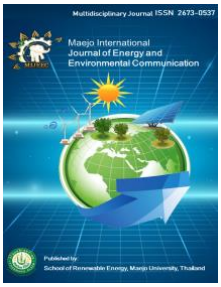
Maejo International Journal of Energy and Environmental Communication

Journal homepage: https://ph02.tci-thaijo.org/index.php/MIJEEC



ARTICLE

Optimization of overliming detoxification of sugarcane bagasse hydrolysate by using response surface methodology for bio-ethanol production

Techut Promta[1], Panomtian Thonkamdee[2], Kittikorn Sasujit[1,*]

[1] School of Renewable Energy, Maejo University, Chiang Mai, 50290, Thailand
[2] Division of Physical System and Environment, Maejo University, Chiang Mai, 50290, Thailand

ARTICLEINFO

Article history:
Received 02 January 2024
Received in revised form
21 January 2024
Accepted 23 January 2024

Keywords:
Sugarcane
Overliming
Detoxification
Optimization
Ethanol

ABSTRACT

This research aimed to study of the sugarcane bagasse was used as a raw material for ethanol production and pretreatment with 2.0% dilute H_2SO_4 at 121ºC for 60 min. *Pichia stipitis* (TISTR 5806) was a microorganism for fermentation. However, acid hydrolysis produces inhibitory compounds like furans, phenolics, and organic acids. Overliming with $Ca(OH)_2$ is a detoxification process that removes these compounds and improves ethanol fermentation. Optimal conditions were determined using RSM and CCD frameworks. The study established specific ranges for the operation conditions: 7–12 for pH, 20–60ºC for reaction temperature, and 30–90 min for reaction time. The results indicated that the optimal conditions for detoxification were a pH of 9.5, a reaction temperature of 40ºC, and a reaction time of 60 min. The analysis revealed the removal of total furans at 38.37% and total phenolics at 50.02%. The fermentation of hydrolysate and detoxified hydrolysate found an ethanol yield of 0.22 and 0.30 $g_{product}/g_{substrate}$, respectively, and a theoretical yield of 43.42% and 57.96%, respectively. Overliming $Ca(OH)_2$ could be an efficient detoxification process for removing and reducing the effects of inhibitory compounds in sugarcane bagasse hydrolysate.

1. Introduction

The global population is expected to grow rapidly by 2050, reaching 10 billion people. Simultaneously, expanding global industrialization leads to a high demand for energy consumption. According to fossil fuel depletion, each country consumes insufficient energy (Khaodee & Chaiworn, 2023). Fossil fuel energy usage has led to global warming and environmental problems. These fuels could release many greenhouse gases and destroy the atmosphere, resulting in lower air quality (Pradechboon & Junluthin, 2022). Renewable energy is the most reliable energy that can be obtained from natural resources. These energy sources could be converted and serve as an alternative source of energy (Ratchawet & Chaiworn, 2022). Energy from biomass has become an increasingly popular alternative for biofuel production to reduce dependence on oil and mitigate global warming (Junluthin et al., 2021).

Lignocellulosic biomass has three major components: cellulose, hemicellulose, and lignin (Dussadeeet al., 2017). Cellulose is a linear homopolymer of glucose linked with glycosidic bonds. Hemicellulose is a branch of heteropolymer units of sugars that mainly consist of

* Corresponding author.
E-mail address: k.sasujit@yahoo.com (Sasujit K.)

pentose (xylose and arabinose) and hexose (glucose, mannose, and galactose). While lignin is the most complex organic compound, it also contains aromatics and phenolics. This structure could support the plant's structure protection (Dussadeeet al., 2022; Trejo et al., 2022).

Biofuel is the most attractive renewable energy source, especially bioethanol. Bioethanol is generally produced from biomass resources, which include agriculture residues such as wheat, sugarcane bagasse, corncob, and rice straw, municipal solid waste, and organic waste (Kaewdiew et al., 2019; Sophanodorn et al., 2020). Bioethanol is commonly used to blend with gasoline for transport fuel such as E85 (gasoline 15%: ethanol 85%) and E10 (gasoline 90%: ethanol 10%). Ethanol has prominent properties such as high heating value for combustion, high octane number, and low cetane number (Germec & Turhan, 2018). Lignocellulosic biomass from sugarcane has one of the most potential substrates for bioethanol production, is rich in sugar content, and is available for feedstock (Bhuyar et al., 2021; Kongchan et al., 2022). There is a composition of 40–45% cellulose, 30–35% hemicellulose, and 20–30% lignin (Sabiha-Hanim & Abd Halim, 2018). Bioethanol production mainly consists of both physical and chemical pretreatment processes to remove the recalcitrant lignocellulose structures, reduce cellulose crystallinity, and increase the high porosity of materials (Cardona et al., 2010). Conventional method for chemical pretreatment of lignocellulosic by acid hydrolysis with HCl, H_2SO_4, and HNO_3. These processes decompose the structures and obtain fermentable sugars (xylose, arabinose, and glucose). Ethanol fermentation will use these sugars as carbon sources. However, there are also inhibitory compounds consisting of furfural, 5-hydroxymethyl furfural (5HMF), phenolics, and organic acids. These compounds, furfural, and 5HMF, are generated from the destruction of fermentable sugars, while phenolics are degraded from the lignin structure. The presence of these products in the hydrolysate substrate could inhibit the growth of microorganisms and reduce their ability to produce ethanol (Gámez et al., 2006).

Detoxification is considered a method to increase ethanol production. Various methods include ion exchange, activated carbon adsorption, membrane separation, and biological treatment. Overliming with $Ca(OH)_2$ shows an effective detoxification process that is widely used and economical. However, the drawback of the overliming process is that sugar loss always occurs. Stoutenburg et al. (2011) reported improved fermentation of sugar maple hydrolysate by *P. stipitis* Y-7124 using overliming detoxification compared to untreated hydrolysate. The hydrolysate was pretreated with 1.0% H_2SO_4 and detoxified by $Ca(OH)_2$ to adjust the pH to 10.5. The experimental results show that after overliming 9.6 g/l of sugar loss, 0.12 and 0.03 $g_{ethanol}/g_{sugar}$ were obtained from the treatment and untreated hydrolysate, respectively. These results indicated that overliming could remove inhibitors from sugar maple hydrolysate (Stoutenburg et al., 2011). Larsson et al. (1999) reported the study of the detoxification of spruce hydrolysate by overliming treated with different alkalis (NaOH and $Ca(OH)_2$). They are pretreated with 0.5%w/w dilute H_2SO_4 and detoxified with alkaline adjusted pH to 10. It was found that ethanol yields of 0.44, 0.42, and 0.32 $g_{ethanol}/g_{sugar}$ were obtained from treated with $Ca(OH)_2$, NaOH and untreated hydrolysate, respectively. This study illustrated that $Ca(OH)_2$ could be more efficient for removing inhibitors than other alkaline (Larsson et al., 1999). Van Zyl et al. (1988) reported the highest ethanol yield compared to overliming detoxification with different alkalis (NH_4OH, NaOH, and $Ca(OH)_2$). In their experiment, sugarcane bagasse was pretreated with 1.8% dilute H_2SO_4 and fermented by *P stipitis* CSIR-Y633. The results showed the highest ethanol yield 0.25 $g_{ethanol}/g_{sugar}$ obtained from treated hydrolysate with $Ca(OH)_2$ to pH 10 (Van Zyl et al., 1988).

