxalic acid pretreatment (CSF = 1.53 ± 0.05). The characterisation of this optimal hydrolysate showed a composition of 16.64 g/dm³ D-glucose, 15.05 g/dm³ D-xylose, 5.16 g/dm³ L-arabinose, 2.87 g/dm³ acetic acid, 3.18 g/dm³ phenolic compounds and 0.96 g/dm³ furans. The adjust of the experimental data let us to calculate at the maximum fermentable sugar recovery a value close to 38.2 g/dm³.

3.2 Sodium borohydride treatment

The hydrolysate, obtained under optimal conditions according to previous treatments, was employed as the basis for the study of variables that could affect the detoxification process with sodium borohydride. Table 1 shows the reductions (%) of phenolic (Y₁) and furans (Y₂) compounds obtained at each assay.

Table 1 2³ central composite design of experiments, with two replicates in the central point, and the corresponding phenolic and furan compounds reduction

Run	time (min)	NaBH ₄ (mM)	pH	Furan reduction (%)	Phenolic reduction (%)
1	10.0	30.0	4.00	24.4	8.0
2	10.0	30.0	6.00	94.0	26.1
3	10.0	90.0	4.00	88.8	25.7
4	10.0	90.0	6.00	93.8	32.6
5	30.0	30.0	4.00	36.0	16.0
6	30.0	30.0	6.00	93.8	37.0
7	30.0	90.0	4.00	90.7	27.5
8	30.0	90.0	6.00	93.3	33.1
9	3.2	60.0	5.00	93.6	28.8
10	36.8	60.0	5.00	96.4	34.5
11	20.0	9.6	5.00	42.7	15.4
12	20.0	110.4	5.00	93.6	31.6
13	20.0	30.0	3.32	30.9	14.6
14	20.0	30.0	6.68	93.4	39.9
15	20.0	30.0	5.00	92.0	35.6
16	20.0	30.0	5.00	88.2	35.8

These results configure the models that fit the two responses studied and that correspond to the equations (3) y (4). Good correlation coefficients have been obtained.

$$Y_1 = 35.82 + 2.25 A + 4.32 B + 6.89 C - 1.72 A^2 - 4.60 B^2 - 3.27 C^2 - 2.08 AB - 3.32 BC \quad R^2_{adj} = 0.974 \quad (3)$$

$$Y_2 = 93.54 + 14.94 B + 17.58 C - 8.13 B^2 - 10.25 C^2 - 14.98 BC \quad R^2_{adj} = 0.977 \quad (4)$$

Where A, B and C represent the corresponding coded values of time, NaBH₄ concentration and pH respectively.

Contour plots of modeled phenolic compounds reductions and response surface for the percentage of removed furans, as a function of B and C at fixed time of 20 min set at the central point, are represented in Fig. 2 and Fig. 3 respectively.

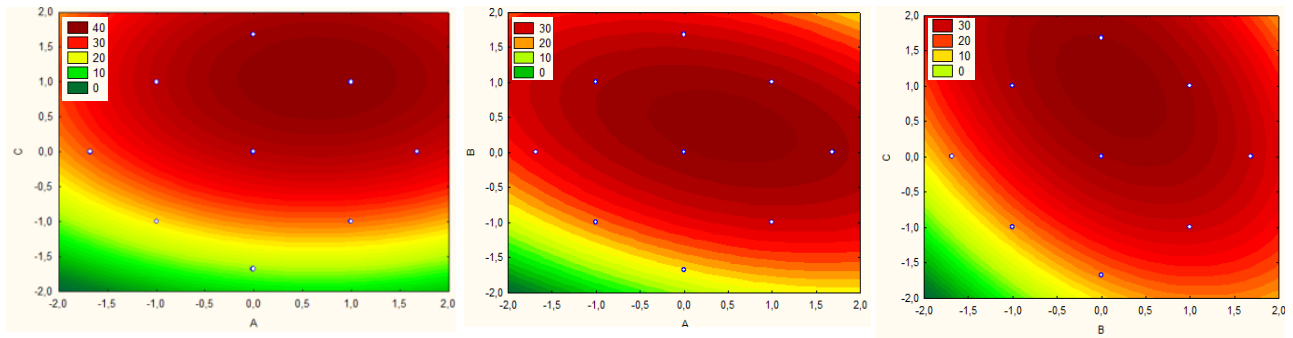


Fig. 2 Contour plots of modeled phenolic compounds reduction at the central point

