

# Enzymatic Hydrolysis of Olive Industry Solid Waste into Glucose, the Precursor of Bioethanol

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**ABSTRACT:** Olive industry solid waste (OISW) is a by-product generated in the process of olive oil extraction. It is a lignocellulosic material consisting of cellulose, hemicelluloses, lignin and other extractives. In this work, a process for hydrolyzing the OISW into its monomers glucose, the precursor of bioethanol was developed. The hydrolysis process involves two stages: in the first stage the OISW was activated by treating it with a material that opened the cellulose structure and made it more accessible for chemical reagents. Several activating materials were evaluated among them are water, NaOH, Ca(OH)<sub>2</sub> and acetic acid. In the second stage, the OISW was subjected to an enzymatic treatment which hydrolyzed it into sugar. A combination of the two enzymes cellulase (endo-1,4-β-glucanase) and β-glucosidase was used in the hydrolysis. Best hydrolysis results were obtained at enzymes dose of 15.0 mg of cellulase and 20.0 mg/g of β-glucosidase per one gram of OISW at 45 °C and a pH of 4.8. A yield of 85.02% sugar was obtained from the hydrolysis of 10% NaOH pre-treated OISW under a positive pressure of air. Whereas, hydrolysis of cellulose extracted from OISW by the Kraft pulping process and bleached using DEHP beaching sequence produced about 95.30% sugar.

**KEYWORDS:** Cellulose; Bioethanol; Enzyme; "Jeft" olive; Waste; Kraft; Pulping; Bleaching.

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## INTRODUCTION

The olive oil industry represents one of the most economically important agro-food sectors in the Mediterranean and Middle Eastern regions, including Palestine. For example, according to the Palestinian Ministry of Agriculture, the West Bank and Jordan produce approximately 135 thousand metric tons of olive fruits every year [1].

In general, olive mill waste consists of about 44% of Olive Industry Solid Wastes (OISW = pomace, also locally known as "jeft" and 56% of olive industry liquid waste (OILW). These wastes are acidic and have an extremely high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) values [2]. The waste materials pose a challenge in waste management to the olive mills and a concern to environmentalists, for it presents a serious disposal problem [2]. The size and magnitude of the olive production worldwide mean that huge amounts of unexploited agronomic wastes are generated [2], thus posing acute environmental problems in the region.

In many countries, OISW is usually burned or left to rot, thus releasing more CO<sub>2</sub> into the atmosphere, which participates in global warming. On the other hand, the OILW is disposed of via the sewage system or unguided release above ground, which has negative implications on wild life, as well as on both surface and underground water quality. Consequently, olive industry waste is a major problem faced by industrialists in view of increasing environmental standards day by day. In addition to this, the olive industry loses economic value by disposing of solid wastes or selling them for a low price.

The challenge is to utilize and convert these waste materials into the material worth of value.

Fig. 1, summarizes all components of OISW, there are four main components of OISW: extractives, lignin (polyphenols), and carbohydrate (cellulose and hemicelluloses). The main components of OISW are cellulose (24.4- 36.6%), hemicelluloses (14.4-36.6) and lignin (20.3 -43.2). These ingredient of OISW making it potentially attractive, low-cost feed material for bioethanol [3].

Ethanol is a valuable material with an enormous number of applications. The most useful among them is as automotive fuel. The production of ethanol for fuel applications is becoming increasingly important in the world to decrease the reliance on un-renewable fossil fuels. Additionally, there is a growing international

concern about the threat of global climate change. Policy makers across the globe are seeking the most effective methods for reducing the buildup of greenhouse gases, that may cause global climate changes. Their main goal is to promote a cleaner environment and reduce dependence on imported petroleum products. Projects in the area of converting the feedstock to bio-ethanol are funded all over the world. For instance, the US Department of Energy (DOE) is promoting the development of ethanol from lignocellulosic feedstock as an alternative to conventional petroleum-based transportation fuels. The US DOE funds both fundamental and applied research in this area [4].

The production of bio-ethanol from OISW has not been fully explored. None of the published work in this field has resulted in the development of commercial processes for the production of bio-ethanol from OISW [5-7]. In this work, efficient technology for converting OISW into bioethanol was developed.