This paper focus on the detoxification of inhibitory compounds from sugarcane bagasse hydrolysate. Hydrolysate would be pretreated by dilute H_2SO_4 and detoxified by overliming with $Ca(OH)_2$. The optimum conditions of overliming detoxification were determined by response surface methodology using a central composite design. *P. stipitis* TISTR5806 (Huang et al., 2009) was used as a microorganism in this experiment due to its high ability to convert both pentose and hexose sugars into ethanol with high ethanol yield.

2. Materials and methods

2.1 Raw material preparation

Sugarcane bagasse was used as the raw material for the experiment at a local Chiang Mai, Thailand market. The raw material was washed to eliminate impurities with distilled water and dried at 105°C for 3 days in a hot air oven. After that, the biomass size is reduced by crushing the machine. For chemical pretreatment through hydrolysis reaction using 2.0% v/v dilute H_2SO_4 at the reaction temperature of 121°C, pressure of 15 psi with 60 min reaction time in an autoclave, and 1:20 for solid/liquid ratio. Next, the sample from the hydrolysis process was separated into liquid and solid by a vacuum pump, which is used as a substrate for detoxification and fermentation, as shown in Figure 1.

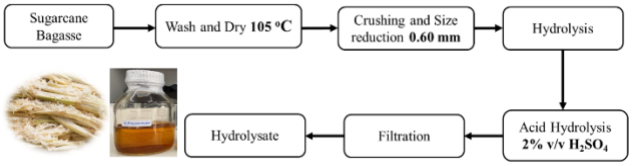


Figure 1. Preparation of sugarcane bagasse hydrolysate process.

2.2 Experimental design and RSM

The optimum detoxification conditions using central composite design (CCD) with Design Expert version 13 (trial version) were used as statistical software. The experimental design is comprised of 3 levels and 3 factors according to independent variables in terms of overcoming detoxification, pH (A), temperature (B), and reaction time (C). Response variables comprise reducing sugar and inhibitory compounds (total furans and total phenolics). This experimental study contained 15 trials with 3 different levels: minimum, mean, and high, as shown in Table 1. The empirical model of the response variables was in the form of quadratic models according to Equation 1.

$$Y=\beta_0+\beta_1 A+\beta_2 B+\beta_3 C+\beta_{12} AB+\beta_{13} AC+\beta_{23} BC+\beta_{11} A^2+\beta_{22} B^2+\beta_{33} C^2 \quad [1]$$

The quadratic term above, where Y represents the response for the

variable, and β_{11} is the regression coefficient. The terms of β_{12}AB, β_{13} AC, β_{23}BC are interaction coefficients. The terms of quadratic coefficients are β_{11} A^2, β_{22} B^2, and β_{33} C^2. This empirical model is used to determine and predict the optimum detoxification conditions.

Table 1. Central composite design on response surface methodology for overliming detoxification of inhibitors.

Independent variables	Minimum	Mean	Maximum
(A) pH	7	10	12.0
(B) Temperature (°C)	20	40	60
(C) Reaction time (min)	30	60	90

2.3 Detoxification methods

The hydrolysate will be reacted with 20% w/v $Ca(OH)_2$ to gradually adjust the pH of the hydrolysate to 7.0, 10.0, and 12.0. The optimum overliming detoxification conditions were obtained from the central composite design (CCD). Under these conditions, inhibitory compounds were detoxified to reduce their negative effects on fermentation, as shown in Figure 2.

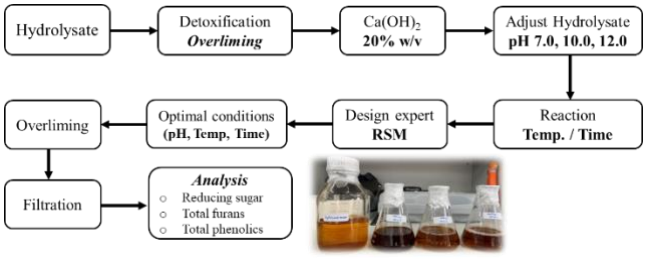


Figure 2. Overliming detoxification of sugarcane bagasse hydrolysate process

2.4 Detoxification methods

P.stipitis TISTR5806 was obtained from the Thailand Institute of Scientific and Technological Research (TISTR) in Bangkok and was used in this experiment. This microorganism culture grew in the agar plate, which contains malt extract, yeast extract, peptone, and glucose, which keeps microorganisms at 30°C in the incubator.

2.5 Inoculum and fermentation

Inoculum of *P. stipitis* TISTR5806 in 1.0 liter of solution that contains 3.0 g of malt extract, 3.0 g of yeast extract, 5.0 g of peptone, and 10.0 g of glucose. This medium will be prepared in 250 ml of a conical flask, sterilized in an autoclave at 121°C for 15 min, cooled down and cultured in this solution. This culture medium will be shaken at 120 rpm in an incubated shaker and controlled at 30°C for 24 h. The fermentation process used 200 ml of hydrolysate substrate. The sample in the flask contained 5.0 g/l of initial cell concentration and hydrolysate (detoxified and non-detoxified). The hydrolysate must be sterilized in an autoclave at 121°C for 15 min and adjusted to a pH of 5.5. All samples were fermented in an incubated shaker at 30°C and 120 rpm. That could be a fermentation time of 96 h and a sample collection of 10 ml every 12 h to analyze cell growth rate, reducing sugar, and ethanol.

2.6 Analytical methods

The study of the hydrolysate and fermentation sample involved determining the total sugar, inhibitory compound, cell growth rate, and ethanol yields. The chemical compositions and ethanol were analyzed using spectrophotometric techniques. Total sugar was determined by phenol-sulfuric acid method at 540 nm (Nielsen, 2010). Reducing sugar was determined by using dinitro salicylic methods at 540 nm (Miller, 1959). Total furan compounds were analyzed by using a spectrophotometer at 284 nm and 320 nm and calculated using a calibration curve (Martinez et al., 2000b). Total phenolic compounds were analyzed using the Folin-ciocalteu assay method at 760 nm (Blainski et al., 2013). Cell concentration in the hydrolysate fermentation sample was measured by using the optical cell density (OD) solution method at wavelength 600 nm (Myers et al., 2013). Dry cell concentration was calculated using a calibration curve of absorbance and dry cell weight. Ethanol concentration was measured by using the dichromate method at wavelength 585 nm (Seo et al., 2009).

