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Research Article

# Effect of Different Parameters on Enzymatic Hydrolysis of Hazelnut Shells

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ABSTRACT

In the last few decades, the increasing levels of environmental pollution have prompted a shift towards alternative energy sources and biobased solutions, such as lignocellulosic biomass. Lignocellulosic biomass (LB) is primarily derived from plants and is composed mainly of polysaccharides, namely cellulose, hemicellulose, and the aromatic polymer lignin. Hazelnut shells (HS), with a high lignin content of 43%, hemicellulose of 30%, and cellulose of 26%, hold promise as a valuable source of LB. In order to process those LB, lignin and hemicellulose are separated using various treatment methods. However, instead of being used solely for combustion, lignin-containing materials can be valorized for a range of purposes, from biomedical applications to the energy sector. In this study, the enzymatic hydrolysis of HS was conducted over different time periods (24, 48, 72, and 96 hours), at different temperature values with varying enzyme concentrations (0.05, 0.1, and 0.25 mL of cellulase/xylanase enzyme cocktail). To enhance the enzymatic hydrolysis, an alkaline pretreatment method using sodium hydroxide (NaOH) was employed. The results demonstrate that the maximum sugar concentration was achieved at 50°C, after 72 hours, and with a cellulase/xylanase cocktail concentration of 0.1 mL.

## 1. Introduction

Over the past few decades, researchers have come to realize that the use of fossil fuel resources is increasingly posing a threat to Earth's ecosystems through the emission of CO2, resulting in rising sea levels and the potential loss of biodiversity. In the worst-case scenario, given the non-renewable nature of fossil fuels, their depletion could lead to a complete collapse of the global energy security system. However, this problem, along with even more dire scenarios, can be avoided by strategically planning the systematic replacement of fossil fuels with renewable, sustainable, environmentally friendly, and high-energy potential alternatives.

One key element in this transition is the utilization of lignocellulosic biomass (LB), a renewable and abundant resource derived from plants, characterized by its polysaccharide and aromatic polymer composition. LB mainly consists of cellulose, hemicellulose, and lignin [1]. To facilitate effective enzymatic hydrolysis and achieve higher bioconversion of fermentable monosaccharides, it has been reported that a pretreatment step is necessary [2].

This pretreatment aims to weaken the naturally heterogeneous and multi-scale structure of the plant cell wall, which poses a challenge for enzymatic hydrolysis of LB [2, 3]. The choice of suitable reagents for pretreatment depends on achieving higher enzymatic hydrolysis of cellulose while maintaining a moderate cost in comparison to potassium hydroxide (KOH) [4, 5]. Alkaline sodium hydroxide (NaOH) pretreatment is a commonly used reagent that effectively removes lignin, thereby eliminating a major obstacle to the structure's recalcitrance and

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enabling better accessibility of enzymes to cellulose and hemicellulose. Lignin is connected to hemicellulose through lignin carbohydrate complexes (LCC) [6, 7].

In herbaceous biomass, the LCC is composed of arabinoxylan and ferulic acid. The location of ferulic acid may vary depending on the specific lignocellulosic sources, but generally, it forms an ester linkage along the hemicellulose backbone. NaOH pretreatment specifically targets the ether and ester bonds within lignin-carbohydrate complexes [5, 8-12]. Ester bond between ferulic acid and the carbohydrate is highly susceptible to alkaline degradation, as the hydroxide ion (resulting from NaOH dissociation) enhances the rate of hydrolysis compared to water [13-15].

There are studies on enzyme hydrolysis for LB, however the values of temperature, enzyme concentration and operation time have not reached a common ground [16, 17]. Although those parameters depend on the enzyme type, enzyme source, enzyme structure along with the LB structure, the optimization of the parameters is necessary to pave the way for further studies. There are several studies that investigate the effects of temperature, enzyme concentration and operation time of enzymatic hydrolysis for basket willow [18], corn starch [19], cotton stalk [20], sugarcane bagasse [21] and crude cellulose [22].

In this study, the optimization of three different parameters to improve enzymatic hydrolysis was investigated. Enzyme concentration (2, 10, and 20 FPU/g), temperature (30, 40, 50, and 60°C), and operation time (24, 48, 72 and 96h) were individually adjusted using the one-variable-at-a-time (OVAT) approach, and the total sugar analysis was considered as the output for evaluation, corresponding to maximum enzyme activity. This is the first paper that examine the enzyme hydrolysis conditions for hazelnut shells.