Lignocellulose biomass should be subjected to the pretreatment stage prior to hydrolysis. The pretreatment stage is important, so as to increase the amorphous region in cellulose, and reduce the amount of hemicellulose and lignin present within the cellulose structure. Several studies have shown that the pretreatment stage greatly affects the rate of cellulose hydrolysis [8]. Pretreatment methods include physical, chemical, and thermal or a combination of the three.

Cellulases are enzymes that are produced by fungi, bacteria, protozoans, plants, and animals. The catalytic effect of cellulases include hydrolyze  $\beta$ -1,4 linking in cellulose chains. The complete hydrolysis process of cellulose required a combination of three types of cellulases: 1- endoglucanases, 2- exoglucanases, 3-  $\beta$ -glucosidase [9].

Endoglucanases is responsible for cleaving internal  $\beta$ -1,4-glycosidic bonds, while exoglucanases plays a role in the release of cellobiose from the reducing and non-reducing ends of cellulose.  $\beta$ -glucosidase has a vital role in the cellulolytic process by hydrolyzing cellobiose molecules to produce glucose monomers and reducing cellobiose's inhibition of endoglucanase and exoglucanase enzymes [10].

In this study, the main component of the "jeft" which is cellulose will be hydrolyzed into its repeat unit glucose the precursor for bioethanol.