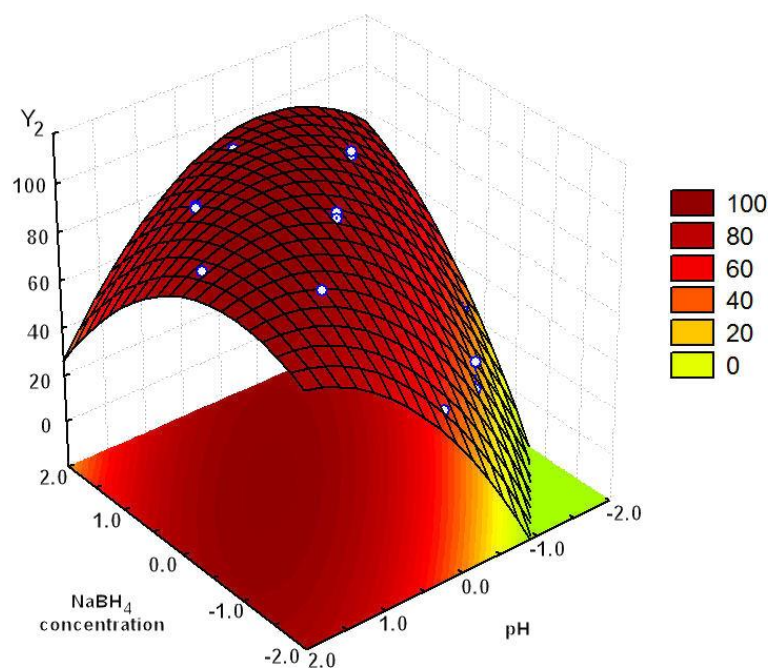


Fig. 3 Response surface modeling the percentage of removed furans as a function of B and C at fixed time of 20 min set at the central point

The use of Design Expert software has allowed us to obtain the corresponding values for the studied variables optimising, at the same time, furans and phenolics reductions jointly.

Finally, the obtained model was verified, carrying out the assays by triplicate, considering optimal conditions for NaBH_4 tests according to the standard model. Table 2 shows data related to the comparative study for response surface modeling confirmation. Minor variations are observed involving a good fit between experimental and theoretical values.

Table 2 Comparative analysis between theoretical values predicted by the model and empirical values obtained considering optimal treatment conditions for both, furans and phenolic compounds reduction

Exp	A	B	C	OPTIMISATION			CONFIRMATION	
				Model Values			Empirical Values	
				% Furans reduction	% Phenol reduction	Desirability	% Furans reduction	% Phenol reduction
O1	0.4	0.1	0.8	98.0	40.0	1	97.9	40.1
O2	0.0	-0.2	0.5	97.9	37.7	1	97.5	34.8
O3	0.7	-1.0	1.0	92.8	36.0	1	94.0	33.4
O4	1.0	-1.0	1.0	92.8	36.5	1	93.8	37.3

3.3 Hydrolysate fermentation

After the optimisation process, it was possible to select different experimental conditions according to the maximum possible desirability. Thus, the experiments O1 to O4 (showed in Table 2) have been selected to be fermented with *P. stipitis* CBS 6054.

Some studies have reported that ethanol fermentation was unsuccessful when the concentration of hydroxymethylfurfural (HMF), furfural and acetic acid in the hydrolysate was more than 1.0, 1.0 and 2.0 g/dm^3 , respectively, [8-10]. Therefore, a detoxification step becomes necessary for the elimination or reduction of the high concentrations of inhibitors such as acetic acid, furfural and HMF [11].

Fig. 4. shows the results (biomass, sugars and ethanol concentrations versus time) for the fermentation of the four optimised selected hydrolysates tests performed, together with the fermentation of the hydrolysate without borohydride treatment.

