Research Article [Araştırma Makalesi]

# Optimization of dilute acid and alkaline peroxide pretreatment to enhance ethanol production from wheat straw

[Buğday saplarından etanol üretimini arttırmak için seyreltik asit ve alkali peroksit önarıtım işlemlerinin optimizasyonu]

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**ABSTRACT**

**Objective:** The main purpose of this study was to determine the optimal pretreatment process conditions for wheat straw to maximize the overall ethanol yield.

**Methods:** The effects of pretreatment conditions for dilute acid and alkaline peroxide pretreatment were investigated using Response Surface Methodology (RSM). Co-fermentation with *S. cerevisiae* and *P. stipitis* was employed to produce ethanol from pretreated solids. Cellulose and xylan composition of wheat straw, degradability of these compounds, as well as ethanol production, were monitored at different conditions.

**Results:** 0.5% $H_2SO_4$ concentration, 15% solid loading, and particle size between 0.75-0.9 mm (20 mesh) was predicted to be optimal by RSM for dilute acid pretreatment and experiment performed at that condition resulted in overall ethanol yield of 12.95%. The maximum overall ethanol yield determined for alkaline peroxide pretreatment is 18.23% and can be achieved when the pretreatment were done with 0.875% $H_2O_2$ at 35 °C for 1 h. Experimental results agreed with the responses.

**Conclusion:** Alkaline peroxide pretreatment of wheat straw was found to be more effective than dilute acid pretreatment for enhancing bioethanol production from wheat straw by co-fermentation.

**Key Words:** bioethanol, co-fermentation, chemical pretreatment, wheat straw, *Pichia stipitis*

**Conflict of interest**: There is no conflict of interest among authors.

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**ÖZET**

**Amaç:** Bu çalışmanın ana amacı buğday saplarından üretilecek nihai etanol miktarını maksimize etmek için en uygun ön arıtma koşullarının tespit edilmesidir.

**Gereç ve Yöntemler:** Hem seyreltik asit hem de alkali peroksit önarıtım yöntemleri için Yüzey Tepki Metodolojisi (YTM) kullanarak ön arıtma koşullarının etkileri araştırılmıştır. Etanol üretimi için ön arıtıma tabi tutulmuş katılara *S. cerevisiae* ve *P. stipitis* ile ko-fermentasyon uygulanmıştır. Buğday saplarının içerdiği selüloz ve ksilanın parçalanabilirliği etanol üretiminde olduğu gibi değişik koşullarda incelenmiştir.

**Bulgular:** Seyreltik asit önarıtımı için YTM tarafından tahmin edilen optimum koşullar %0.5 $H_2SO_4$ konsantrasyonu, %15 katı yükleme oranı ve 0.75-0.9 mm partikül boyutu olarak bulunmuş ve bu koşullarda yapılan deney sonucunda %12.95 nihai etanol üretimi gerçekleşmiştir.  Alkali peroksit önarıtımı için tespit edilen en yüksek etanol miktarı ise %18.23 olarak bulunmuş ve bu değere 35 °C 'de 1 saat %0.875 $H_2O_2$ ile ön arıtım gerçekleştirildiğinde ulaşılmıştır.

**Sonuçlar:** Deneysel sonuçlar YTM ile elde edilen sonuçlarla uyum içindedir. Buğday saplarından kofermentasyonla etanol üretiminin iyileştirilmesinde alkali peroksit ile ön arıtım  işleminin seyreltik asit ön arıtımına kıyasla daha etkili olduğu tespit edilmiştir.

**Anahtar Kelimeler:** biyoetanol, ko-fermentasyon, kimyasal ön arıtım, buğday sapı, *Pichia stipitis*

**Çıkar Çatışması**: Yazarların çıkar çatışması bulunmamaktadır.

## Introduction

There is a soaring increase in world energy consumption, which also increases concerns about climate change. Biomass-based renewable energy, such as cellulosic ethanol, is considered to be one solution to the energy crisis and global warming [1]. Ethanol is one of the most important alternatives to gasoline and it can also be used as a fuel supplement in gasoline. Lignocellulosic waste materials including various agricultural residues, fruit and vegetable wastes, woods, municipal solid waste, waste from the pulp and paper industry, and herbaceous energy crops are the most abundant renewable source of biomass [2]. Among them, wheat straw is known to be the largest biomass feedstock in Europe and the second largest in the world after rice straw [3]. Because of increasing demand of human food consumption, global production of wheat straw needs to be increased, therefore, it has a great potential as feedstock for ethanol production in 21st century [4, 5]. It is estimated that approximately 41 million tons are produced annually in Turkey [6]. Wheat straw contains 30-45% cellulose, 20-35%hemicellulose and 8-16% lignin [7, 8]. Because of its abundance and high amount of cellulose and xylan content wheat straw is an attractive and promising source for bioethanol production.

Enzymatic hydrolysis of cellulose to glucose is of central importance in conversion of lignocellulose to bioethanol[8]. For large scale bioethanol production, the biochemical pathways applied at moderate process conditions using cellulase enzyme to convert cellulosic polymers to fermentable sugars are the most promising ones [9]. However, recalcitrance of lignocellulosic biomass to chemical and enzymatic digestion limits efficient production of cellulosic ethanol [1].

Pretreatment is now regarded as a critical step in lignocellulose processing. It removes the structural barriers (lignin and hemicelluloses) and alters the physical ones (surface area, crystallinity, pore size distribution, degree of polymerization) and by the way improves the accessibility of enzyme for hydrolysis. Due to the fact that pretreatment is among the most expensive steps in the bioethanol conversion process, an efficient, less energy intensive and cost effective pretreatment method is needed to decrease the cost of ethanol production [10].

An effective pretreatment should meet following main requirement: (a) free cellulose and hemicellulose from their complex structure with lignin complex so that high amount of sugars can be obtained by enzymatic hydrolysis, (b) avoiding formation of inhibitors for enzymes and microorganisms, (c) reducing the enzyme demand. Several methods have been applied for pretreatment of lignocellulosic waste materials. The pretreatment by dilute acid and alkaline peroxide have proven to be efficient process for wide range of lignocellulosic waste materials [11-13].

Conversion of dilute acid and alkaline peroxide pretreated wheat straw into bioethanol has been previously investigated [8, 12]. Most of these studies reported the conditions for efficient hydrolysis of wheat straw without detailing the specific effects of individual treatment parameters on saccharification yields. Due to the high xylan content in wheat straw, a proper pretreatment method and use of suitable yeast species for fermentation of xylose to ethanol may increase overall yield of ethanol.