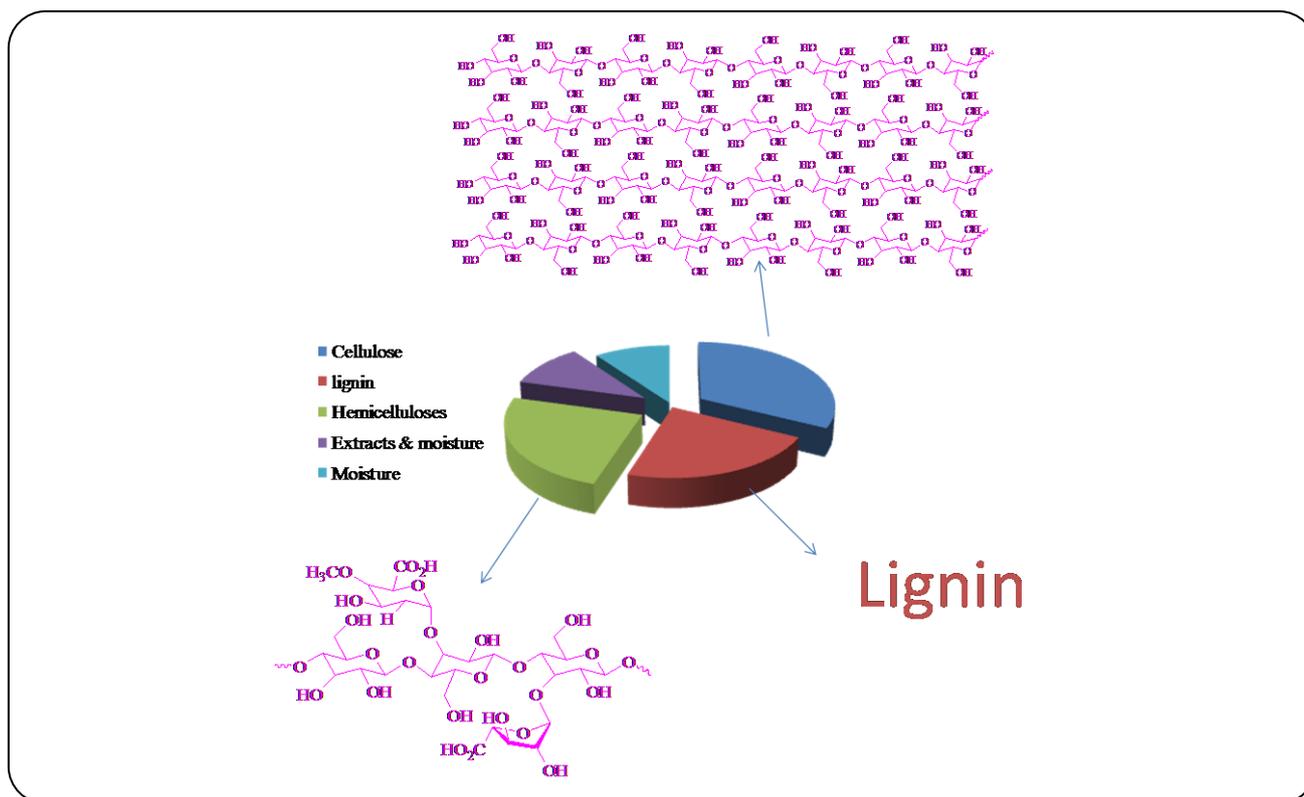


Fig. 1: components of OISW.

The primary objective of this work is to define the optimum enzymatic conditions for conversion of olive waste ("jeft") or cellulose extracted from "jeft" into the monomeric sugar glucose. In this project the following process for conversion of "jeft" or cellulose to glucose will be evaluated. The process involves several treatments that together form a unique reaction network.

## EXPERIMENTAL SECTION

### General experimental

All reagents were purchased from the Aldrich Chemical Company and were used without any further purification unless otherwise specified. Kraft pulping and prehydrolysis treatment were performed using a high Parr Reactor model: Buchiglasuste, bmd 300. OISW used in this work was obtained from an olive factory from Tulkarm city in Palestine and stored in a freezer at about  $-5^{\circ}\text{C}$ .

Sugar concentration was performed using a calibrated blood glucose meter with the brand name ACCU-CHEK® perform Nano. It is a trademark of the Roche

Company, made in the USA. A calibration curve was prepared according to the following procedure: A series of standard solutions of sugar ranging from concentrations 300.0 mg/dl to 700 mg/dl were prepared and represented as Actual Sugar Concentration (ASC). The solutions were analyzed by a blood glucose meter to determine their sugar concentrations and represented as Measured Sugar Concentration (MSC). The calibration curve was generated by plotting Actual Sugar Concentration (ASC) against Measured Sugar Concentration (MSC). The calibration curve was used to overcome the error margin in the meter which was about 13.0%.

All OISW and cellulose samples were washed with deionized water to neutral pH before use.

### Effect of enzyme concentration on the rate of hydrolysis

The enzyme concentrations that produced the highest rate of hydrolysis for OISW pre-hydrolyzed with water (1:4 ratio) was determined to be 15.0 mg/g OISW of cellulase enzyme and 20.0 mg/ g OISW. Using these doses of the enzyme, a 32% conversion was achieved after about 8.0 hr.

### Hydrolysis of olive industry solid waste into glucose

#### Pre-hydrolysis of OISW

A suspension of OISW and the pre-hydrolysis agent in water (10% NaOH(aq), 10% Ca(OH)<sub>2</sub> (aq) or 2% AcOH(aq) in a 1:1) was prepared in a reactor vessel (1L). The reactor was sealed, pressurized to 50 psi using N<sub>2</sub> and heated. The temperature was raised to 160 °C in about 30 min and maintained at this temperature for 1 h. The suspension was then cooled to room temperature, filtered by suction filtration, washed with distilled water (3 x 200 mL) and stored in a plastic bag in the refrigerator for future use.

#### Enzymatic hydrolysis pre-hydrolyzed OISW

The hydrolysis was performed using the enzymes cellulase (endo-1,4-β-glucanase) and β-glucosidase in a buffer acetate solution with a pH of 4.8 at a 10% consistency. The buffer solution was prepared by dissolving 2 g of sodium acetate salt in 100 mL distilled water and adding acetic acid until a pH of 4.8 was reached.

A sample of prehydrolyzed OISW (10.0 g) was suspended in 90 mL of buffer solution in a 250 mL glass bottle. The required amount of enzymes cellulase and β-glucosidase was then added to the mixture. The bottle was capped and clamped to a shaker and placed in a water bath at about 45 °C. A sample of the mixture was withdrawn from the suspension after 2, 4, 6 and 8 h, placed in a test tube, centrifuged to remove the suspension and the sugar content was determined using a blood glucose meter and the prepared calibration curve.

