Abstract

In this study, the effect of hydrogen peroxide concentration (0%-1.25% and 2.5%) combined with 1% NaOH and temperature (30°C, 45°C and 60°C) on the composition of pre-treated sunflower stalks and fermentable sugars after enzymatic hydrolysis was investigated. Cellulose content ranged from 39.50% to 45.46%, and it increased by 15% after pre-treatment with 1.25% H₂O₂ + 1% NaOH mixture compared to that obtained with 1% NaOH pre-treatment. For cellulose content, the addition of H₂O₂ in the 1% NaOH solution resulted in a decrease of almost 40% in lignin content. Total reduced sugar in pre-treated biomass increased by 39% when the H₂O₂ concentration increased from 0% H₂O₂ + 1% NaOH to 2.5% H₂O₂ + 1% NaOH. Glucose recovery (g glucose/100 g initial cellulose) was maximum (63%) in the solid pre-treated with 1.25% H₂O₂ + 1% NaOH at 45°C for 6 hours (h).

Keywords: Hydrogen, Cellulose.

1. Introduction

Lignocellulosic agricultural residues are a promising natural renewable resource for bioethanol. Lignocellulosic materials consist of cellulose, hemicellulose and lignin. Enzymatic saccharification of cellulose and further fermentation of sugars to ethanol are the main processes of bioethanol production. Enzymatic hydrolysis is a more difficult step than the fermentation process. The porosity of biomass, crystallinity of cellulose and the complex structure of lignin and hemicellulose affect cellulose hydrolysis into fermentable sugars. Holocellulose components are generally covered with lignin structure. Therefore, an effective pre-treatment method is required to increase accessibility of cellulose and remove lignin from biomass. Different pre-treatment methods, including chemical, biological, and physical ones have been developed for lignocellulosic waste pre-treatment [1]. Among chemical pre-treatment processes, alkaline pre-treatment is generally known to be more effective in the pre-treatment of bio-mass since alkaline

removes lignin (Zhao et al)\[^2\]. In many studies, positive results were reported for biomass pre-treatment with a combination of NaOH and hydrogen peroxide solution (Karagöz et al., 2012)\[^3\]. This process is also known as alkaline peroxide oxidation (APO) and promotes the depolymerization of lignin via reacting lignin and related phenolics (Gould, 1984)\[^4\]. This pre-treatment has been successfully applied to corn stover (Selig et al., 2009)\[^5\], cashew apple bagasse (Correia et al., 2013)\[^6\], sugarcane bagasse (Rabelo et al., 2008)\[^7\], rice straw (Patel and Bhatt, 1992)\[^8\], and other biomass (Ayeni et al., 2013)\[^9\].

With more than one million tons in annual production (TUIK, 2013)\[^10\], sunflower is the largest oil seed source for Turkey. After seed harvesting, sunflower stalks can be considered as readily available raw material for bio-ethanol production. Several studies on the effect of pre-treatment methods on the enzymatic hydrolysis of sunflower cellulose have been carried out. In pre-treatment with dilute sulphuric acid, 33 g of glucose and xylose per 100 g of raw material have been produced at the pre-treatment conditions of 167°C and 1.3% sulphuric acid concentration (Ruiz et al., 2013)\[^11\]. In another study, sunflower stalks were pre-treated by steam explosion and sodium hydroxide, and subsequently saccharified enzymatically. Steam explosion at 1.05 kg/cm² pressure for 1.5 h was reported to be the optimum pre-treatment condition for the saccharification of sunflower stalks (Sharma et al., 2002)\[^12\]. Sulphuric acid, hydrochloric acid, maleic acid and acetic acid were used in separating sugars from safflower stalks, and sulphuric acid was found to be the best separation reagent. The optimum hydrolysis parameters of sunflower stalks using sulphuric acid were reported as a 1/30 ratio of solid to acid, reaction time 60 min, and reaction temperature 120°C (Du et al., 2012)\[^13\].