*Saccharomyces cerevisiae* is the yeast that most commonly used in bioethanol production. Nevertheless, it is not capable of converting xylose to ethanol. For fermentation of both of glucose and xylose sugars, various xylose fermentable microorganisms including *Candida* shehatea [14], *Candida guilliermondi* [15], *Pichia stipitis* [14, 16], *Zymomonas mobilis* [17], *Pachysolen tannophilus* [14], *Kluyveromyces marxianus* [18, 19], *Mucor indicus* and *Rhizopus oryzae* [20] have been used. Among these yeasts, *P. stipitis* is one of the most promising species to ferment xylose to ethanol owing to its low by-product formation.

In this study, enzymatic digestibility of cellulose and hemicellulose from alkaline peroxide and dilute acid pretreated wheat straw is reported. The key parameters: (1) peroxide concentration, (2) residence time, (3) temperature for alkaline peroxide pretreatment and key parameters: (1) acid concentration, (2) solid loading, (3) size of the wheat straw for dilute acid pretreatment were examined. The effect of these parameters on the responses: cellulose and xylose digestibility, enzymatic hydrolysis and co-fermentation yields, through response surface methodology (RSM) was detected. Pretreated and enzyme saccharified wheat straw was fermented by *P. stipitis* and *S. cerevisiae*.

## Materials and Methods

### Materials

Post grain harvested wheat straw was collected from Bolu, Turkey. Dried wheat straw was grounded in a rotary mill and passed through different size screens. The particle sizes between 0.9-0.75 mm (20 mesh), 0.5-0.3 mm (40 mesh) and 0.3-0.2 mm (60 mesh) were used in this study. Carbohydrate and lignin composition of the wheat straw was analyzed by National Renewable Energy Laboratory procedures [21].

### Pretreatment

The effects of pretreatment methods, alkaline peroxide and dilute acid pretreatment, were tested. The effect of pretreatment parameters including: acid concentration, solid mass loading and size of the solids were evaluated for dilute acid pretreatment; and the parameters including: temperature, residence time and peroxide concentration were evaluated for alkaline peroxide pretreatment. Box-Behnken methodology was used for the experimental

design and Response Surface Modeling was applied to determine the effect of the pretreatment parameters on saccharification and co-fermentation.

## Dilute acid pretreatment

Dilute acid pretreatment was performed in an autoclave at 140 °C for 90 minutes. After autoclave the remaining solid residues were separated by vacuum filtration using 10 µm pore-sized filters and the solid fractions washed with de-ionized water until they reached neutral pH. Before analyzing the cellulose, hemicellulose and lignin content of pretreated wheat straw, remaining solids were dried in an oven at 50 °C for 48 h, and then dried solids were used for bioethanol production. Solid recovery yield was calculated as dry weight of water insoluble solid remaining after pretreatment referred to 100 g of untreated raw material.

## Alkaline peroxide pretreatment

Air-dried wheat straw was sieved to size of 20 mesh. Unless otherwise noticed, 5 g of 20 mesh-sized solids were transferred in to 100 ml flasks containing 100 ml of alkaline peroxide solutions at pH 11.5. Flask were placed in an orbital shaker at a speed range of 180 rpm and pretreated until desired retention time has been reached. At the end of the retention time flasks were removed from the shaker and the solid and liquid fractions were filtered through 10 µm pore-sized filters. The solid fractions were washed with de-ionized water until they reached neutral pH. After washing, the solid samples were dried in an oven at 50 °C for 48 h and used as carbon source for Same Vessel Saccharification and Fermentation (SVSCF).

## Enzymes and cells

Cellulase mixture including: endo-glucanases, exo-glucanases, cellobiohydrolases , ß-glucosidases from *Trichoderma reesei* (Celluclast-1.5L) and cellobiase from *Aspergillus niger* (Novozyme 188) were kindly provided by Novo Nordisk A/S, Denmark.

*Saccharomyces cerevisiae ATCC 26602* was purchased from American Type Culture Collection, USA and *Pichia stipitis DSM 3651* was purchased from German Collection of Microorganisms and Cell Cultures. For inocula preparation, the *S. cerevisiae* and *P. stipitis* cells were incubated in Yeast Medium containing 1% glucose, 0.3% yeast extract, 0.3% malt extract, and 0.5% peptone at 37 °C for 24 and 48 h, respectively.

## Enzymatic saccharification and co-fermentation procedure

SVSCF of the dilute acid and alkaline peroxide pretreated samples (at 5% solid loading) was performed as previously described [22]. The first stage of the SVSCF process was started with the addition of the enzymes Celluclast 1.5 L and Novozyme 188. 500 µl Novozyme 188 and 200 µl Celluclast 1.5 L enzyme

loading was applied for per gram of dry mater. At the end of the first stage performed at 50°C for 24 h temperature decreased to 37 °C and second stage was started with adding yeast cells. To provide the appropriate conditions for yeast cells, first stage slurry was supplemented with (g/L): yeast extract, 5; $(NH_4)_2 SO_4$, 3.75; $KH_2PO_4$, 2.1; $MgSO_4.7H_2O$, 0.375; $CaCl_2.2H_2O$, 0.5 before adding the yeast cells.

## Analytical procedures

The efficiency of the SVSCF process was monitored for 72 h by taking 1 ml samples periodically. Samples were centrifuged at 6000 rpm for 5 minute and the change of the glucose, xylose and ethanol concentrations in supernatants were analyzed as described in [22].

## Calculations

The term of the digestibility of the cellulose and xylan in the pretreated solid were defined as the glucose or xylose released via enzymatic hydrolysis divided by the glucose or xylose in the pretreated solid and it was calculated from Eq. 1 and Eq. 2.

The term of ethanol yield was defined as g ethanol produced from 100 g of pretreated solid and calculated from Eq. 3. The efficiency of the SVSCF process was calculated from Eq. 4.

