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Application of Fenton's Reaction to Steam Explosion Prehydrolysates from Poplar Biomass

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Abstract

The application of Fenton's reaction to enhance the fermentability of prehydrolysates obtained from steam explosion pretreatment of poplar biomass was studied. Reaction conditions of temperature and H_2O_2 and Fe(II) concentrations were studied. The fermentability of prehydrolysate treated by Fenton's reaction was tested by using different inoculum sizes of thermotolerant strain *Kluyveromyces marxianus* CECT 10875. The highest percentages of toxic compound degradation (ranging from 71 to 93% removal) were obtained at the highest H_2O_2 concentration tested (50 m*M*). However, a negative effect on fermentability was observed at this H_2O_2 concentration at the lower inoculum loading. An increase in inoculum size to 0.6 g/L resulted in an enhanced ethanol fermentation yield of 95% relative to control.

Index Entries: Fenton's reaction; detoxification; poplar prehydrolysate; ethanol production; *Kluyveromyces marxianus.*

Introduction

For an efficient ethanol production by biologic processing of lignocellulose, feedstock must be pretreated to more effectively recover the hemicellulosic fraction and, concurrently, to make the cellulose more accessible to enzymatic hydrolysis. Among the different pretreatment options, autohydrolysis steam explosion has been proved to be an adequate pretreatment method for lignocellulosic materials such as hardwoods and has the advantage of being developed at a commercial scale *(1)*. In steam explosion pretreatment, which is often performed at temperatures about 200°C and acid conditions, some degradation products from carbohydrates and lignin are formed that inhibit fermentation of hemicellulosic prehydrolysates *(2,3)*. Although the concentration of such inhibitory compounds varies greatly with the operational pretreatment conditions and the raw material

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used, the toxic substances may be divided into three main groups based on their origin: weak acids (mainly acetic acid), degradation products of sugars such as furfural (dehydration product of pentoses) and hydroxymethylfurfural (HMF) (dehydration product of hexoses), and phenolic compounds from lignin (aromatic acids, alcohols such as catechol, and aldehydes such as 4-hydroxybenzaldehyde and vanillin) *(4,5)*.

Various biologic, physical, and chemical detoxification methods have been employed to reduce the toxicity of lignocellulosic hydrolysates. Selection of the most adequate method will depend on the composition of prehydrolysate and the species of microorganism employed in the fermentation step, because each type of hydrolysate has a different degree of toxicity and each species of microorganism has a different degree of tolerance to inhibitors *(6,7)*. Other key features to be considered are the cost of the method and the simplicity of integrating it into the overall process.

Mussatto and Roberto *(8)* have recently reviewed these detoxification alternatives, including the application of different combinations of treatment methods, which seems to be the better approach. Nevertheless, there is no reference in the literature about the application of treatments based on the total oxidation of toxic organic compounds by using advanced oxidation processes (AOPs), which appear to be very promising in the treatment of contaminated effluents containing natural phenolic pollutants *(9,10)*. The AOPs potentially destroy toxic organic compounds in wastewater effluents such as unsaturated aliphatic and aromatic compounds *(11)*. The efficiency of these systems is based on the production of strong oxidant species, such as hydroxyl radicals, which are able to oxidize almost all organic pollutants. These radicals are generated in different ways and are highly reactive and unselective oxidants.

One of the most effective AOPs used to remove organic pollutants in aqueous solutions is Fenton's reaction *(12)*, which involves the reduction of H_2O_2 by a ferrous ion into OH^- and $OH^{\cdot}$ (hydroxyl radical). The reagent supplies a source of $OH^{\cdot}$ that can react with organic compounds to give oxidation products:

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

This reaction has recently been used to degrade lignin from steam explosion of beech *(13)* and organic contaminants from agroindustrial effluents *(14)*.