## 2. Materials and Methods

### 2.1.Materials

HS were sourced from the local market in Turkey and grinded (Lavion HC-100 Cereal Grinder). Sodium hydroxide (NaOH) was purchased from Sigma-Aldrich Supelco (CAS1310732), while Trichoderma reesei cellulase and Trichoderma viride xylanase were obtained from Sigma-Aldrich with enzyme activities of 700 U/g (CAS9012548) and xylanase activity ranging from 100-300 units/mg (CAS9025574), respectively.

### 2.2. Sample preparation and pre-treatment

For performing alkaline pretreatment, 2.5 g of HS was weighed and dissolved in 2% NaOH-water and NaOH-glycerol solutions. Subsequently, a 0.05 M acetate buffer with a pH of 4.8 was added to stabilize the pH. The mixture was then transferred to falcon tubes and incubated at 60°C for 2 hours. After the incubation period, the samples were centrifuged at 5000 rpm for 5 minutes, and the supernatant was carefully transferred to new pre-weighed falcon tubes. The solid residue obtained from the centrifugation was used for enzymatic hydrolysis.

### 2.3.Enzymatic hydrolysis

For the enzymatic hydrolysis process, 1g of pre-treated HS was added to 50 mL falcon tubes containing 10 mL of 0.05M acetate buffer at pH 4.8. The enzyme cocktail consisting of cellulase, and xylanase was loaded at a volume of 0.1 mL, which corresponds to approximately 20 FPU/g cellulose and xylan. The enzymatic hydrolysis was carried out under different parameters while maintaining a constant stirring speed of 150 rpm. Enzyme concentration (2, 10, and 20 FPU/g), temperature (30, 40, 50, and 60°C), and operation time intervals (24, 48, 72 and 96h) were selected based on a previous study by [1].

Different enzyme loads were tested, including 0.05, 0.1, and 0.25 mL (equivalent to 2, 10, and 20 FPU/g) of the cellulase and xylanase cocktail. Each sample was incubated for 72 hours at 50°C with continuous stirring at 150 rpm. The spectrophotometric analysis was performed at 490 nm to determine the total sugar concentration.

After determining the optimal enzyme concentration, the study proceeded to investigate the effect of different temperatures. The temperatures considered were 30°C, 40°C, 50°C, and 60°C. The parameters held constant during these experiments were a shaking speed of 150 rpm, a hydrolysis time of 72 hours, and an enzyme concentration of 0.1 mL. Furthermore, the impact of different hydrolysis times was examined. The durations tested were 24 hours, 48 hours, 72 hours, and 96 hours. The temperature, enzyme concentration, and shaking speed remained constant at 50°C, 0.1 mL, and 150 rpm, respectively.

### 2.4.Total sugar analysis

To determine the total sugars, phenol-sulfuric acid method was used [23] and a calibration curve was created by measuring increasing glucose concentrations. For each sample, 0.5 mL of the supernatant was transferred to glass test tubes, followed by the addition of 0.5 mL of 5% phenol solution and 2.5 mL of concentrated sulfuric acid ($H_2SO_4$). The tubes were then placed in a water bath for 15 minutes. After cooling down, the samples were analyzed spectrophotometrically at a wavelength of 490 nm. All results are the means of three samples and error bars represent the variability of data. Total sugar analysis was performed as an indirect indicator for specific enzyme activities.

## 3. Results and Discussion

### 3.1. Effect of increasing enzyme concentration

In this study, samples were enzymatically hydrolyzed at 150 rpm, 72 h, with varying enzyme concentrations 0.05 mL, 0.1mL, 0.25 mL and Figure 1 shows the effect of increasing enzyme concentration.

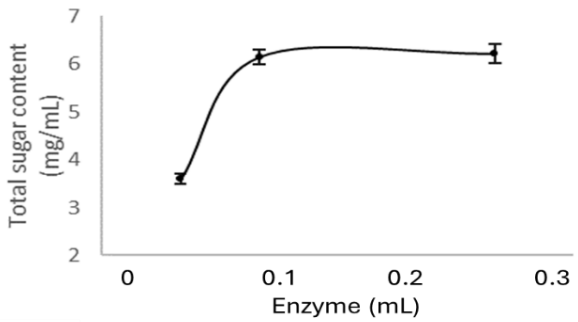


**Figure 1.** Effect of increasing enzyme concentration on enzymatic hydrolysis

Enzyme concentrations between 0.05 and 0.10 mL, the increase of sugar content was very sharp and at higher enzyme concentrations the total sugar content tended to stay constant. Moreover, the higher concentration of 0.25 mL increases the total sugar content slightly. However, in the long term this can be resulted as unnecessary enzyme consumption. Similar results were shown with different LB [24-26].