*Cellulose digestibility*

$$= \frac{\begin{array}{c} cellulose\ in\ the\ pretreated\ solid\ (as\ glu\cos e) - \\ glu\cos e\ in\ the\ remaining\ solid\ after\ SVSCF \end{array}}{cellulose\ in\ the\ pretreated\ solid\ (as\ glu\cos e)} \text{ (Eq. 1)}$$

*Xylan digestibility*

$$= \frac{\begin{array}{c} xylan\ in\ the\ pretreated\ solid\ (as\ xylose) - \\ xylose\ in\ the\ remaining\ solid\ after\ SVSCF \end{array}}{xylose\ in\ the\ pretreated\ solid\ (as\ xylose)} \text{ (Eq. 2)}$$

$$Ethanol\ yield = \frac{ethanol\ produced}{glu\cos e\ and\ xylose\ in\ the\ pretreated\ solid} \times 100 \text{ (Eq. 3)}$$

$$\%Overall\ yield = \frac{ethanol\ produced}{dried\ raw\ material} \times 100 \text{ (Eq. 4)}$$

## Statistical analysis

The establishment of the experimental design and the analysis of all the results have been carried out using the Matlab 2009 software. The second-order polynomial coefficient was also determined using this software. By using RSM, the experimental response obtained was

analyzed with the following second-order polynomial, Eq. 5:

$$Y = \beta_0 + \beta_1\chi_1 + \beta_2\chi_2 + \beta_3\chi_3 + \beta_{1,1}\chi_1^2 + \beta_{2,2}\chi_2^2 + \beta_{3,3}\chi_3^2 + \beta_{1,2}\chi_1\chi_2 + \beta_{1,3}\chi_1\chi_3 + \beta_{2,3}\chi_2\chi_3 \quad \text{(Eq. 5)}$$

Where Y is the response (for example, ethanol yield), $x_1$, $x_2$ and $x_3$ are the coded levels of the three variables (for example, particle size, acid concentration and solid loading, respectively in dilute acid pretreatment) and $\beta_i$ is the model coefficient calculated from experimental data.

## Results

### Raw material

The carbohydrate and lignin composition of the wheat straw was analyzed as described previously. The chemical composition of the raw material is (% w/w, dry weight basis): cellulose (as glucose): 42.81±0.7; xylan (as xylose): 23.83±0.2; other hemicellulosic compounds (as sum of arabinose, galactose and mannose): 1.37±0.1; acid-soluble lignin: 1.39±0.4; acid-insoluble lignin: 13.71±0.7; ash: 3.73±0.3; and other extractives: 10.5±0.25. The high cellulose and hemicellulose content make this biomass an appropriate raw material for bioethanol production.

### Optimization of dilute acid pretreatment

In the literature, the effects of three variables of dilute acid pretreatment, time, and acid concentration[7, 13, 25] were widely examined for optimization of lignocellulosic ethanol production processes. Whereas, studies on the effect of the solid loading (lignocellulosic biomass/ liquid ratio) and biomass size on pretreatment are rare.

In the present paper, wheat straw was subjected to dilute acid pretreatment under different process conditions. The variables were studied in the following ranges: 0.5-1.5% acid concentration; 5-15% w/v solid loading; 20–60 mesh particle size.

Optimum levels for these parameters were determined using a statistical $2^3$ full factorial design. The experimental design matrix and effect of the variables on solid recovery and sugar loss were given in Table 1. In terms of solid recovery and composition, applied dilute acid pretreatment conditions resulted in a wide variety of pretreated solid and liquid fractions. Amount of sugars leaving the raw material and entering the liquid fraction changed with respect to the pretreatment condition. As seen in Table 1, pretreatment of 40 mesh sized wheat straw at 15% solid loading (Run #15) and 20 mesh sized wheat straw with 10% solid loading (Run #9) with 1.5% (w/w) $H_2SO_4$ resulted in highest HMF (0.82g/L) and furfural (2.13 g/L) generation, respectively. Increasing acid concentration or solid loading resulted in increase especially in furfural concentration (Table 1). The highest solid recovery yield was obtained when 15% (w/w) solid with 40 mesh particle size pretreated with 0.5% (w/w) $H_2SO_4$ (Run #14). High solid recovery also resulted in high cellulose and xylan digestibility, 92.54% and 92.42%, respectively (Table 2). As seen in Table 1, solid loading was found to be more important than particle size for improving solid recovery. In addition, to the increase in the solid recovery and enzymatic saccharification, increasing the sugar conversion rate (for both glucose and xylose) to ethanol is crucial for obtaining higher ethanol yields. Pretreated wheat straw contain remarkable amount of xylan which could not be fermented by *S. cerevisiae*. In this study, the pretreated straw was subjected to co-fermentation using *Saccharomyces cerevisiae* and xylose fermenting *Pichia stipitis*.

It was found that glucose and xylose were totally consumed at the end of the co-fermentation. Even though the highest ethanol yield (21.95%, g ethanol/g sugar) was obtained at low solid loading, the highest overall yield (g ethanol/g raw material, 11.87%) was obtained at high solid loading (Table 2). The influence of solid loading and acid concentration on overall ethanol yield was shown in Figure 1 (A). The effects of the acid concentration and particle size, on overall yield at optimum solid loading value (15%) were shown in Figure 1 (B). It was clear that decreasing acid concentration caused overall yield of ethanol to increase when PS was incresead from 60 mesh to 20 mesh. The RSM predicted the maximum overall yield as 12.87% when 20 mesh size wheat straw is pretreated with 0.5% $H_2SO_4$ at a solid loading of 15%. In order to test the reliability of this result, an additional experiment was carried out at that predicted optimum condition.The experimentally observed actual overall yield was found as 12.95% and this value is so close to value predicted by the model (%12.87).

### Optimization of alkaline peroxide pretreatment

In order to optimize the alkaline peroxide pretreatment of wheat straw the effect of three variables ($H_2O_2$ concentration, temperature and time) on the composition of the solid residue and ethanol yield of fermentation process was examined. 5% solid loading was employed for alkaline peroxide pretreatment. Table 3 shows the experimental variables for alkaline pretreatment of wheat straw and effect of the variables on solid recovery, water-insoluble solid and liquid fraction compositions.

The solid recovery for the alkaline peroxide pretreatment ranged from 40.80% to 51.20% with respect to the pretreatment conditions. The solid recovery was improved by decreasing pretreatment time and temperature (Table 3). The cellulose content of the raw wheat straw was 42.81%, and it increased to 71.30% after alkaline peroxide pretreatment with 0.875% (w/v) $H_2O_2$ at 55 °C for 4 h.