Quantification of the main toxic compounds present in prehydrolysates generated when pretreating poplar biomass at 210°C and 4 min was performed in the present study. The application of Fenton's reaction was then evaluated to degrade such toxic compounds and to enhance fermentability of prehydrolysates. Reaction conditions of temperature and H_2O_2 and Fe(II) concentrations were optimized in order to degrade toxic compounds identified in the prehydrolysate. The fermentability of prehydrolysate treated by Fenton's reaction using thermotolerant strain *Kluyveromyces marxianus* CECT 10875, at different inoculum sizes, was tested.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma (St. Louis, MO), except ferrous sulfate ($FeSO_4 \cdot 7H_2O$) and H_2O_2 (33%) (w/v), which were supplied by Panreac (Barcelona, Spain).

Preparation of Hemicellulose Prehydrolysate

Chipped poplar biomass (*Populus nigra*) was provided by the Renewable Energy Development Center at Lubia (Soria, Spain). The steam explosion tests were performed in a batchwise-operated reactor as described previously *(15)*. The pretreated material was suction filtered, and the filtrate (prehydrolysate) was collected (approx 1 L) and analyzed.

Experimental Procedure for Fenton's Reaction Treatment

Experiments to achieve the reduction or complete removal of toxic organic compounds were conducted in 100-mL Sovirel flasks. The reaction mixture, consisting of 75 mL of prehydrolysate (pH 3.8) and the precise amount of reagent (H_2O_2 and heptahydrated ferrous sulfate), was continuously stirred and incubated at the desired temperature in a water bath for 2 h.

A 2^3 factorial experimental design based on with central point replicate *(16)* was performed considering two levels for temperature (60 and 70°C), Fe(II) (10 and 90 ppm), and H_2O_2 (10 and 50 m*M*) using the software Statagraphics Plus 5.0 (Table 2). The percentage of toxic compound removal after 2 h of reaction was used as the response factor. Low, high, and central levels have been designated –1, +1, and 0, respectively.

Microorganism and Fermentation Experiments

K. marxianus CECT 10875, a thermotolerant yeast strain, was used in fermentation experiments. Active cultures for inoculation were prepared by growing the microorganism overnight on a rotary shaker at 150 rpm and 42°C in a growth medium (initial pH of 5.5) containing 5 g/L of yeast extract, 2 g/L of NH_4Cl, 1 g/L of KH_2PO_4, 0.3 g/L of $MgSO_4 \cdot 7H_2O$, and 30 g/L of glucose.

To study the effect of inoculum size on fermentability, a 2^4 factorial experimental design with central point replicate was performed considering two levels of inoculum concentration (0.2 and 1 g/L) (Table 3). Fermentation experiments were carried out in 100-mL Erlenmeyer flasks with each containing 25 mL of detoxified prehydrolysates supplemented with the medium just described and incubated at 42°C and 150 rpm. Flasks were inoculated at biomass concentrations of 0.2, 0.6, and 1 g/L under nonsterile conditions. Ethanol concentration was determined at 24 h from the onset of fermentation. Fermentation tests were evaluated in terms of ethanol yield

($Y_{p/s}$). $Y_{p/s}$ was calculated by dividing the ethanol produced in 24 h by the initial glucose concentration and expressed as a percentage of a control without any toxic compound.

Analytical Procedures

4-Hydroxybenzoic acid, vanillin, syringaldehyde, syringyl alcohol, 4-hydroxybenzaldehyde, catechol, guaiacol, syringic acid, and vanillic acid analysis was performed on a high-performance liquid chromatography (HPLC) system with a photodiode-array detector. Acetic, levulinic, and formic acid analysis was carried out with an HPLC system with a refractive index detector. Ethanol was measured by gas chromatography with a flame ionization detector. A more detailed description of the analytical procedure has been described previously *(7)*.

Results and Discussion

Prehydrolysate Composition and Fermentability

Table 1 shows the results of the quantitative determination of degradation compounds identified in the prehydrolysate from steam pretreatment of poplar at 210°C and a 4-min residence time. Acetic acid (1400 mg/L), derived from acetyl groups present in the hemicellulose of hardwood poplar; furfural (500 mg/L), from degradation of pentoses; and formic acid (400 mg/L), from furfural and HMF degradation, were the main compounds present in the prehydrolysate. HMF concentration (100 mg/L) is lower because of the low quantity of hexoses in hemicellulose of hardwood and their slower degradation.