### 3.2. Effect of temperature

Temperature is an important factor for enzymatic reactions. Generally, cellulase and xylanase operate at different temperatures of 45-55 °C and 60°C, respectively [15]. However, cellulase and xylanase optimum temperatures can vary depending on the biomass structure and source adaptations [25]. Beyond the optimum operation temperatures, enzymes start to denaturate. As shown in Figure 2, temperatures up to 50°C, increasing temperature led to increased sugar release and between 40-50°C, ability to release saccharides of enzyme cocktail exponentially increased. At higher temperatures than 50°C, cellulase enzyme activity restricted due to the denaturation, while xylanase activity relatively increased and maximum concentration of total sugar obtained at 50°C as 11.19 mg/mL.

Figure 2 indicates that a temperature of 50 °C has a substantial impact, as the total sugar concentration observed was approximately 50–80% higher compared to temperatures of 40 °C and 60 °C. Increasing the temperature beyond the optimal range can lead to enzyme denaturation, thereby halting sugar production. Conversely, lowering the temperature below the optimal level may reduce the enzyme's energy, hindering its ability to interact with the substrate. Similar sharp changes in enzyme activity with temperature variations have been documented in other studies [26, 27]. Moreover, in a previous study where the effect of different parameters on enzymatic hydrolysis of wheat straw was reported, the optimum temperature was found to be 55 ºC and the higher and lower temperature values was reported to decrease the enzymatic hydrolysis by 1/3 fold [24].

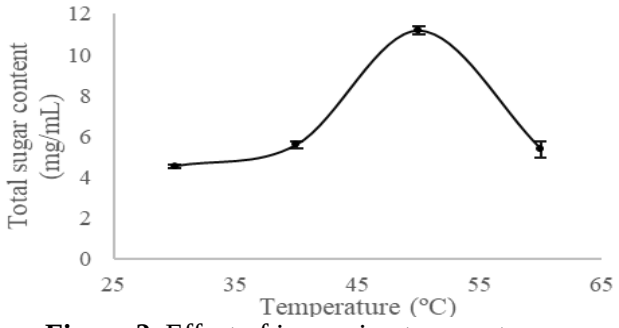


**Figure 2.** Effect of increasing temperature on enzymatic hydrolysis

### 3.3. Effect of different operation times

After the optimum temperature and enzyme concentrations were determined, the pre-treated nutshells mixed with enzyme cocktail was operated for 24 to 96 hours in order to examine the effect of operation time. Results were presented in Figure 3. It was shown that up to 48 h, total sugar concentration was increased two-fold and slightly increased to 11.19 mg/mL at 72h. After the first 72h, total sugar content was gradually decreased which can be explained by the accumulation of hydrolysis products resulting in enzymatic inhibition. Similar results were obtained elsewhere [24] which investigate alkaline pretreatment on wheat straw and enzymatic hydrolysis.

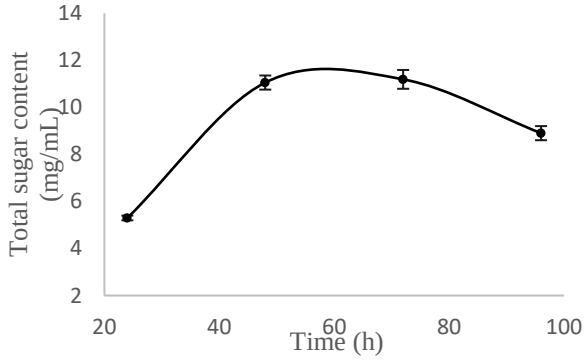


**Figure 3.** Effect of different process time on enzymatic hydrolysis

## 4. Conclusion

Efficient enzymatic hydrolysis achieved by highly accessible substrates to enzymes cellulase and xylanase. Main factor that affects hydrolysis is the accessible surface area of substrates, lignin content of LB and crystallinity. The main purpose of pretreatment is removing lignin and hemicellulose, while doing that, accessible substrate surface of cellulose tremendously increased, crystallinity of cellulose decreased, porosity increased and as a result of this total sugar content increased.

Our results showed that the optimum operation conditions for the alkaline pretreatment integrated enzymatic hydrolysis were 0.10~10FPU/g for 72 h incubation at 50°C while stirring at 150 rpm. This research will serve as a reference guide for further studies on enzymatic hydrolysis of hazelnut shells.

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The authors contributed equally to the study.

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This study does not require ethics committee permission or any special permission.

***The Declaration of Research and Publication Ethics***
The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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