Alkaline peroxide pretreatment appeared to be suitable method for obtaining high xylan recovery. In contrast

**Table 1.** Box-Behnken experimental design of the three variables at three levels for dilute acid pretreatment of wheat straw and effect of the variables on solid recovery, water-insoluble solids (WIS) and liquid fraction compositions

| Run | Size (mesh) | Solid loading (%, w/v) | $H_2SO_4$ conc. (%, w/v) | Solid recovery (%, w/w) | WIS composition (%, w/w) | | | Liquid fraction composition (g/L) [%][b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Cellulose | Xylan | Lignin | Glucose | Xylose | HMF | Furfural |
| 1 | 40 (0)[a] | 10 (0) | 1 (0) | 50.60 | 66.23 | 11.91 | 22.68 | 1.92 [1.92] | 2.15 [2.15] | 0.20 [0.20] | 0.70 [0.70] |
| 2 | 40 (0) | 10 (0) | 1 (0) | 51.40 | 66.23 | 9.59 | 23.11 | 2.13 [2.13] | 2.08 [2.08] | 0.20 [0.20] | 0.66 [0.66] |
| 3 | 40 (0) | 10 (0) | 1 (0) | 51.80 | 65.61 | 10.30 | 22.57 | 2.11 [2.11] | 2.07 [2.07] | 0.21 [0.21] | 0.73 [0.73] |
| 4 | 20 (-1) | 5 (-1) | 1 (0) | 44.40 | 64.53 | 10.04 | 22.74 | 1.51 [3.02] | 1.66 [3.32] | 0.12 [0.24] | 0.67 [1.34] |
| 5 | 20 (-1) | 15 (+1) | 1 (0) | 56.53 | 60.71 | 5.48 | 18.96 | 3.37 [2.24] | 5.32 [0.33] | 0.45 [0.30] | 1.07 [0.71] |
| 6 | 60 (+1) | 5 (-1) | 1 (0) | 42.40 | 63.15 | 7.92 | 22.96 | 1.67 [3.34] | 2.11 [4.22] | 0.15 [0.30] | 0.76 [1.52] |
| 7 | 60 (+1) | 15 (+1) | 1 (0) | 55.87 | 60.73 | 9.37 | 22.75 | 3.15 [2.10] | 5.24 [3.49] | 0.62 [0.41] | 1.22 [0.81] |
| 8 | 20 (-1) | 10 (0) | 0,5 (-1) | 53.80 | 64.97 | 11.04 | 21.83 | 2.59 [2.59] | 3.95 [3.95] | 0.37 [0.37] | 0.85 [0.85] |
| 9 | 20 (-1) | 10 (0) | 1,5 (+1) | 50.40 | 43.03 | 5.14 | 29.16 | 5.10 [5.10] | 1.96 [1.96] | 0.18 [0.18] | 2.13 [2.13] |
| 10 | 60 (+1) | 10 (0) | 0,5 (-1) | 52.40 | 62.73 | 11.25 | 21.66 | 2.88 [2.88] | 4.26 [4.26] | 0.49 [0.49] | 0.96 [0.96] |
| 11 | 60 (+1) | 10 (0) | 1,5 (+1) | 49.00 | 59.15 | 7.44 | 28.95 | 3.91 [3.91] | 1.72 [1.72] | 0.19 [0.19] | 1.51 [1.51] |
| 12 | 40 (0) | 5 (-1) | 0,5 (-1) | 45.20 | 65.59 | 6.00 | 23.57 | 2.26 [4.52] | 2.75 [5.50] | 0.20 [0.40] | 0.66 [1.32] |
| 13 | 40 (0) | 5 (-1) | 1,5 (+1) | 40.00 | 62.73 | 5.73 | 27.26 | 3.46 [6.92] | 0.97 [1.94] | 0.21 [0.42] | 1.34 [2.68] |
| 14 | 40 (0) | 15 (+1) | 0,5 (-1) | 59.33 | 59.31 | 7.29 | 19.31 | 3.07 [2.04] | 5.48 [3.65] | 0.73 [0.48] | 0.79 [0.53] |
| 15 | 40 (0) | 15 (+1) | 1,5 (+1) | 55.33 | 58.17 | 7.03 | 28.10 | 7.64 [5.09] | 3.45 [2.30] | 0.82 [0.55] | 0.90 [0.60] |

[a] numbers in parentheses are coded values of variables in the experimental design.
[b] values in brackets are the g of released monomeric sugars and generated inhibitor compounds in the liquid fraction from 100 g raw material.