As a first step in the study of the fermentability of the prehydrolysate from steam explosion pretreatment of poplar, the medium was adjusted to pH 5.5 with 2 *N* NaOH and supplemented with 30 g/L of glucose (pH 5.5). Growth and ethanol production by *K. marxianus* CECT 10875 were evaluated. Fermentation was completely inhibited at any inoculum concentration tested (data not shown).

Degradation of Toxic Compounds by Fenton's Reaction

The Fenton's reaction was applied to prehydrolysate to reduce the initial concentration of toxic compounds. Table 2 provides the factorial experimental design, the relation between codified and real values, and the percentage of toxic compound degradation. The choice of the particular values for the three factors studied (temperature, H_2O_2, and ferrous iron) was based on results obtained previously *(17)*.

As can be seen in Table 2, Fenton's reaction was an effective method to reduce overall concentration of furans and phenolic compounds in the prehydrolysate. For all compounds tested, the highest percentages of degradation were obtained at the highest H_2O_2 concentration tested.

Table 1
Composition of Degradation Products in Liquid Fraction Obtained After Steam Explosion Pretreatment of Poplar Biomass at 210°C, 4 min

Compound	Concentration (mg/L)[a]
Acetic acid	1400
Formic acid	400
Levulinic acid	n.q.
Furfural	500
HMF	100
4-Hydroxybenzaldehyde	n.q.
4-Hydroxybenzoic acid	100
Catechol	23
Guaiacol	n.q.
Syringaldehyde	35
Syringic acid	n.q.
Vanillin	22
Vanillic acid	n.q.

[a] n.q., not quantified.

Increased catechol, HMF, and vanillin degradations were obtained at low temperatures and the highest values for H_2O_2. For removal of furfural, higher H_2O_2 concentrations and temperatures and low iron loads were more adequate. Concentration of sugars (10.1 g/L in the form of oligomers) and aliphatic acids in the prehydrolysate (1.4 and 0.4 g/L of acetic and formic acids, respectively) was not affected by Fenton's reaction at the conditions studied (data not shown). Similarly, 4-hydroxybenzoic acid was more recalcitrant to oxidation than aldehydes and catechol. Although *K. marxianus* is able to use all monosaccharides present in the prehydrolysate, they are found at low concentration (about 10% of total sugars) in comparison with oligosaccharides. Thus, in this study, only the effect of Fenton's reaction on the oligosaccharides was considered.

Figures 1–5 show the response surface plots for catechol, furfural, HMF, 4-hydroxybenzoic acid, and vanillin, respectively, when iron concentration and temperature parameters are fixed. The model applied could not explain syringaldehyde reduction by Fenton's reaction and, therefore, it is not represented.

As can be seen in Fig. 1, catechol removal increased significantly by Fenton's reaction (an average of 81.5% removal). H_2O_2 was the most important factor in removing catechol, and no effects for temperature and Fe(II) concentration were observed.

Fenton's reaction caused significant furfural degradation in the prehydrolysate for all conditions tested (Fig. 2). For a favorable decrease in furfural, the major effect was owing to H_2O_2, but temperature also played a significant role. A negative interaction between temperature and H_2O_2 was observed.

Table 2
Factorial Experimental Design of Prehydrolysates Treated by Fenton's Reaction and Removal of Toxic Compounds

	Codified value			Variable level			Removal of toxic compounds					
Experiment no.	T	H_2O_2	Fe^{2+}	T (°C)	H_2O_2 (mM)	Fe^{2+} (ppm)	Furfural (%)	HMF (%)	4-Hydroxy benzoic acid (%)	Catechol (%)	Vanillin (%)	Syringaldehyde (%)
1	–1	–1	–1	60	10	10	51	30	14	65	27	40
2	1	–1	–1	70	10	10	66	32	8	61	36	46
3	–1	1	–1	60	50	10	91	80	61	92	64	60
4	1	1	–1	70	50	10	91	75	56	87	68	71
5	–1	–1	1	60	10	90	58	33	11	78	32	31
6	1	–1	1	70	10	90	67	21	7	74	36	34
7	–1	1	1	60	50	90	91	81	69	92	77	83
8	1	1	1	70	50	90	93	76	71	92	73	74
9	0	0	0	65	30	50	77	69	28	87	46	57
10	0	0	0	65	30	50	78	71	25	87	50	95