3. Results and Discussion

The optimization conditions of this study involve overliming detoxification of inhibitory compounds in sugarcane bagasse hydrolysate by using response surface methodology (RSM) to enhance ethanol fermentation. For the detoxification process, $Ca(OH)_2$ was used as a chemical in the form of a solution to remove inhibitory compounds. This research aims to study the effect of independent variables, such as pH, temperature, and reaction time, on inhibitory compounds and sugar reduction. The study will compare the results before and after the experiment. *P. stipitis* TISTR5806 would ferment sugarcane bagasse hydrolysate in an ethanol fermentation process.

3.1 Chemical compositions of raw materials

The characteristics of sugarcane bagasse were high sugar content, availability for feedstock, cheap raw materials cost and a high potential substrate for ethanol production. Generally, sugarcane bagasse comprises cellulose and hemicellulose, which could degrade into monomeric sugar (pentose and hexose). These sugars would be used as carbon sources for ethanol fermentation. While lignin degraded into a complex polymer comprised of aromatic compound (Sabiha-Hanim & Abd Halim, 2018).

3.2 Detoxification methods

The physical and chemical pretreatment process of sugarcane bagasse was provided for fermentation, which breaks down both cellulose and hemicellulose structures to obtain fermentable sugar that consists of pentose (xylose and arabinose) and hexose (glucose, mannose, and galactose) (Chandel et al., 2011; Mosier et al., 2005). Microorganisms use these sugars as carbon sources in ethanol fermentation. The sugarcane bagasse was pretreated using a 2.0% v/v dilute H2SO4 solution at a temperature of 121°C for 60 min. After acid breaks down, chemicals are made that stop the process from happening. These include phenolics, furan derivatives (furfural and 5-hydroxymethyl furfural (5HMF)), and weak acids (formic acid and acetic acid). These compounds would reduce ethanol fermentability, resulting in a lower ethanol yield. A hydrolysate was produced and utilized as a fermentation substrate. The chemical composition of sugarcane bagasse hydrolysate is shown in Table 2.

Table 2. Chemical compositions of sugarcane bagasse hydrolysate

Compositions	Amount
Total sugar (g/l)	69.39
Reducing sugar (g/l)	58.20
Total Furans (mg/l)	16.94
Total Phenolics (µg/l)	21.25

3.3 Response surface models for detoxification

Overliming with $Ca(OH)_2$ was selected to detoxify these compounds (furfural, 5HMF, phenolics, and weak acids). The overliming method had a high efficiency for removing inhibitors, especially furan compounds, which could precipitate toxic compounds at high pH conditions, and a low cost of operation (Antunes et al., 2012). A central composite design (CCD) on RSM was used to design an experiment. The experiment on response models for chemical compositions in sugarcane bagasse hydrolysate includes reducing sugar and inhibitory compounds (furans and phenolics). The quadratic model was used as a response surface design for these responses to predict results compared with the experimental results. These regression models obtained from the RSM design were used to determine the optimum conditions for detoxifying inhibitory compounds. The experimental design included three independent variables: pH (A), temperature (B), and reaction time (C). These variables affect the removal of inhibitory compounds. The equation for quadratic models is shown in Equation 2-4.

Where Y was the predicted concentration response. Variables A, B, and C were pH, temperature (ºC), and reaction time (min), respectively. The analysis of variance (ANOVA) was used as a statistical tool to express the response models. The summarized analysis of variance is shown in Table 3, which was analyzed by the statistical program of Design-Expert version 13 (trial version). The results indicated that the response model was highly significant. The P-value, R^2, and F-value were used to describe the significance of each independent variable that resulted in the response model. From the experiment, the response of the reducing sugar model had a P-value = 0.0478, R^2 = 0.8978, and F-value = 4.88. The response of inhibitory compounds models furans had P-value = 0.0138, R^2 = 0.9405, and F-value = 8.78, and phenolics had P-value = 0.0372, R^2 = 0.9806 and F-value = 5.52. These models have a p-value <0.05, which illustrates that these models fit well with experimental data. These independent variables were statistically significant for reducing sugar, total furans, and total phenolics. The interaction between these variables in the response model is shown in Table 4-5.

Reducing sugar (g/l)

$$Y_{reducing\ sugar} = -19.9299 + 14.9395A + 0.122582B + 0.425613C - 0.0672AB - 0.0285AC + 0.000017BC - 0.878439A^2 + 0.00671419B^2 - 0.00129587C^2 \quad [2]$$

Total furans(mg/l)

$$Y_{total\ furans} = 21.8905 + 0.744943A - 0.264709B + 0.00374048C + 0.0380845AB + 0.012025AC - 0.000287583BC - 0.238914A^2 - 0.00169104B^2 - 0.00127183C^2 \quad [3]$$

Total Phenolics (µg/l)

$$Y_{total\ phenolics} = 18.4696 - 1.35363A + 0.0514547B + 0.0485564C + 0.0014405AB + 0.00378533AC - 0.000085BC + 0.0157154A^2 - 0.000728222B^2 - 0.000789716C^2 \quad [4]$$

Table 3. Summarized analysis of variance (ANOVA) of the experimental results of the quadratic model for the chemical compositions in hydrolysate during overliming detoxification

Source	Reducing sugar (g/l)			Total Furans (g/l)			Total Phenolics (g/l)		
	Sum of Squares	F-value	p-value	Sum of Squares	F-value	p-value	Sum of Squares	F-value	p-value
Model	3906.3	4.88	0.0478	298.92	8.78	0.0138	59.63	5.52	0.0372
A-pH	3227.17	36.29	0.0018	204.95	54.18	0.0007	50.65	42.21	0.0013
B-Temperature	2.72	0.0306	0.868	16.79	4.44	0.089	0.0176	0.0147	0.9082
C-Reaction time	7.40E-06	8.32E-08	0.9998	26.17	6.92	0.0465	2.29	1.91	0.226
AB	90.32	1.02	0.3598	29.01	7.67	0.0394	0.0415	0.0346	0.8598
AC	36.55	0.411	0.5497	6.51	1.72	0.2466	0.6448	0.5374	0.4964
BC	0.0008	9.00E-06	0.9977	0.2382	0.063	0.8118	0.0207	0.0172	0.9007
A^2	182.45	2.05	0.2115	13.5	3.57	0.1175	0.0584	0.0487	0.8341
B^2	43.66	0.4909	0.5148	2.77	0.7322	0.4313	0.5136	0.428	0.5418
C^2	8.23	0.0926	0.7732	7.93	2.1	0.2073	3.06	2.55	0.1713
Residual	444.68			18.91			6		
P-value	Significant			Significant			Significant		
R^2	0.8978			0.9405			0.9086		