The objective of the current study is to investigate the effect of H\(_2\)O\(_2\) concentration in 1% NaOH solution and of temperature on the composition of pre-treated sunflower stalks and fermentable sugars after enzymatic hydrolysis.

### 2. Material and Methods

#### 2.1 Material

Sunflower stalks used in this study were collected from the Trakya region of Turkey. Raw material was air dried down to 10% moisture. The dried materials were ground and screened with a sieve shaker to obtain particle sizes between 0.224-0.850 mm. Samples were stored in plastic bags at +4°C for future use. Celluclast 1.5 L and Novoyme 188 were purchased from Sigma Aldrich (St. Louis, USA). Aminex HPX 87P column

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was purchased from Bio-Rad Laboratories (California, USA). All chemicals used were standard analytical grades.

2.2 Pre-treatment and enzymatic digestion of sunflower stalk

Dried and milled sunflower stalks were pre-treated with H$_2$O$_2$ (0, 1.25% and 2.5%, v/v) in 1% NaOH solution at different temperatures (30-45-60°C) for 6 h in a water bath. The solid to liquid ratio was 1/20.

Enzymatic hydrolysis was carried out in stoppered conical flasks (50 mL). The pH was adjusted to 4.8 with acetate buffer, and a mixture of cellulase (60 FPU/g of dry biomass) and β-Glucosidase (40 CBU/g of dry biomass) was added to the pre-treated substrate in a total working volume of 20 ml. The hydrolysis reactions were carried out at 50°C in an incubator for 48 h by shaking at 150 rpm. The reactions were stopped in a boiling water bath for 15 min and hydrolysates were clarified by centrifuging at 5000 rpm for 5 min. The supernatants were analysed for glucose and xylose using HPLC.

2.3. Analytical methods

The chemical composition of raw and pre-treated sunflower stalks were determined according to National Renewable Energy Laboratory (NREL) methods (Sluiter et al., 2008a)\[14\]. 0.3 g solid was hydrolysed by 3 mL of 72% (w/w) H$_2$SO$_4$ at 30°C for 60 min then the reaction mixture was diluted to 4% (w/w) and autoclaved at 121°C for 60 min. Lignin was determined by solid residue, cellulose and hemicellulose amounts were determined from filtrate by using High Performance Liquid Chromatography (Agilent 1100). The HPLC system was mainly equipped with a Bio-Rad Aminex HPX-87P column (300 mm × 7.8 mm), and a refractive index detector. The analytical column was operated at 80°C with 0.2 μm filtered HPLC grade water as the mobile phase. The mobile phase flow rate was 0.6 mL/min.

Total reducing sugars were determined by the dinitrosalicylic acid (DNS) method (Miller, 1959).\[15\] Enzyme activity of Celluclast 1.5L® was determined by NREL protocols and reported as Filter Paper Unit (FPU) (Adney and Baker, 2008)\[16\]. One unit of FPU is defined as the amount of enzyme required to liberate 1 μmol of glucose from Whatman no: 1 filter paper per minute at 50°C. One cellobiose unit (CBU) is the amount of enzyme that converts 1 μmol of cellobiose to 2 μmol of glucose per minute. Glucose recovery (%) is calculated as follows:

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\text{Glucose Recovery (mg/g) =} \frac{\text{Amount of glucose produced} \times 0.9}{\text{Amount of cellulose in unpretreated solid}} \times 100
\]

3. Results and Discussion

3.1. Composition of sunflower stalks

The composition (% dry basis) of the sunflower stalks used in this study was shown in Figure 1. Cellulose was the most abundant fraction (32.44±1.96%), followed by hemicellulose (19.12 ± 1.22%) and lignin (15.57 ± 1.18%). These values were consistent with those previously reported for sunflower stalks from other sources (Monlau et al., 2012)\[17\].