**Table 2.** Effect of the dilute acid pretreatment variables on enzymatic digestibility and ethanol yield

| Run | Cellulose digestibility (%) | | Xylan digestibility (%) | | Ethanol Yield (%) | | Overall ethanol yield (%) | |
|---|---|---|---|---|---|---|---|---|
| | Experimental | Predicted | Experimental | Predicted | Experimental | Predicted | Experimental | Predicted |
| 1 | 83.19 | 82.87 | 83.37 | 82.81 | 18.80 | 18.96 | 9.51 | 9.72 |
| 2 | 83.14 | 82.87 | 82.90 | 82.81 | 18.78 | 18.96 | 9.65 | 9.72 |
| 3 | 82.27 | 82.87 | 82.16 | 82.81 | 19.29 | 18.96 | 9.99 | 9.72 |
| 4 | 53.18 | 53.77 | 53.61 | 54.27 | 21.95 | 21.13 | 9.75 | 9.31 |
| 5 | 80.19 | 88.68 | 80.86 | 88.93 | 20.99 | 19.87 | 11.87 | 11.31 |
| 6 | 74.49 | 66.01 | 64.16 | 66.08 | 20.73 | 21.85 | 8.79 | 9.34 |
| 7 | 92.49 | 91.89 | 92.66 | 92.00 | 18.61 | 19.43 | 10.40 | 10.83 |
| 8 | 85.75 | 81.65 | 86.10 | 82.09 | 20.73 | 21.74 | 11.15 | 11.71 |
| 9 | 85.03 | 80.04 | 85.54 | 80.82 | 14.74 | 15.67 | 7.43 | 7.85 |
| 10 | 87.42 | 92.41 | 87.89 | 92.61 | 20.29 | 19.36 | 10.63 | 10.21 |
| 11 | 80.63 | 84.73 | 81.18 | 85.19 | 19.33 | 18.32 | 9.47 | 8.91 |
| 12 | 59.85 | 63.36 | 59.91 | 63.26 | 20.79 | 20.60 | 9.40 | 9.27 |
| 13 | 48.72 | 53.11 | 49.67 | 53.73 | 17.81 | 17.70 | 7.12 | 7.13 |
| 14 | 92.54 | 88.15 | 92.42 | 88.36 | 19.31 | 19.42 | 11.46 | 11.45 |
| 15 | 92.62 | 89.11 | 92.56 | 89.21 | 15.01 | 15.20 | 8.31 | 8.44 |
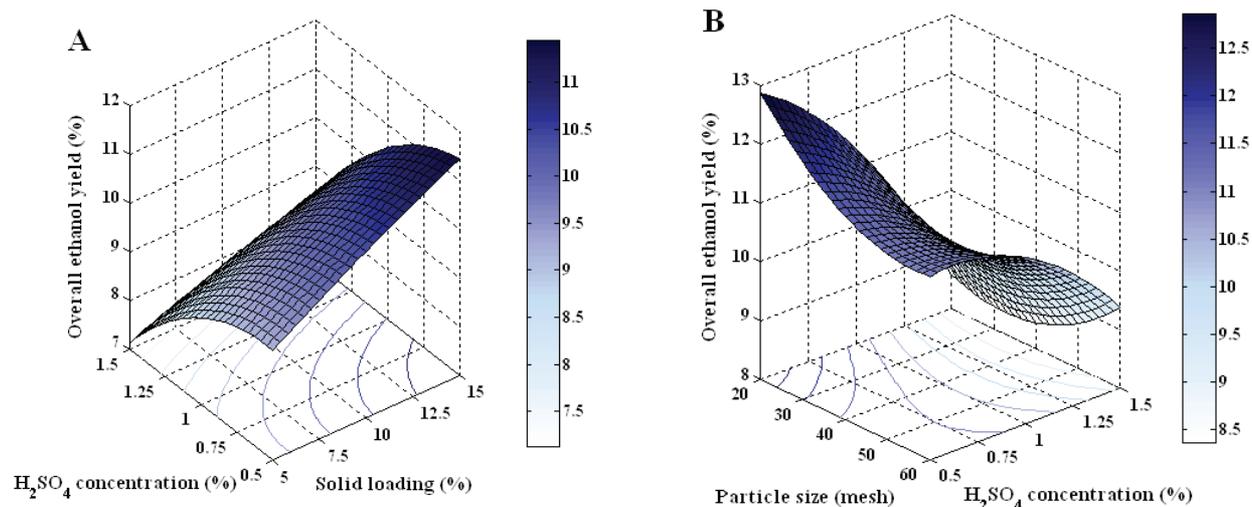


**Figure 1.** Response surface of overall ethanol yield as a function of (A) $H_2SO_4$ concentration and solid loading when sized of the solid particle was between 0.3-0.5 mm (40 mesh); (B) particle size and $H_2SO_4$ concentration when solid loading was fixed at 10%.

to acid pretreatment, higher amount of xylan was recovered after alkaline peroxide pretreatment. The maximum xylan content of recovered solid was found to be 11.91% for acid pretreatment while it was 23.83 % for alkaline peroxide pretreated wheat straw. Similarly, cellulose content of the recovered solids was higher for alkaline pretreated wheat straw as compared to the acid pretreated one. Increase in the cellulose and xylan content of the pretreated solid increased the level of glucose and xylose, the main fermentable sugars, obtained from enzymatic saccharification. Decreased lignin content in alkaline peroxide pretreatment seems to be the main reason of increased cellulose and xylan content. In the present study, lignin removal after alkaline peroxide pretreatment ranged from 65.59% to 80.55% (Table 3). As the amount of removed lignin increased higher amount of sugar was lost into the liquid fraction (Run #1, Table 3).

**Table 3.** Box-Behnken experimental design of the three variables at three levels for alkaline pretreatment of wheat straw and effect of the variables on solid recovery, water-insoluble solids (WIS) and liquid fraction compositions

| Run | Temperature (°C) | Time (h) | $H_2O_2$ conc. (%, w/v) | Solid recovery (%, w/w) | WIS composition (%, w/w) | | | Liquid fraction composition (g/L) [%] | | Lignin removal (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Cellulose | Xylan | Lignin | Glucose | Xylose | |
| 1 | 55 (0)[a] | 4 (0) | 0.875 (0) | 45.20 | 70.63 | 20.31 | 6.03 | 2.72 [5.54] | 0.09 [0.18] | 79.97 |
| 2 | 55 (0) | 4 (0) | 0.875 (0) | 45.80 | 70.14 | 20.59 | 5.94 | 2.11 [4.22] | 0.09 [0.18] | 79.57 |
| 3 | 55 (0) | 4 (0) | 0.875 (0) | 44.40 | 71.30 | 20.37 | 6.10 | 2.35 [3.70] | 0.08 [0.16] | 80.09 |
| 4 | 35 (-1) | 1 (-1) | 0.875 (0) | 51.20 | 63.59 | 23.60 | 9.14 | 1.30 [2.60] | 0.08 [0.16] | 65.59 |
| 5 | 35 (-1) | 7 (+1) | 0.875 (0) | 47.20 | 65.39 | 22.49 | 7.92 | 0.80 [1.60] | 0.05 [0.10] | 72.54 |
| 6 | 75 (+1) | 1 (-1) | 0.875 (0) | 45.20 | 65.41 | 20.76 | 7.12 | 1.79 [3.58] | 0.06 [0.12] | 76.33 |
| 7 | 75 (+1) | 7 (+1) | 0.875 (0) | 44.40 | 69.60 | 23.65 | 6.31 | 1.84 [3.68] | 0.05 [0.10] | 79.59 |
| 8 | 35 (-1) | 4 (0) | 0.35 (-1) | 48.40 | 63.81 | 18.45 | 8.55 | 0.40 [0.80] | 0.05 [0.10] | 69.59 |
| 9 | 35 (-1) | 4 (0) | 1.4 (+1) | 47.60 | 68.03 | 22.24 | 7.14 | 1.20 [2.40] | 0.08 [0.16] | 73.51 |
| 10 | 75 (+1) | 4 (0) | 0.35 (-1) | 44.40 | 66.51 | 16.28 | 9.80 | 1.11 [2.22] | 0.07 [0.14] | 68.02 |
| 11 | 75 (+1) | 4 (0) | 1.4 (+1) | 40.80 | 65.39 | 23.83 | 7.53 | 2.08 [4.16] | 0.07 [0.14] | 77.42 |
| 12 | 55 (0) | 1 (-1) | 0.35 (-1) | 46.00 | 64.81 | 18.86 | 9.15 | 0.39 [0.78] | 0.05 [0.10] | 69.04 |
| 13 | 55 (0) | 1 (-1) | 1.4 (+1) | 44.40 | 58.20 | 20.76 | 9.73 | 0.20 [0.40] | 0.06 [0.12] | 68.23 |
| 14 | 55 (0) | 7 (+1) | 0.35 (-1) | 45.60 | 68.03 | 19.66 | 9.73 | 0.38 [0.76] | 0.04 [0.08] | 67.40 |
| 15 | 55 (0) | 7 (+1) | 1.4 (+1) | 41.20 | 65.89 | 23.36 | 6.42 | 0.50 [1.00] | 0.08 [0.16] | 80.55 |