Remark: P-value < 0.05 Significant

Table 4. RSM for analysis of reducing sugar from overliming detoxification of inhibitory compounds in sugarcane bagasse hydrolysate

Run	Std	Factors			Response		
		pH	Temperature (°C)	Reaction time (min)	Reducing Sugar (g/l)		
					Experimental	Predicted	Residual
1	5	7	20	90	48.77	47.22	1.55
2	13	9.5	40	9.55	38.98	34.19	4.79
3	2	12	20	30	16.15	23.21	-7.06
4	3	7	60	30	50.63	50.55	0.08
5	14	9.5	40	110.45	37	34.2	2.8
6	11	9.5	6.36	60	47.37	44.34	3.03
7	1	7	20	30	44.46	42.96	1.5
8	9	5.30	40	60	42.36	47.82	-5.46
9	12	9.5	73.64	60	50.4	45.84	4.56
10	8	12	60	90	6.24	13.11	-6.87
11	15	9.5	40	60	36.19	37.49	-1.3
12	7	7	60	90	56.54	54.85	1.69
13	4	12	60	30	10.44	17.36	-6.92
14	10	13.70	40	60	9.16	-3.89	13.05
15	6	12	20	90	13.47	18.92	-5.45

Table 5. RSM for analysis of inhibitory compounds from overliming detoxification in sugarcane bagasse hydrolysate

Run	Std	Factors			Response					
		pH	Temperature (°C)	Reaction time (min)	Total furans (mg/l)			Total Phenolics (µg/l)		
					Experimental	Predicted	Residual	Experimental	Predicted	Residual
1	5	7	20	90	12.48	11.85	0.6286	11.13	10.91	0.22
2	13	9.5	40	9.55	10.9	9.48	1.41	8.5	9.17	-0.67
3	2	12	20	30	1.23	2.72	-1.49	8.84	7.63	1.21
4	3	7	60	30	10.97	10.4	0.57	12.14	12.22	-0.08
5	14	9.5	40	110.45	3.17	4.83	-1.66	7.74	7.8	-0.06
6	11	9.5	6.36	60	11.69	10.35	1.34	8.58	9.61	-1.02
7	1	7	20	30	15.22	16.08	-0.86	12.31	12.19	0.12
8	9	5.30	40	60	11.58	12.69	-1.1	14.52	14.01	0.51
9	12	9.5	73.64	60	5.03	6.62	-1.59	10.03	9.73	0.30
10	8	12	60	90	4.38	3.35	1.04	7.99	7.6	0.40
11	15	9.5	40	60	10.44	10.39	0.04	10.62	10.49	0.13
12	7	7	60	90	7.14	5.48	1.66	10.04	10.74	-0.69
13	4	12	60	30	4.2	4.66	-0.46	8.25	7.95	0.30
14	10	13.70	40	60	0.52	-0.35	0.86	6.3	7.53	-1.24
15	6	12	20	90	1.7	2.1	-0.40	8.08	7.48	0.59

3.4 Optimization of detoxification

Figure 3 shows the response surface curve of the interaction between pH, temperature, and reaction time in reducing sugar concentration. It was found that increasing the pH using the $Ca(OH)_2$ affects the decrease of reducing sugar while raising the reaction temperature effect on releasing reducing sugar. The response surface curve illustrated that increasing these two variables resulted in the release of a high amount of reducing sugar. However, with the increasing pH, sugar loss also occurred due to the ability of $Ca(OH)_2$ to react and precipitate, reducing sugar. From these results, The experiment with high reaction temperature and reaction time shows high reducing sugar. The high reaction temperature shows that structures of both cellulose and hemicellulose will be broken down into monomeric sugar according to a long reaction time caused by lignocellulose structure contacted with acid between hydrolysis reaction that could obtain a high amount of fermentable sugar (Excoffier et al., 1991). The results are related to Mustafa Germec et al. (2016), studied ethanol production from rice hull by response surface methodology to determine the optimum conditions for reducing high-reducing sugar. It was found that increasing temperature and time could be obtained from high fermentable sugar. Baig and Dharmadhikari (2014) studied the optimization of detoxification of cotton stalk hydrolysate by overliming 75% H_2SO_4 hydrolysis. The results show that increasing the pH of hydrolysate using CaO results in the loss of fermentable sugar. This study found that at pH 7.0, 10.0, and 12.0, loss of fermentable sugar occurred 6.68, 14.57, and 25.30%, respectively.

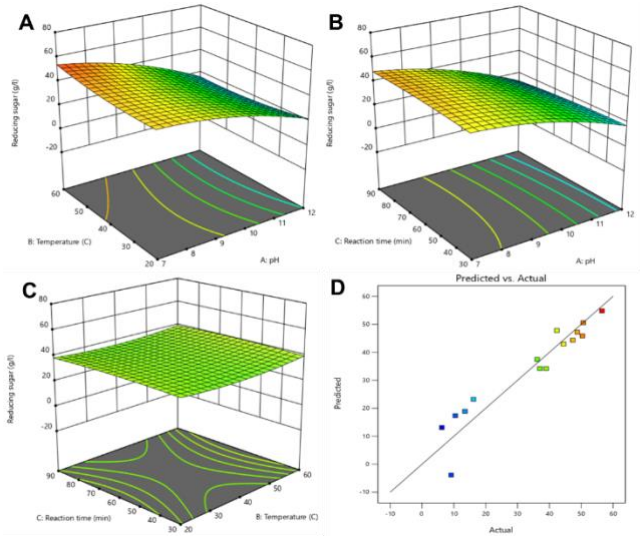


Figure 3. 3D response surface curve of reducing sugar on overliming detoxification process: (A) effect of pH and temperature, (B) the effect of pH and reaction time, (C) the effect of temperature and reaction time and (D) the actual and predicted value of reducing sugar.

Figure 4 shows the response surface curve of total furans on overliming detoxification. Increasing pH and reaction temperature results in lower total furans contents, which means that the total furans could be removed at high pH and reaction temperature. Meanwhile, increasing reaction temperature and time shows the removal of total furans. These results indicated that increasing these variables could affect the elimination of total furans from the hydrolysate.