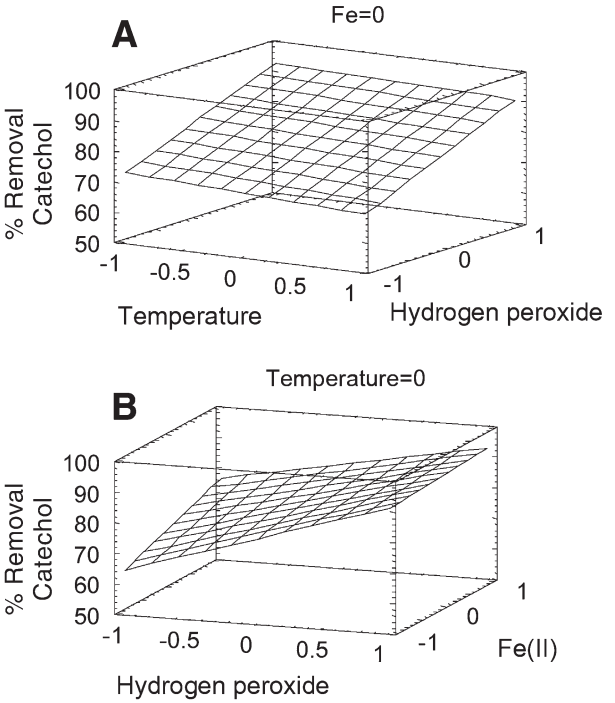

Fig. 1. Three-dimensional (3D) representation of response surface corresponding to percentage of catechol removal after 2 h of reaction. **(A)** Iron concentration and **(B)** temperature parameters are fixed at central values (Fe^{2+} = 50 ppm and T = 65°C).

H_2O_2 concentration was the only important factor for HMF removal (Fig. 3). At higher H_2O_2 concentration, HMF removal increased, but no effect of temperature and ferrous ion concentration was observed.

4-Hydroxybenzoic acid was only partly degradated by Fenton's reaction (average 35% removal). H_2O_2 concentration had, again, the major effect on 4-hydroxybenzoic acid degradation (Fig. 4), independently of temperature and iron concentration. For vanillin removal (Fig. 5), H_2O_2 concentration was also the most important factor.

The results showed that H_2O_2 was the most important factor for inhibitor removal. The highest percentages of toxic compound degradation (ranging from 71 to 93% removal) were obtained at the highest H_2O_2 concentration tested (50 m*M*). However, an increase in Fe(II) concentration from 10 to 90 ppm had no effect on toxic removal, suggesting that further optimization of this treatment should include lower Fe(II) concentration or even the lack of Fe(II).

Fermentation Experiments of Detoxified Prehydrolysates

To test the fermentability of the prehydrolysates after treatment at different Fenton's reaction conditions, fermentation experiments of detoxified broths, supplemented with nutrients and 30 g/L of glucose, were performed

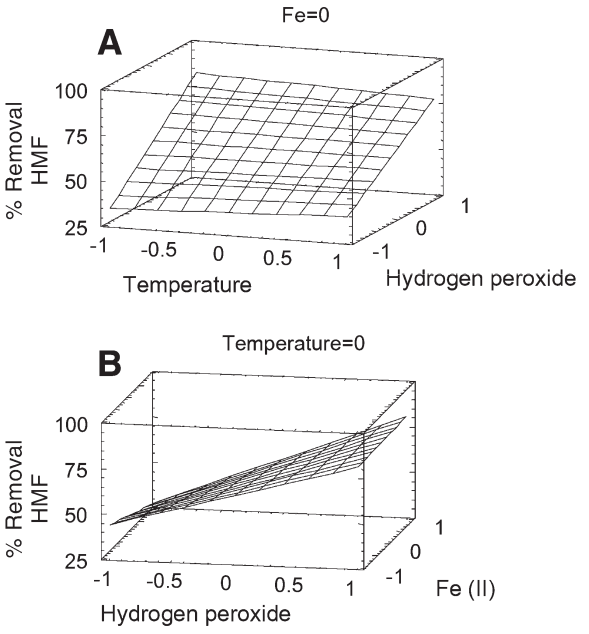

Fig. 3. 3D representation of response surface corresponding to percentage of HMF removal after 2 h of reaction. **(A)** Iron concentration and **(B)** temperature parameters are fixed at central values (Fe^{2+} = 50 ppm and T = 65°C).