Surface responses showing the effect of the pretreatment conditions on overall ethanol yield is depicted in Fig. 2. It is clear from Fig. 2 (A) and (B) that decreasing the pretreatment time and temperature had an enhancing effect on overall yield. This increase is mainly due to the high solid recovery yields obtained at low temperatures and short pretreatment durations. To obtain high overall yields, peroxide concentrations should be kept between 0.35% and 1.1% (w/v) and applied at low temperatures and short pretreatment durations.

As seen in Table 4, the calculated values of digestibility of main carbohydrates, and ethanol yields and overall yields agreed well with those determined experimentally. When the alkaline peroxide pretreatment was carried out at 35 °C for 4 h, increasing the peroxide concentration from 0.35% to 1.4% increased the digestibility of cellulose from 75.44% to 93.13%. Nonetheless, increasing peroxide concentration to 1.4% did not favor ethanol production. The experimentally determined highest overall yield was 18.23% with 0.875% $H_2O_2$ at 35 °C for 1 h. The highest overall yield was 12.95% for the dilute acid pretreated wheat straw.
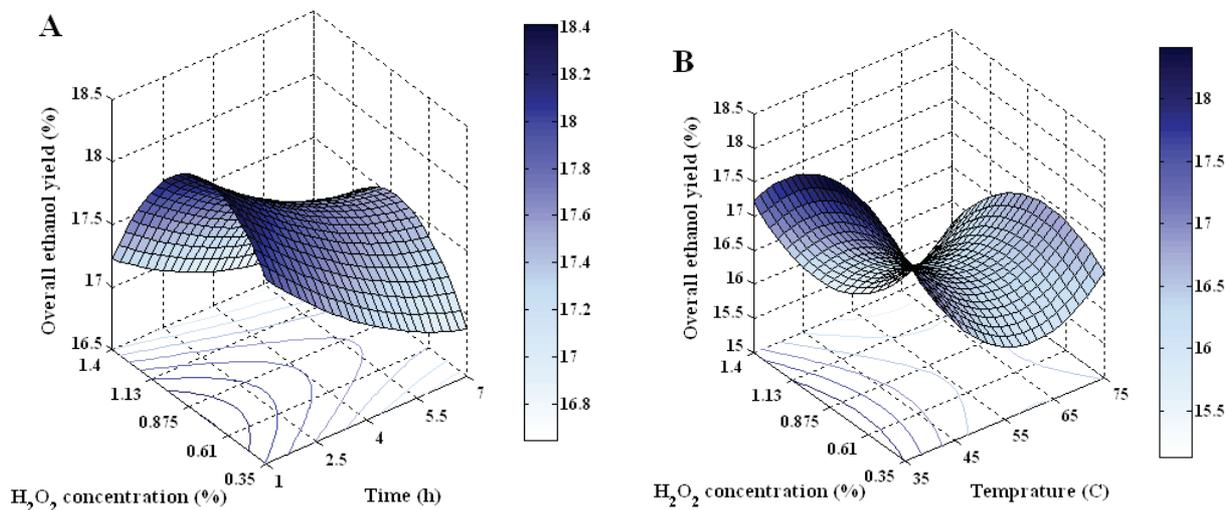


**Figure 2.** Response surface of overall ethanol yield as a function of (A) hydrogen peroxide concentration and time when the pretreatment temperature was fixed at 35 °C; (B) peroxide concentration and temperature when the pretreatment time was fixed at 1 h.

**Table 4**. Effect of the alkaline peroxide pretreatment variables on enzymatic digestibility and ethanol yield

| Run | Cellulose digestibility (%) | | Xylan digestibility (%) | | Ethanol Yield (%) | | Overall ethanol yield (%) | |
|---|---|---|---|---|---|---|---|---|
| | Experimental | Predicted | Experimental | Predicted | Experimental | Predicted | Experimental | Predicted |
| 1 | 88.00 | 81.02 | 78.04 | 78.39 | 35.13 | 35.65 | 15.88 | 16.09 |
| 2 | 81.24 | 81.02 | 78.23 | 78.39 | 35.27 | 35.65 | 16.16 | 16.09 |
| 3 | 78.90 | 81.02 | 78.89 | 78.39 | 36.56 | 35.65 | 16.23 | 16.09 |
| 4 | 82.34 | 83.39 | 82.33 | 83.38 | 35.61 | 35.86 | 18.23 | 18.36 |
| 5 | 86.21 | 87.01 | 86.20 | 87.00 | 36.69 | 36.83 | 17.32 | 17.52 |
| 6 | 82.50 | 81.70 | 82.49 | 81.69 | 37.77 | 37.63 | 17.07 | 16.87 |
| 7 | 84.40 | 83.35 | 84.38 | 83.33 | 39.00 | 38.75 | 17.16 | 17.03 |
| 8 | 75.44 | 76.44 | 75.44 | 76.43 | 36.66 | 35.63 | 17.74 | 17.27 |
| 9 | 93.13 | 90.28 | 93.12 | 90.27 | 34.93 | 35.56 | 16.62 | 16.77 |
| 10 | 74.11 | 76.96 | 74.11 | 76.95 | 37.15 | 36.51 | 16.49 | 16.34 |
| 11 | 85.41 | 84.41 | 85.40 | 84.40 | 37.35 | 38.37 | 15.24 | 15.71 |
| 12 | 73.45 | 71.40 | 73.44 | 71.39 | 34.17 | 34.95 | 15.72 | 16.07 |
| 13 | 85.18 | 86.98 | 85.17 | 86.97 | 34.96 | 34.08 | 15.52 | 15.25 |
| 14 | 80.77 | 78.97 | 80.76 | 78.96 | 33.33 | 34.22 | 15.20 | 15.47 |
| 15 | 82.63 | 84.68 | 82.62 | 84.67 | 37.67 | 36.89 | 15.52 | 15.17 |

## Optimization and validation of the models

For the adequacy of the models analysis of variance (ANOVA) and validation were justified. $Y_1$ and $Y_2$ represent the overall ethanol yields obtained by the fermentation of dilute acid and alkaline peroxide pretreated wheat straw, respectively. The F-test value of the models was calculated as 6.2145 for $Y_1$ and 5.7632 for $Y_2$, with a low probability value ($p<0.05$). A probability of $p=0.1$, $p=0.05$ and $p=0.001$ means 90%, *95%* and 99% probability of making a correct statement and $p=0.05$ is usually acceptable for biological processes. Thus, it can be concluded that in this work the generated models were significant at 95% confidence level. The coefficients of determination ($R^2$) of the models were determined as follows: 0.9179 for $Y_1$ and 0.9121 for $Y_2$.