Figure 1. Effect of H$_2$O$_2$ concentration on the composition of alkaline pre-treated sunflower stalks at 45°C for 6 hours.
3.2. Effect of peroxide concentration on the composition of pre-treated sunflower composition

The effect of peroxide concentration on the composition of alkaline pre-treated sunflower at 45°C for 6 h is shown in Figure 1. Pre-treatment with the mixture of 1.25% H₂O₂+1% NaOH resulted in an increase in the composition of carbohydrate (cellulose and hemicellulose) content. Cellulose content ranged from 39.50% to 45.46%, and increased by 15% after pre-treatment with 1.25% H₂O₂+1% NaOH mixture compared to the result obtained with 1% NaOH pre-treatment. Similar behaviour was observed in the hemicellulose composition. Hemicellulose content varied between 20.81% and 26.11%. Pre-treatment with 1.25% H₂O₂ + 1% NaOH raised hemicellulose content in the pre-treated solid by 25% compared to results obtained with 1% NaOH. On the other hand, for carbohydrate content, the H₂O₂ addition in the 1% NaOH solution caused a decrease of almost 36% in lignin content compared to its content in the solid pre-treated with 1% NaOH. A further increase of H₂O₂ concentration from 1.25% to 2.50% resulted in a slight decrease of the composition of both carbohydrate and lignin composition.

3.3. Effect of temperature on pre-treated sunflower composition

Figure 2 shows the effect of temperature on the sunflower composition pre-treated with a 1.25% H₂O₂+1% NaOH solution for 6 h. Temperature did not affect the cellulose content but lignin content decreased at 45°C. Cellulose content was around 46% in all samples pre-treated at different temperatures. Hemicellulose content slightly decreased (6%) with temperature increases. However, when temperature was increased from 30°C to 45°C, lignin content dropped by almost 44%.

3.4. Effect of pre-treatment conditions on enzymatic hydrolysis of sunflower stalks

The effects of H₂O₂ concentration and temperature on total reduced content, glucose and xylose content of pre-treated sunflower stalks are shown in Figure 3 and Figure 4, respectively. With the enzyme combination used in this study (a mixture of 60 FPU cellulase/g dry biomass and 40 CBU β-Glucosidase/g dry biomass), both temperature and H₂O₂ concentration and temperature on total reduced content positively impacted on fermentable sugar production after enzymatic hydrolysis. While total reduced sugar content ranged from 579 mg/g to 789 mg/g in the sunflower stalks pre-treated with H₂O₂-alkaline at 45°C for 6 h,
glucose and xylose contents were between 271 mg/g and 318 mg/g, and 71 mg/g and 103 mg/g, respectively. Total reduced sugar in pre-treated biomass increased by 39% when H₂O₂ concentration increased from 0% H₂O₂+1% NaOH to 2.5% H₂O₂+1% NaOH. This increase was only 17% for glucose content; whereas it reached 40% in hemicellulose content. In terms of total sugar content, pre-treatment at 60°C resulted in maximum total sugar of 792 mg/g (Figure 4). However, glucose content did not change when temperature was increased from 45°C to 60°C. Recovery of glucose (g glucose/100 g initial cellulose) was maximal (63%) in the solid pre-treated with 1.25% H₂O₂+1% NaOH at 45°C for 6 h (Figure 5). Further increases in the temperature in the pre-treatment condition resulted in a decrease of the glucose recovery due to dropping solid recovery.

**Conclusion**

The effects of temperature and percentage of H₂O₂ in the alkaline peroxide oxidation pre-treatment process of sunflower stalks after enzymatic hydrolysis showed that extensive lignin degradation (up to 60%) was achieved. An improvement in cellulose content in pre-treated solid occurred after the addition of H₂O₂. The condition of 1.25% H₂O₂+1% NaOH at 45°C for 6 hours resulted in a 63% glucose yield after enzymatic hydrolysis of sunflower stalks. Further research is needed to optimize the process conditions of alkaline oxidation of sunflower stalks.