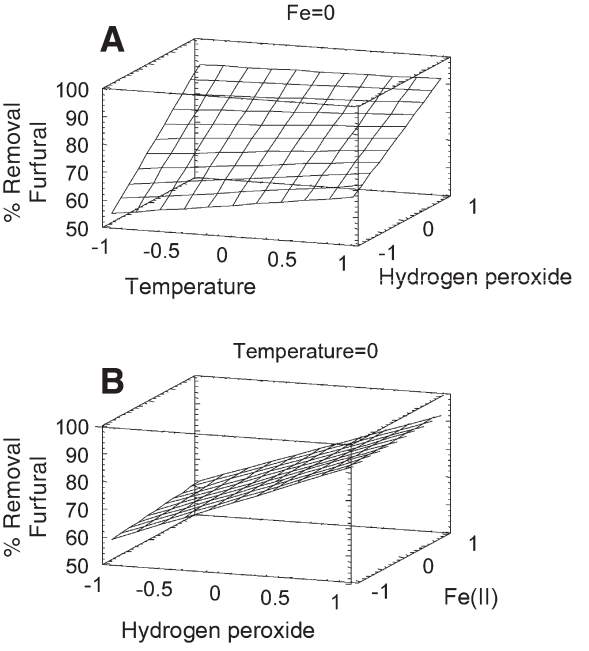

Fig. 2. 3D representation of response surface corresponding to percentage of furfural removal after 2 h of reaction. **(A)** Iron concentration and **(B)** temperature parameters are fixed at central values (Fe^{2+} = 50 ppm and T = 65°C).

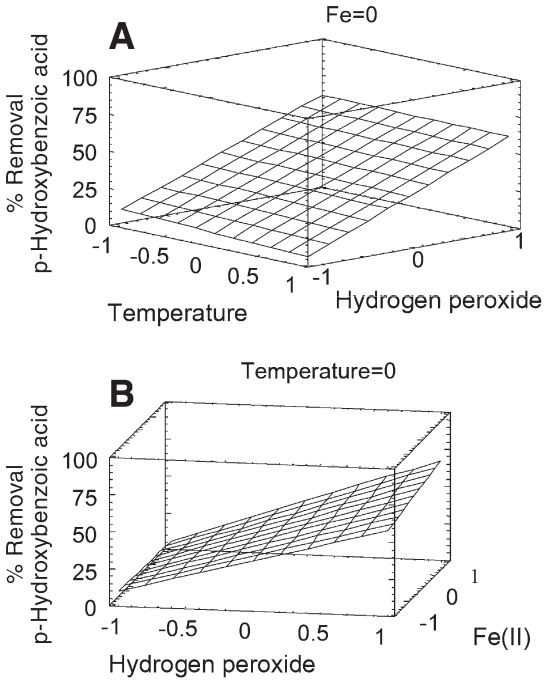

Fig. 4. 3D representation of response surface corresponding to percentage of 4-hydroxybenzoic acid removal after 2 h of reaction. **(A)** Iron concentration and **(B)** temperature parameters are fixed at central values (Fe^{2+} = 50 ppm and T = 65°C).

by using different inoculum concentrations of *K. marxianus* CECT 10875. Table 3 provides the factorial experimental design, the relation between codified and real values, and the ethanol yield at 24 h from the onset of fermentation. As can be seen, Fenton's reaction enhanced prehydrolysate fermentability in most conditions tested. Because by Fenton's reaction furans and phenolics are specifically removed (concentrations of aliphatic acids were not affected), this implies that these compounds are major inhibitors in lignocellulosic prehydrolysates.

At lower H_2O_2 concentration and inoculum load, ethanol yields above 90% of the control were obtained. At this low inoculum load an increase in H_2O_2 concentration caused a total inhibition of fermentation (Table 3). An excess of H_2O_2 seems to cause a detrimental effect on the microorganism. At higher inoculum sizes (0.6 and 1 g/L), ethanol production was about 92–99% of the control, even at the highest H_2O_2 concentration.