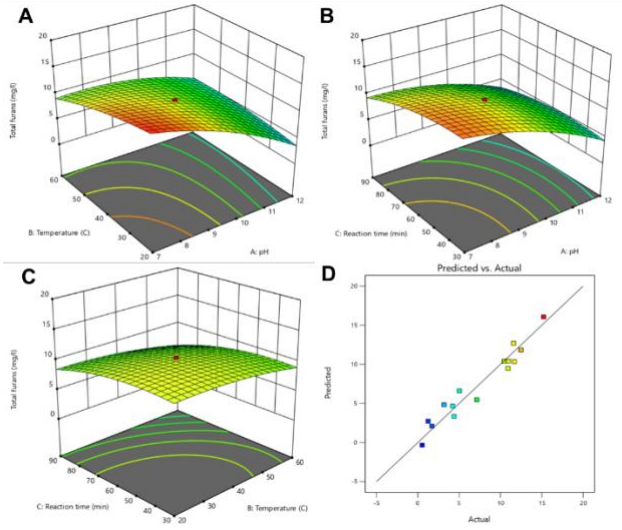


Figure 4. 3D response surface curve of total furans on overliming detoxification process: (A) effect of pH and temperature, (B) effect of pH and reaction time, (C) effect of temperature and reaction time and (D) actual and predicted value on reducing sugar.

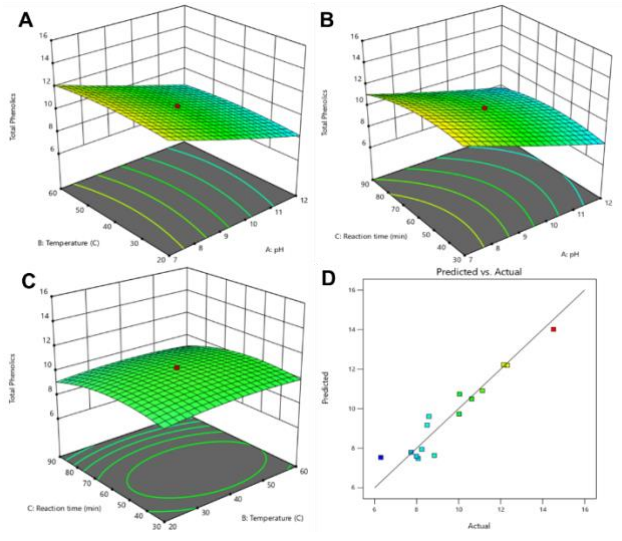


Figure 5. 3D response surface curve of total phenolics on overliming detoxification process. (A) effect of pH and temperature, (B) the effect of pH and reaction time, (C) the effect of temperature and reaction time and (D) the actual and predicted value of reducing sugar.

Figure 5 shows the response surface curve for total phenolics on overliming detoxification. It was found that increasing pH and reaction temperatures showed lower total phenolic content. These high concentrations could help eliminate total phenolics. Previous studies conclude that increasing these variables, total furans and total phenolics has a high removal efficiency. The optimum concentration of total furans removed was 92.69% by overliming detoxification. The overliming detoxification with $Ca(OH)_2$ showed a higher percentage of elimination of inhibitory compounds than another alkali studied by Mateo et al. (2013). Meanwhile, Mohagheghi et al. reported the effects of pH from 9 to 11 on overliming with $Ca(OH)_2$. It was found that pH 10 enabled the highest overall ethanol yield (Mohagheghi et al., 2006). Central composite design (CCD) was used as a tool for the experiment design. The three independent variables and the level of factors for the response of reducing sugar and inhibitory compounds are shown in Tables 4 and 5. The optimum conditions for removing inhibitory compounds from sugarcane bagasse hydrolysate were selected at a pH of 7.0, 10.0, and 12.0. The results showed that reducing sugar was 56.54 g/l at a pH of 7.0, a reaction temperature of 60ºC, and a reaction time of 90 min. This optimum condition was suitable for detoxification due to the high amounts of reducing sugar. Total furans had a maximum concentration of 15.22 mg/l with the treated sample at pH 7.0, 20ºC temperature, and 30 min for reaction time. Total phenolics had a maximum concentration of 14.52 µg/l, with the treated sample at pH 5.30, reaction temperature of 40ºC, and 60 min for reaction time. Two of these conditions for inhibitory compounds were inappropriate for the detoxified condition, which showed the lowest removal of inhibitors.

In contrast, the lowest amount of reducing sugar was observed at a concentration of 6.24 g/l when the sample was handled at a pH of 12, reaction temperature of 60ºC, and a reaction time of 90 min. This scenario demonstrates that an elevated pH during overliming reduces sugar content, which is an unfavourable situation for detoxification as it results in a lower sugar yield. The minimum of total furans for the inhibitory compounds was 0.52 mg/l found at the treated sample of pH 13.70, 40ºC temperature, and 60 min for reaction time. The minimum of total phenolics was 6.3 µg/l, found at the same condition as total furans. These conditions were suitable for detoxifying these compounds due to the lowest content of inhibitory compounds. In addition, the effect of reaction temperatures of 20, 40, 60, and reaction times of 30, 60, and 90 min was investigated. The results showed that increasing these two variables affects the elimination of these compounds while a high amount of reducing sugar could be released in this condition. These results are related to the Alriksson et al. (2006) and Millati et al. (2002).

3.5 Optimization conditions for overliming detoxification

The experimental design on overliming detoxification using central composite design (CCD) and the effects of independent variables on detoxification are above. The previous experimental results showed the independent variables (pH, temperature, and reaction time) that affect the reduction of sugar and inhibitory compounds (total furans and total phenolics). The experiment found that increasing these variables affects the removal of inhibitory compounds from hydrolysate while at high pH for hydrolysate, and the treatment resulted in sugar loss. These results indicated they could obtain a high reducing sugar concentration at a lower pH. To minimize

these detoxification effects, the optimum conditions were selected to overliming detoxification conditions. From previous experimental results obtained, the optimization conditions were the most efficient for overliming the detoxification of inhibitors, as shown in Table 6. The optimal conditions of detoxification were reducing sugar concentrations of 36.19 g/l, total furans of 10.4354 mg/l, and total phenolics of 10.6186 µg/l with the treatment condition pH of 9.5, reaction temperature of 40oC, and reaction time of 60 min. This study of optimum conditions is similar to the experiment of Deshavath et al. (Deshavath et al., 2017), which explained that hydrolysate detoxification showed lower inhibitor and high ethanol yield. Furthermore, it was found that at the optimum conditions, overliming could eliminate total furans of 38.37% and total phenolics of 50.02%, While this detoxified method resulted in 37.81% of sugar loss. Sugar loss has always occurred since hydroxide degradation from Ca(OH)2 between detoxified inhibitors could convert sugar into unfermentable compounds. These compounds could be the organic acid group (acetic acid, formic acid, and levulinic acid) during fermentable degradation of sugars in hydrolysate substrate (Carvalheiro et al., 2005; Cheng et al., 2008; Lanka et al., 2011).