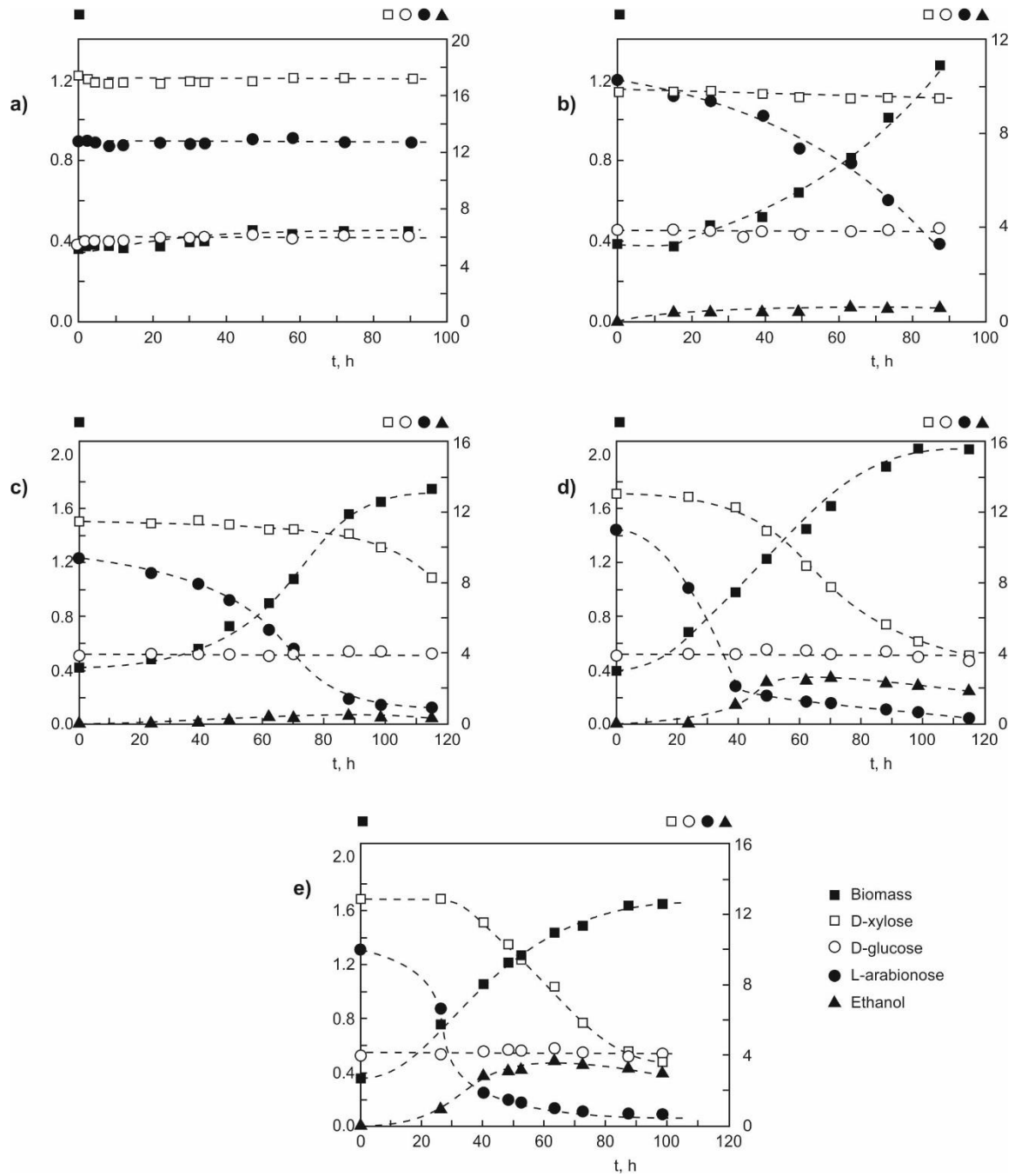


Fig. 4 Fermented profiles of the hydrolysates a) without NaBH₄ treatment; b) O1; c) O2; d) O3; e) O4. All concentrations expressed in g/dm³

The ethanol yield was calculated attending to Eq. (5)

$$Y_{E/S} = \frac{\text{Ethanol produced (kg)}}{\text{Total substrate consumed (kg)}} \quad (5)$$

As a consequence of the experimental data obtained, Table 3, it is noted that although the treated hydrolysates provide the best detoxification conditions, the fermentation responses (ethanol yield mainly) are not quite good. It opens a new stage that will have to be developed in the future.