Figure 6 shows the response surface plot for ethanol yield when temperature and iron and temperature and H_2O_2 parameters are fixed at central values. In ethanol yield, the more important effects are owing to initial inoculum (positive) and H_2O_2 concentration (negative). A positive effect in the interaction between the factor H_2O_2 and inoculum concentrations was also observed. As can be seen in Fig. 6A, Fe(II) concentrations, in the range studied, had a slight effect on ethanol yield. On the other

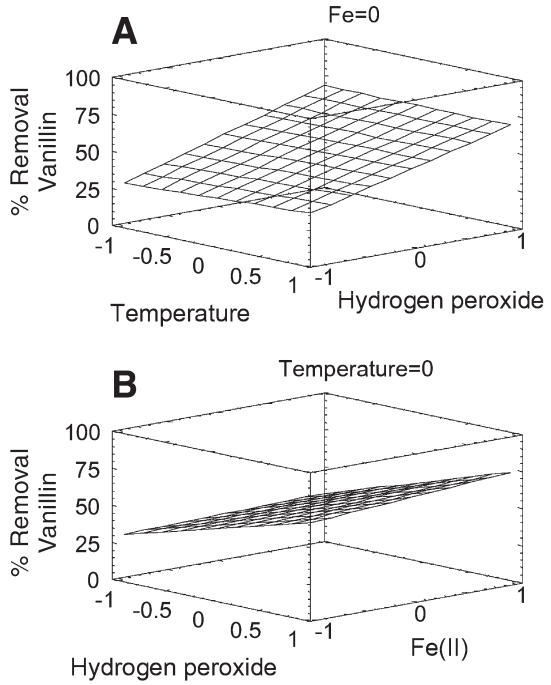

Fig. 5. 3D representation of response surface corresponding to percentage of vanillin removal after 2 h of reaction. **(A)** Iron concentration and **(B)** temperature parameters are fixed at central values (Fe^{2+} = 50 ppm and T = 65°C).

hand, the effect of inoculum concentration on ethanol production was more pronounced.

Bacteria and yeasts have been shown to metabolize furans *(18,19)* and aromatic aldehydes *(20,21)* although enzymes involved in the metabolic pathways remain unknown in most cases. In a previous work, Oliva et al. *(22)* demonstrated that *K. marxianus* CECT 10875 exhibits higher aldehyde assimilation rates in comparison with other fermentating microorganisms, although the reduction of aldehydes to their corresponding alcohols was necessary to start ethanol production by the yeast. As a consequence, the presence of aldehydes caused a significant increase in lag phase before ethanol production started. At the higher inoculum sizes used in their study, the period of aldehyde assimilation was shortened and, consequently, the lag phase. Thus, the optimum inoculum concentration, and the adaptation of the microorganism to toxic compounds, should be considered important variables in increasing fermentability of prehydrolysates.

Higher H_2O_2 concentrations (Fig. 6B), which were more effective in removing toxic substances, produced a decrease in ethanol production. Higher H_2O_2 concentrations had a pernicious effect on ethanol yield, showing that an excess of H_2O_2 has an inhibitory effect on the microorganism. By using a large yeast inoculum the toxic effect of H_2O_2 could be overcome.

Table 3
Factorial Design for Fermentation Experiments of Prehydrolysates Treated by Fenton's Reaction[a]

Experiment no.	Codified value				Variable level				
	T	H_2O_2	Fe^{2+}	Inoculum	*T* (°C)	H_2O_2 (ppm)	Fe^{2+} (m*M*)	Inoculum (g/L)	$Y_{p/s}$ (%)
1	–1	–1	–1	–1	60	10	10	0.2	92
2	1	–1	–1	–1	70	10	10	0.2	93
3	–1	1	–1	–1	60	50	10	0.2	0
4	1	1	–1	–1	70	50	10	0.2	0
5	–1	–1	1	–1	60	10	90	0.2	65
6	1	–1	1	–1	70	10	90	0.2	99
7	–1	1	1	–1	60	50	90	0.2	0
8	1	1	1	–1	70	50	90	0.2	36
9	–1	–1	–1	1	60	10	10	1	99
10	1	–1	–1	1	70	10	10	1	96
11	–1	1	–1	1	60	50	10	1	94
12	1	1	–1	1	70	50	10	1	92
13	–1	–1	1	1	60	10	90	1	97
14	1	–1	1	1	70	10	90	1	98
15	–1	1	1	1	60	50	90	1	92
16	–1	1	1	1	70	50	90	1	96
17	0	0	0	0	65	30	50	0.6	95
18	0	0	0	0	65	30	50	0.6	95