Models were validated at additional experimental conditions different from those employed to generate the models. The results of these additional experiments showed good correlation with predicted values (Table 5).

## Discussions

This paper describes the effects of the pretreatment conditions on ethanol yield. In this study we examined different pretreatment conditions for two different pretreatment technologies: dilute acid and alkaline peroxide pretreatment. We determined that the content of the raw material is in agreement with the previous studies [13, 14].

For dilute acid pretreatment we focused on three pretreatment conditions: particle size, solid loading and acid concentration. According to Hendriks and Zeeman [26], decreasing particle size increases the specific surface area and reduces the degree of polymerization. Thus, particle size of the biomass has a strong effect on hydrolysis [27]. Because size reduction requires significant amount of energy, it is important to determine the optimum particle size to decrease the energy cost of grinding. According to HPLC results of the liquid fraction obtained from dilute acid hydrolysis, decreasing the particle size has increased the formation of furfural and HMF formations which have been reported as inhibitory compounds for yeast fermentation [28, 29]. Because they are generated from the degradation of glucose and xylose during steam and acid pretreatment processes, furfural and HMF production represent monomeric sugar loss [30].

Delgenes[31], reported that a concentration of 1 g/L HMF and 0.5 g/L furfural has an inhibitory effect on *S. cerevisiae* cells. In another study with baker's yeast, it was reported that when 0.9 g/L and 4 g/L furfural found in the media, the ethanol yield decreased by 18% and 79%, respectively [32].

Alkaline peroxide pretreatment is another pretreatment method can be applied to the lignocellulosic biomass. This method enhances enzymatic conversion of lignocellulosic biomass through oxidative delignification and reduction of cellulose crystallinity [33]. Increased lignin removal is the reason for enhanced enzymatic saccharification [34, 35]. However, high amount of lignin removal could result in loss of cellulose. Sun[34], performed alkaline peroxide pretreatment of rye straw (with 2% $H_2O_2$ at pH 11.5 and 50 °C for 12 h) and obtained dissolution of 83.1% of lignin and 70% of hemicelluloses. Qi[35], conducted the alkaline peroxide pretreatment of wheat straw (with 0.3% $H_2O_2$ and 1.5% NaOH at 50 °C for 6 h) and found that the cellulose content of the wheat straw increased to 60.17% from 40.98% and in contrast, the percent of hemicellulose and lignin content decreased. In this paper we applied alkaline peroxide pretreatment to wheat straw and examine the effects of $H_2O_2$ concentration, temperature and time on the overall ethanol yield. From the results we saw that alkaline peroxide pretreatment is quick and efficient method even at low temperatures. Furthermore no furfural and HMF were observed in the liquid fraction.

**Table 5.** Validation of the models

| Pretreatment method | $X_1$ | $X_2$ | $X_3$ | $Y_{actual}$ | $Y_{predicted}$ |
|---|---|---|---|---|---|
| Dilute acid | 40 | 5.41 | 0.75 | 11.2711 | 9.2658 |
| | 20 | 10 | 0.875 | 11.3516 | 10.8180 |
| | 20 | 6.66 | 1 | 11.2533 | 9.6787 |
| Alkaline peroxide | 75 | 7 | 0.875 | 16.7679 | 17.0332 |
| | 35 | 1 | 1.05 | 17.03947 | 18.1514 |
| | 45 | 4 | 1.225 | 17.33334 | 16.1215 |

$X_1$: Particle size (mesh) for acid pretreatment; temperature (°C) for alkaline peroxide pretreatment
$X_2$: Solid loading (%) for acid pretreatment; time (h) for alkaline peroxide pretreatment
$X_3$: $H_2SO_4$ concentration (%) for acid pretreatment; $H_2O_2$ concentration (%) for alkaline peroxide pretreatment

Results shows that, alkaline peroxide pretreatment method is more suitable than dilute acid pretreatment for subsequent enzymatic saccharification and co-fermentation by *S. cerevisiae* and *P. stipitis*. Although higher solid recovery was achieved with dilute acid pretreatment, alkaline peroxide pretreatment resulted in higher ethanol yield. This might be due to the well protection of the structural integrity of carbohydrates and more efficient lignin removal during alkaline peroxide pretreatment.

## Conclusions

In this study the optimal conditions for alkaline and acid pretreatment of wheat straw was determined and the efficiency of these pretreatment methods in terms of cellulose and xylan digestibility and ethanol production yield was compared. When the wheat straw pretreated at the optimal conditions for dilute acid ($H_2SO_4$: 0.5%, solid loading: 15%, particle size: 20 mesh), 12.87% predicted and 12.95% observed overall ethanol yields were achieved. Because of the efficient lignin removal by alkaline peroxide pretreatment, the pretreated solid contains less lignin and more cellulose as compared to acid pretreated solid. Thus, higher overall yields of ethanol were obtained by the alkaline peroxide pretreatment method. The optimized alkaline peroxide pretreatment condition was found to be 0.875% (w/v) $H_2O_2$ at 35 °C for 1 h and resulted in predicted and observed overall yields of 18.36% and 18.23%, respectively.

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## References

[1] Wan C, Zhou Y, Li Y. Liquid hot water and alkaline pretreatment of soybean straw for improving cellulose digestibility. Bioresour Technol 2011; 102(10): 6254–6259.

[2] Krishna SH, Chowdary GV. Optimization of Simultaneous Saccharification and Fermentation for the Production of Ethanol from Lignocellulosic Biomass. J Agric Food Chem 2000; 48(5): 1971–1976.

[3] Kim S, Dale BE. Global potential bioethanol production from wasted crops and crop residues. Biomass Bioenerg 2004; 26: 361–375.