3.6 Ethanol fermentation

Table 7 shows the ethanol yield from fermentation by *P.stipitis*. During ethanol fermentation with non-detoxified hydrolysate, about 96 h showed a decrease in reducing sugar while ethanol production increased. Which sugar could be converted to ethanol by yeast. Ethanol production increased quickly from during time of 12 h until the time of 96 h. The highest recorded ethanol production at this time was 9.27 g/l. The experiment revealed that the ethanol yield was 0.22 $g_{product}/g_{substrate}$ and a theoretical yield of 43.42%. The ethanol production rate of the hydrolysate without detoxification was 0.09 g/l-h, and the specific growth rate of yeast was determined to be 0.0073 h^{-1} during the fermentation process at 96 h, as shown in Figure 6. The ethanol fermentation of detoxified hydrolysate from the overliming process. It was found that the maximum ethanol production was 11.55 g/l, and the ethanol productivity was 0.19 g/l-h, which shows an ethanol yield of 0.30 $g_{product}/g_{substrate}$ and ethanol yield of 57.96%, respectively. The specific growth rate of detoxified hydrolysate measured during the fermentation process was 0.0179 h^{-1}, as shown in Figure 7.

Table 6. Chemical compositions from overliming detoxification in sugarcane bagasse hydrolysate at optimal conditions

Sample	Chemical compositions		
	Reducing sugar (g/l)	Total Furans (mg/l)	Total Phenolics (µg/l)
Hydrolysate (non-detoxification)	58.20	16.94	21.25
Hydrolysate (overliming)	36.19	10.44	10.62
% Removal	37.81	38.37	50.02

Figure 8 shows hydrolysate dry cell concentration and overliming detoxification on ethanol fermentation from *P. stipitis* .. The growth rate of the detoxified hydrolysate was greater than that of the non-detoxified hydrolysate. The results demonstrated that inhibitors in the hydrolysate can inhibit yeast metabolism during fermentation, leading to a decrease in ethanol production. Kinetic parameters, including the rate of sugar consumption and ethanol productivity, could support the experiment. The higher values of these kinetic parameters during fermentation imply that the yeast can efficiently convert reducing sugars into ethanol without producing harmful byproducts. Furthermore, inhibitors impacted the lower kinetic parameters of the non-detoxified hydrolysate.

Table 7. Parameter results of ethanol fermentation from *Pichia stipitis* TISTR5806

Details	Hydrolysate	Overliming Detoxification
Specific growth rate (1/h)	0.0073	0.0179
Initial reducing sugar (g/L)	41.78	38.98
Final reducing sugar (g/L)	35.60	8.34
Usage of reducing sugar (%)	14.79	78.60
Ethanol concentration (g/l)	9.27	11.55
Time for maximum ethanol (h)	96	60
Ethanol yield ($g_{product}/g_{substrate}$)	0.22	0.30
Theoretical yield (%)	43.42	57.96
Ethanol productivity (g/L/h)	0.09	0.19

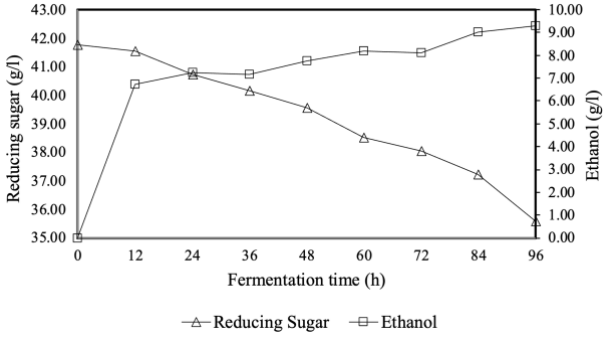


Figure 6. Reducing sugar and ethanol concentration production from sugarcane bagasse hydrolysate by *Pichia stipitis* TISTR5806

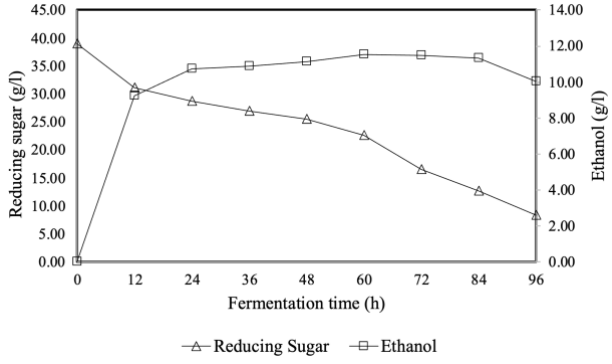


Figure 7. Reducing sugar and ethanol concentration production from overliming detoxification of sugarcane bagasse hydrolysate by *Pichia stipitis* TISTR5806

Total furans could affect intracellular respiration and inhibit the growth of microorganisms (Palmqvist & Hahn-Hägerdal, 2000). Phenolic compounds had antimicrobial activity, destroyed the integrity of cell membranes, and inhibited the average growth of microbial cells, which reduced fermentation efficiency (Chen et al., 2020; Gupta et al., 2023). In addition, a specific growth rate could indicate the ethanol yield. Moreover, the byproduct of this process contains gypsum, which could be used as a mixture of concrete

(Martinez et al., 2000a). Similar results had been reported by Amartey and Jeffries (Amartey & Jeffries, 1996) that improved fermentation of corncob by *P. stipitis* CBS 6054 resulted in a total sugar of 18% and an ethanol yield of 0.21 gproduct/gsubstrate for untreated hydrolysate.

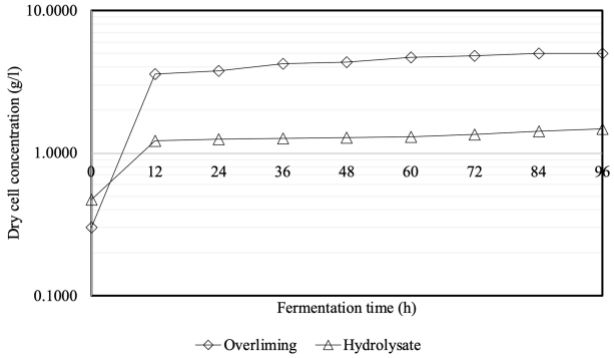


Figure 8. Dry cell concentration of hydrolysate and overliming detoxification on ethanol fermentation from *Pichia stipitis* TISTR5806

The total sugar was 82% and the ethanol yield was 0.32 $g_{product}/g_{substrate}$ for the treated hydrolysate with $Ca(OH)_2$. The overliming detoxification treatment with $Ca(OH)_2$ could efficiently eliminate inhibitors from rice straw hydrolysate and improve higher ethanol fermentation. It was found that ethanol yields were 0.43, 0.37, and 0.34 $g_{product}/g_{substrate}$ of overliming, ammonia-neutralized, and NaOH-neutralized, respectively (Lin et al., 2016). Ge et al. (2011) reported the comparison of ethanol fermentation of overliming and concentrated acid hydrolysate of corncob hydrolysate. The results show that 0.39 and 0.30 $g_{product}/g_{substrate}$ ethanol yield 34.0% and 4.3% usage of sugar of overliming and hydrolysate, respectively. Furthermore, Brito et al. (2018) reported the optimization of hydrolysis palm press fiber hydrolysate using RSM. The optimum hydrolysis conditions were 5% H_2SO_4 at 121ºC for 60 min and fermented by *Pichia stipitis* NRRLY 7124. Activated charcoal and overliming detoxification were applied to the treated hydrolysate. Their experimental results and detoxified condition were nearly the results of this study.