[a]$Y_{p/s}$ was relative to control (100% control equivalent to 0.42 g/g).

The response surfaces clearly show that an H_2O_2 concentration of 50 m*M*, at which more toxic compounds were removed, is not adequate to obtain high ethanol production. From the results, it can be concluded that the overall elimination of toxic compounds present in the prehydrolysates is not necessary to alleviate inhibition.

Conclusion

Historically, a number of detoxification methods have been proposed to transform inhibitors into inactive compounds or to reduce their concentration. The effectiveness of a detoxification method depends not only on reducing the presence of inhibitors in hemicellulosic prehydrolysates, but also on the microorganism strains and fermentation conditions. Fenton's reaction has been shown to be an effective method for removing toxic compounds generated during steam explosion of biomass. H_2O_2 was the most important factor for inhibitor removal. The highest H_2O_2 concentration tested (50 m*M*) was most effective in removing inhibitors. However, this concentration resulted in lower ethanol yield when using a lower inoculum size (0.2 g/L). A larger inoculum size (0.6 g/L) was required to overcome

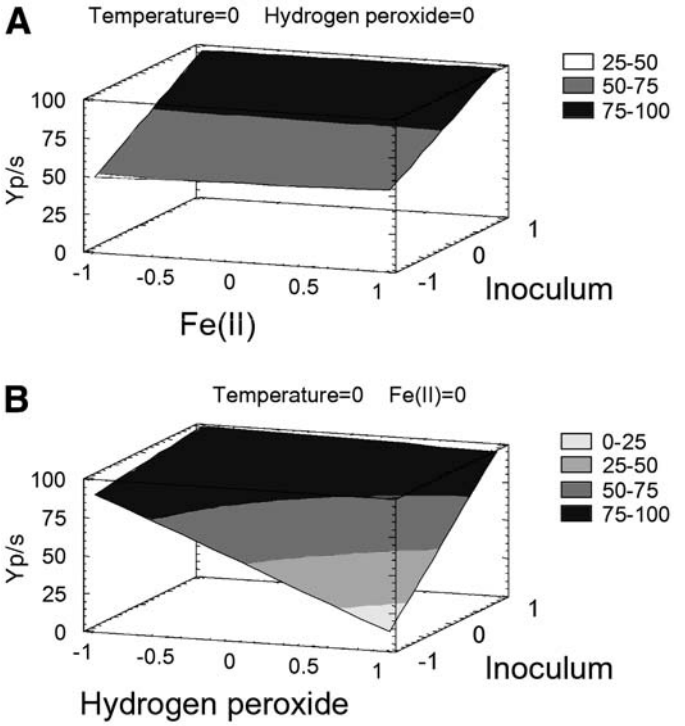

Fig. 6. 3D representation of response surface corresponding to ethanol yields after 24 h of fermentation. **(A)** Temperature and H_2O_2 concentration and **(B)** temperature and iron parameters are fixed at central values (T = 65°C, H_2O_2= 30 m*M*, and Fe^{2+} = 50 ppm). Data of ethanol yield are shown as a percentage of a control without toxic compounds.

the negative effect of H_2O_2. On the other hand, lower H_2O_2 concentration (10 m*M*) and inoculum load (0.2 g/L) resulted in ethanol yields above 90% of the control. From the results, it can be concluded that it is not necessary to completely eliminate toxic compounds present in the prehydrolysates to alleviate inhibition. An adequate inoculum load and the adaptation of the yeast to toxic compounds seem to provide other interesting methods for improving prehydrolysate fermentability.

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