Table 3 Fermentation parameters

Exp	Detoxification conditions			Fermentation parameters		
	t, min	NaBH ₄ , mM	pH	Y _{E/s} , g/g	t, h	Total sugar consumed, %
O1	24	63	5.8	0.059	73	22.4
O2	20	54	5.5	0.077	62	18.6
O3	27	30	6.0	0.186	62	46.3
O4	30	30	6.0	0.271	63	45.8

It is noteworthy, however, that the hydrolysates treated with a lower concentration of NaBH₄ have provided better ethanol yields; thus, the maximum Y_{E/s} value was obtained in the fermentation of the hydrolyzate O4 (0.271 g/g) at 63 h and consuming almost half of the initial total sugars.

4. Conclusion

The use of NaBH₄ has demonstrated to be a good method to reduce phenolic compounds and furans concentrations in order to improve the fermentation process. The better conditions were able to reduce even totally the furans content and around 40% of phenolic compounds. This treatment has improved the fermentability of the acid hydrolysates.

References

- [1] Mateo S., Roberto I.C., Sánchez S., Moya A.J. (2013). Detoxification of hemicellulosic hydrolyzate from olive tree pruning residue. *Ind. Crops Prod.* 49, 196-203.
- [2] Overend, R.P., Chornet, E., Gascoigne, J.A. (1987). Fractionation of lignocellulosics by steam-aqueous pretreatments and discussion. *Philos. Trans. Roy. Soc. London A* 321, 523–536.
- [3] Moya A.J., Peinado S., Mateo S., Fonseca, B.G., Sánchez S. (2016). Improving bioethanol production from olive pruning biomass by deacetylation step prior acid hydrolysis and fermentation processes *Bioresour. Technol.* 220, 239–245.
- [4] Kabel, M.A., Bos, G., Zeevalking, J., Voragen, A.G.J., Schols, H.A. (2007). Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. *Bioresour. Technol.* 98, 2034–2042.
- [5] Lindegren, C.C., Nagai, S., Nagai, H. (1958). Induction of respiratory deficiency in yeast by manganese, copper, cobalt and nickel. *Nature* 182, 446–448.
- [6] Martinez, A., Rodriguez, M.E., York, S.W., Preston, J.F., Ingram, L.O. (2000). Use of UV absorbance to monitor furans in dilute acid hydrolysates of biomass. *Biotechnol. Prog.* 16, 637–641.
- [7] Fonseca, B.G., Puentes, J.P., Mateo, S., Sánchez, S., Moya, A.J., Roberto, I.C. (2013). Detoxification of rice straw and olive tree pruning hemicellulosic hydrolysates employing *Saccharomyces cerevisiae* and its effect on the ethanol production by *Pichia stipitis*. *J. Agric. Food Chem.* 61, 9658–9665.
- [8] Guo, G.L., Chen, W.H., Chen, W.H., Men, L.C., Hwang, W.S. (2008). Characterization of dilute acid pretreatment of silvergrass for ethanol production. *Bioresour. Technol.* 99, 6046–6053.
- [9] Palmqvist, E., Hahn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. *Bioresour. Technol.* 74, 25–33.
- [10] Wei, W., Wu, S., Liu, L. (2012). Enzymatic saccharification of dilute acid pretreated eucalyptus chips for fermentable sugar production. *Bioresour. Technol.* 110, 302–307.
- [11] Trinh, L.T.P., Kundu, C., Lee, J.W., Lee, H.J. (2014). An integrated detoxification process with electro dialysis and adsorption from the hemicellulose hydrolysates of yellow poplars. *Bioresour. Technol.* 161, 280–287.