5. Conclusion

In conclusion, the detoxification procedure, including overliming with $Ca(OH)_2$, was shown to be highly successful in eliminating inhibitory chemicals from sugarcane bagasse hydrolysate. The hydrolysate was pretreated using a 2.0% v/v dilution of H_2SO_4. During the detoxification process, sugar was significantly lost, especially with high pH concentrations. The optimal overliming parameters, pH, temperature, and reaction time were determined using the central composite design (CCD) in response surface methodology (RSM). These conditions were effective in enhancing ethanol fermentation by *P. stipitis* TISTR5806. Increasing all parameter effects on treatment inhibitors and showing high elimination of inhibitors. The optimum conditions for overliming detoxification were a reaction time of 60 min and a reaction temperature 40ºC at a pH of 9.5. Consequently, the overliming detoxification process was suitable for eliminating inhibitory compounds and could enhance ethanol fermentability from sugarcane bagasse hydrolysate.

Acknowledgement

The authors thank the funding from the School of Renewable Energy, Maejo University, Thailand. Thailand Institute of Scientific and Technological Research: TISTR Culture Collection for *Pichia stipites* TISTR5806 in this research.

References

Alriksson, B., Sjöde, A., Nilvebrant, N.-O., & Jönsson, L. J. (2006). Optimal conditions for alkaline detoxification of dilute-acid lignocellulose hydrolysates. Applied Biochemistry and Biotechnology, 130, 599-611.

Amartey, S., & Jeffries, T. (1996). An improvement in Pichia stipitis fermentation of acid-hydrolysed hemicellulose achieved by overliming (calcium hydroxide treatment) and strain adaptation. World Journal of Microbiology and Biotechnology, 12, 281-283.

Antunes, F., Milessi, T., Oliveira, I., Chandel, A., & Silva, S. (2012). Characterization of sugarcane bagasse hemicellulosic hydrolysate after detoxification with overliming and activated charcoal. Proceedings of the 20th European Biomass Conference and Exhibition.

Baig, M. Z., & Dharmadhikari, S. (2014). Optimization of detoxification with over liming and charcoal treatment for increasing the fermentability of cotton stalk hydrolyzate. Indian Journal of Applied Research, 4(07), 08-10.

Blainski, A., Lopes, G. C., & De Mello, J. C. P. (2013). Application and analysis of the folin ciocalteu method for the determination of the total phenolic content from Limonium brasiliense L. Molecules, 18(6), 6852-6865.

Brito, P. L., de Azevedo Ferreira, C. M., Silva, A. F. F., Pantoja, L. d. A., Nelson, D. L., & dos Santos, A. S. (2018). Hydrolysis, detoxification and alcoholic fermentation of hemicellulose fraction from palm press fiber. Waste and Biomass Valorization, 9, 957-968.

Bhuyar, P., Trejo, M., Dussadee, N., Unpaprom, Y., Ramaraj, R., & Whangchai, K. (2021). Microalgae cultivation in wastewater effluent from tilapia culture pond for enhanced bioethanol production. Water Science and Technology, 84(10-11), 2686-2694.

Cardona, C., Quintero, J., & Paz, I. (2010). Production of bioethanol from sugarcane bagasse: status and perspectives. Bioresource Technology, 101(13), 4754-4766.

Carvalheiro, F., Duarte, L., Lopes, S., Parajó, J., Pereira, H., & Gırio, F. (2005). Evaluation of the detoxification of brewery's spent grain hydrolysate for xylitol production by Debaryomyces hansenii CCMI 941. Process Biochemistry, 40(3-4), 1215-1223.

Chandel, A. K., da Silva, S. S., & Singh, O. V. (2011). Detoxification of lignocellulosic hydrolysates for improved bioethanol production. Biofuel production-recent developments and prospects, 10, 225.

Chen, X., Zhai, R., Li, Y., Yuan, X., Liu, Z.-H., & Jin, M. (2020). Understanding the structural characteristics of water-soluble phenolic compounds from four pretreatments of corn stover and their inhibitory effects on enzymatic hydrolysis and fermentation. Biotechnology for Biofuels, 13, 1-13.

Cheng, K.-K., Cai, B.-Y., Zhang, J.-A., Ling, H.-Z., Zhou, Y.-J., Ge, J.-P., & Xu, J.-M. (2008). Sugarcane bagasse hemicellulose hydrolysate for ethanol production by acid recovery process. Biochemical Engineering Journal, 38(1), 105-109.

Dussadee, N., Ramaraj, R., & Cheunbarn, T. (2017). Biotechnological application of sustainable biogas production through dry anaerobic digestion of Napier grass. 3 Biotech, 7, 1-9.

Dussadee, N., Reansuwan, K., Ramaraj, R., & Unpaprom, Y. (2022). Removal of CO_2 and H_2S from biogas and enhanced compressed bio-methane gas production from swine manure and elephant grass. Maejo International Journal of Energy and Environmental Communication, 4(3), 39-46.

Deshavath, N. N., Mohan, M., Veeranki, V. D., Goud, V. V., Pinnamaneni, S. R., & Benarjee, T. (2017). Dilute acid pretreatment of sorghum biomass to maximize the hemicellulose hydrolysis with minimized levels of fermentative inhibitors for bioethanol production. 3 Biotech, 7, 1-12.

Excoffier, G., Toussaint, B., & Vignon, M. R. (1991). Saccharification of steam-exploded poplar wood. Biotechnology and Bioengineering, 38(11), 1308-1317.

Gámez, S., González-Cabriales, J. J., Ramírez, J. A., Garrote, G., & Vázquez, M. (2006). Study of the hydrolysis of sugar cane bagasse using phosphoric acid. Journal of Food Engineering, 74(1), 78-88.

Ge, J.-P., Cai, B.-Y., Liu, G.-M., Ling, H.-Z., Fang, B.-Z., Song, G., Yang, X.-F., & Ping, W.-X. (2011). Comparison of different detoxification methods for corn cob hemicelluose hydrolysate to improve ethanol production by Candida shehatae ACCC 20335. African Journal of Microbiology Research, 5(10), 1163-1168.