[4] Ortiz R, Sayre KD, Govaerts B, Gupta R, Subbarao GV, et al. Climate change: Can wheat beat the heat? Agric Ecocst Environ 2008; 126: 46–58.

[5] Talebnia F, Karakashev D, Angelidaki I. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. Bioresour Technol 2010; 101(13): 4744–4753.

[6] Deniz I, Kırcı H, Ates S. Optimisation of wheat straw Triticum drum kraft pulping. Ind Crops Prod 2004; 19(3): 237–243.

[7] Saha BC, Iten LB, Cotta MA, Wu YV. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Proces Biochem 2005; 40: 3693–3700.

[8] Chen H, Han Y, Xu J. Simultaneous saccharification and fermentation of steam exploded wheat straw pretreated with alkaline peroxide. Proces Biochem 2008; 43 (12): 1462–1466.

[9] Kumar P, Barrett DM, Delwiche MJ, Stroeve P. Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. Ind Eng Chem Res 2009; 48(8): 3713–3729.

[10] Curreli N, Agelli M, Pisu B, Rescigno A, Sanjust E, et al. Complete and efficient enzymic hydrolysis of pretreated wheat straw. Proces Biochem 2002; 237(9): 937–941.

[11] Saha BC, Cotta MA. Enzymatic hydrolysis and fermentation of lime pretreated wheat straw to ethanol. J Chem Technol Biotechnol 2007; 82: 913–919.

[12] Qureshi N, Saha BC, Hector RE, Cotta M. Removal of fermentation inhibitors from alkaline peroxide pretreated and enzymatically hydrolyzed wheat straw: Production of butanol from hydrolysate using Clostridium beijerinckii in batch reactors. Biomass Bioenerg 2008; 32(12): 1353–1358.

[13] Castro E, Díaz MJ, Cara C, Ruiz E, Romero I, et al. Dilute acid pretreatment of rapeseed straw for fermentable sugar generation. Bioresour Tech 2011; 102(2): 1270–1276.

[14] Abbi M, Kuhad RC, Singh A. Bioconversion of pentose sugars to ethanol by free and immobilized cells of Candida shehatae (NCL-3501): Fermentation behaviour. Proces Biochem 1996; 31(6): 555–560.

[15] Schirmer-Michel AC, Flôres SH, Hertz PF, Matos GS, Ayub MAZ. Production of ethanol from soybean hull hydrolysate by osmotolerant Candidaguilliermondii NRRL Y-2075 Bioresour Tech 2008; 99(8): 2898–2904

[16] Buaban B, Inoue H, Yano S, Tanapongpipat S, Ruanglek V, et al. Bioethanol production from ball milled bagasse using an on-site produced fungal enzyme cocktail and xylose-fermenting Pichia stipitis. J Biosci Bioeng 2010; 110(1): 18–25.

[17] Yamashita Y, Kurosumi A, Sasaki C, Nakamura Y. Ethanol production from paper sludge by immobilized Zymomonas mobilis. Biochem Eng J 2008; 42: 314–319.

[18] Lark N, Xia Y, Qin CG, Gong CS, Tsao GT. Production of ethanol from recycled paper sludge using cellulase and yeast, Kluveromyces marxianus. Biomass Bioenergy 1997; 12(2): 135–143.

[19] Ballesteros M, Oliva JM, Negro MJ, Manzanares P, Ballesteros I. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with Kluyveromyces marxianus CECT 10875. Proces Biochem 2004; 39: 1843–1848.

[20] Karimi K, Emtiazi G, Taherzadeh MJ. Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with Mucor indicus, Rhizopus oryzae, and Saccharomyces cerevisiae. Enzym Microb Technol 2006; 40: 138–144.

[21] Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, et al. Determination of structural carbohydrates and lignin in biomass. National Renewable Energy Laboratory, Golden, CO. 2008. Available at http://www.nrel.gov/biomass/pdfs/42618.pdf

[22] Karagöz P, Rocha IV, Özkan M, Angelidaki I. Alkaline peroxide pretreatment of rapeseed straw for enhancing bioethanol production by Same Vessel Saccharification and Co-Fermentation. Bioresour Technol 2012; 104: 349–357.

[23] Curreli N, Fadda MB, Rescigno A, Rinaldi AC, Soddu G, et al. Mild alkaline/oxidative pretreatment of wheat straw. Proces Biochem 1997; 32(8): 665–670.

[24] Saha BC, Cotta MA. Ethanol production from alkaline peroxide pretreated enzymatically saccharified wheat straw. Biotechnol Prog 2006; 22(2): 449–453.

[25] Hsu TC, Guo GL, Chen WH, Hwang WS. Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. Bioresour Tech 2010; 101(13): 4907–4913.

[26] Hendriks AT, Zeeman G. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour Tech 2009; 100(1): 10–18.

[27] Maache-Rezzoug Z, Pierre G, Nouviaire A, Maugard T, Rezzoug SA. Optimizing thermomechanical pretreatment conditions to enhance enzymatic hydrolysis of wheat straw by response surface methodology. Biomass Bioenerg 2011; 35(7): 3129–3138.

[28] Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. Bioresour Technol 2000; 74(1): 25–33.

[29] Taherzadeh MJ, Gustafsson L, Niklasson C, Lidén G. Inhibition effects of furfural on aerobic batch cultivation of *Saccharomyces cerevisiae* growing on ethanol and/or acetic acid. J Biosci Bioeng 2000; 90(4): 374–80.

[30] Lau MW, Gunawan C, Dale BE. The impacts of pretreatment on the fermentability of pretreated lignocellulosic biomass: a comparative evaluation between ammonia fiber expansion and dilute acid pretreatment. Biotechnol Biofuel 2009; 2(30): 1–11.

[31] Delgenes J. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae*. Enzym Microb Technolnol 1996; 19(3): 220–225.

[32] Palmqvist E, Almeida JS, Hahn-Hagerdal B. Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. Biotechnolnol Bioeng 1999; 62: 447–454.

[33] Gould JM. Studies on the mechanism of alkaline peroxide delignification of agricultural residues. Biotechnol Bioeng 1985; 27: 225-231.

[34] Sun RC, Fang JM, Tomkinson J. Delignification of rye straw using hydrogen peroxide. Ind Crop Prod 2000; 12(2): 71–83.

[35] Qi B, Chen X, Shen F, Su Y, Wan Y. Optimization of Enzymatic Hydrolysis of Wheat Straw Pretreated by Alkaline Peroxide Using Response Surface Methodology. Ind Eng Chem Res 2009; 48(15): 7346–7353.