Germec, M., Kartal, F. K., Bilgic, M., Ilgin, M., Ilhan, E., Güldali, H., Isci, A., & Turhan, I. (2016). Ethanol production from rice hull using *Pichia stipitis* and optimization of acid pretreatment and detoxification processes. Biotechnology progress, 32(4), 872-882.

Germec, M., & Turhan, I. (2018). Ethanol production from acid-pretreated and detoxified rice straw as sole renewable resource. Biomass Conversion and Biorefinery, 8, 607-619.

Gupta, V. C., Singh, M., Prasad, S., & Mishra, B. N. (2023). Minimization of Inhibitor Generation in Rice Straw Hydrolysate Using RSM Optimization Technique. Agriculture, 13(7), 1431.

Huang, C.-F., Lin, T.-H., Guo, G.-L., & Hwang, W.-S. (2009). Enhanced ethanol production by fermentation of rice straw hydrolysate without detoxification using a newly adapted strain of Pichia stipitis. Bioresource technology, 100(17), 3914-3920.

Junluthin, P., Pimpimol, T., & Whangchai, N. (2021). Efficient conversion of night-blooming giant water lily into bioethanol and biogas. Maejo International Journal of Energy and Environmental Communication, 3(2), 38-44.

Kaewdiew, J., Ramaraj, R., Koonaphapdeelert, S., & Dussadee, N. (2019). Assessment of the biogas potential from agricultural waste in northern Thailand. Maejo International Journal of Energy and Environmental Communication, 1(1), 40-47.

Khaodee, W., & Chaiworn, P. (2023). Sustainable management of rice straw addressing burning issues and harnessing bioethanol potential. Maejo International Journal of Energy and Environmental Communication, 5(3), 43-51.

Kongchan, W., Unpaprom, Y., Dussadee, N., & Ramaraj, R. (2022). Bioethanol production from low-grade konjac powder via combination of alkaline and thermal pretreatments. Maejo International Journal of Energy and Environmental Communication, 4(3), 27-31.

Lanka, S., Adivikatla, V. R., Shaik, N., Kothagauni, S. Y., Panda, S. H., Yenumula, G. P., & Linga, V. R. (2011). Studies on different detoxification methods for the acid hydrolysate of lignocellulosic substrate *Saccharum spontaneum*. Dynamic Biochemistry, Process Biotechnology and Molecular Biology, 5, 1-5.

Larsson, S., Reimann, A., Nilvebrant, N.-O., & Jönsson, L. J. (1999). Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce. Applied Biochemistry and Biotechnology, 77, 91-103.

Lin, T.-H., Guo, G.-L., Hwang, W.-S., & Huang, S.-L. (2016). The addition of hydrolyzed rice straw in xylose fermentation by *Pichia stipitis* to increase bioethanol production at the pilot-scale. Biomass and Bioenergy, 91, 204-209.

Lynd, L. R., & Wang, M. Q. (2003). A product-nonspecific framework for evaluating the potential of biomass-based products to displace fossil fuels. Journal of Industrial Ecology, 7(3-4), 17-32.

Martinez, A., Rodriguez, M. E., York, S. W., Preston, J. F., & Ingram, L. O. (2000a). Effects of Ca (OH)2 treatments ("overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates. Biotechnology and Bioengineering, 69(5), 526-536.

Martinez, A., Rodriguez, M. E., York, S. W., Preston, J. F., & Ingram, L. O. (2000b). Use of UV absorbance to monitor furans in dilute acid hydrolysates of biomass. Biotechnology Progress, 16(4), 637-641.

Mateo, S., Roberto, I. C., Sánchez, S., & Moya, A. J. (2013). Detoxification of hemicellulosic hydrolyzate from olive tree pruning residue. Industrial Crops and Products, 49, 196-203.

Millati, R., Niklasson, C., & Taherzadeh, M. J. (2002). Effect of pH, time and temperature of overliming on detoxification of dilute-acid hydrolyzates for fermentation by *Saccharomyces cerevisiae*. Process Biochemistry, 38(4), 515-522.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31(3), 426-428.

Mohagheghi, A., Ruth, M., & Schell, D. J. (2006). Conditioning hemicellulose hydrolysates for fermentation: effects of overliming pH on sugar and ethanol yields. Process Biochemistry, 41(8), 1806-1811.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology, 96(6), 673-686.

Myers, J. A., Curtis, B. S., & Curtis, W. R. (2013). Improving accuracy of cell and chromophore concentration measurements using optical density. BMC Biophysics, 6, 1-16.

Nielsen, S. S. (2010). Phenol-sulfuric acid method for total carbohydrates. Food analysis laboratory manual, 47-53.

Pradechboon, T., & Junluthin, P. (2022). Alkali pretreatment and enzymatic saccharification of blue-green alga Nostochopsis lobatus for bioethanol production. Maejo International Journal of Energy and Environmental Communication, 4(1), 23-28.

Palmqvist, E., & Hahn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresource Technology, 74(1), 25-33.

Ratchawet, A., & Chaiworn, P. (2022). Pretreatment of barley straw with acid and alkaline solutions to boost the efficiency of fermentable yield enzymatic degradation in the separated fermentation technique for ethanol production. Maejo International Journal of Energy and Environmental Communication, 4(2), 64-69.

Sophanodorn, K., Unpaprom, Y., Homdoung, N., Dussadee, N., & Ramaraj, R. (2020). Thermochemical pretreatment method followed by enzyme hydrolysis of tobacco stalks for bioethanol production. Global Journal of Science & Engineering, 5, 6-10.

Sabiha-Hanim, S., & Abd Halim, N. A. (2018). Sugarcane bagasse pretreatment methods for ethanol production. Fuel ethanol production from sugarcane, 63-79.

Seo, H.-B., Kim, H.-J., Lee, O.-K., Ha, J.-H., Lee, H.-Y., & Jung, K.-H. (2009). Measurement of ethanol concentration using solvent extraction and dichromate oxidation and its application to bioethanol production process. Journal of Industrial Microbiology and Biotechnology, 36(2), 285-292.

Stoutenburg, R. M., Perrotta, J. A., & Nakas, J. P. (2011). Overliming inhibitors in a hemicellulosic hydrolysate: improving fermentability by feedstock detoxification and adaptation of Pichia stipitis. Journal of Industrial Microbiology and Biotechnology, 38(12), 1939-1945.

Trejo, M., Bhuyar, P., Unpaprom, Y., Dussadee, N., & Ramaraj, R. (2022). Advancement of fermentable sugars from fresh elephant ear plant weed for efficient bioethanol production. Environment, Development and Sustainability, 24(5), 7377-7387.

Van Zyl, C., Prior, B. A., & Du Preez, J. C. (1988). Production of ethanol from sugar cane bagasse hemicellulose hydrolyzate by Pichia stipitis: Scientific note. Applied Biochemistry and Biotechnology, 17, 357-369.