#### Extraction of cellulose form OISW

##### Kraft pulping

The pulping process followed in this work is reported previously by our laboratory. [11].

##### Soxhlet Extraction of OISW

Soxhlet extraction was performed on OISW (200.0 g, OD weight 80%) to remove extractives such as residual olive oil. The extraction was carried out for 4 hr using ethyl acetate (500 mL).

##### Kraft pulping

Kraft pulping was conducted in a high Parr Reactor of one Liter capacity. The pulping conditions are shown in Table 1.

Table 1: Pulping conditions.

Pulping variable	
Cooking liquor to OISW ratio	4:1
Cooking Temperature	160 °C
Temperature rising time	30 min
Holding time	90 min
Pressure	50 psi
% Active alkali charge	12.5
%sulfidity	8

Active alkali charge [NaOH+Na<sub>2</sub>S], and sulfidity [Na<sub>2</sub>S/(NaOH+Na<sub>2</sub>S)], where expressed as g/L Na<sub>2</sub>O. At the end of cooking, the produced pulp (brown cellulose) was collected by suction filtration, washed several times with water, air dried at room temperature, and stored in plastic bags for further use.

##### Pulp Bleaching

Obtained brown cellulose was subjected to bleaching using the sequence DEHP. The individual stages of the bleaching sequence were carried out as shown below. After each stage the produced cellulose was collected by suction filtration, washed with water until neutralization and used in the next stage without drying.

D-stage: Conducted at 10% consistency in a plastic bag for 1.0 hr at 70 °C. Reagent used was 1.0% ClO<sub>2</sub> (based on pulp weight), with an end pH of approximately 2.5.

E-stage: Conducted in a plastic bag at 10% consistency for 90 min at 60 °C using 5% NaOH (5% based on pulp weight). After the completion of the treatment the produced pulp was filtered and washed several times with water until the neutral filtrate was obtained.

H-stage: Conducted in a plastic bag at 10% consistency for 60 min at 60 °C and at a pH of 10. Hypochlorite charge of 2.5% based on pulp weight. NaClO was obtained from a stock solution that contained 5% of NaClO.

P-stage: Conducted in a plastic bag at 10% consistency, for 60 min, at 60 °C and a pH of 9 to 11 and with 2% H<sub>2</sub>O<sub>2</sub>, 0.5% MgSO<sub>4</sub>.7H<sub>2</sub>O, and 3.0% NaOH (based on pulp weight).

##### Hydrolysis of extracted cellulose from OISW into glucose

The pre-hydrolysis and hydrolysis processes of cellulose extracted from OISW to glucose was performed as shown above

**Hydrolysis of OISW under a positive pressure of air**

To a 100.0 g of OISW pre-treated with 10% NaOH (OD wt. 80.0 g) or pre-treated with only water in a reactor vessel (1L, described previously), 400.0 mL of the buffer solution (pH 4.8) was added, followed by the two enzymes. The reactor was sealed, pressurized to 5 psi with air and mixed at a low speed for the time shown in table 2.49. The product was filtered and analyzed for sugar content.

**RESULT AND DISCUSSION**

Cellulose hydrolysis means depolymerization of cellulose polymer into glucose monomers. Cellulose hydrolysis can be carried out using several methods such as acid hydrolysis and enzymatic hydrolysis.

Cellulose consists of polymeric chains that associate with each other through H-bonding. It consists of two regions: amorphous and crystalline. The amorphous region is the accessible part of the cellulose structure. However, the crystalline region is not accessible by mild reagents. Therefore, complete hydrolysis of the cellulose chain into its monomeric glucose must be preceded by an activation stage to convert the crystalline region into an amorphous region. In this work, the enzymatic hydrolysis stage was preceded by an activation stage. Several reagents were evaluated at the activation stage, such as water, sodium hydroxide, calcium hydroxide and acetic acid. The activation stage was called in this manuscript as a "pre-hydrolysis stage". The pre-hydrolysis stage was followed with enzymatic treatment.

The enzymatic hydrolysis was performed on OISW and on cellulose extracted from OISW using published method [12]. It was carried out at 45°C in a thermostat water bath at a pH of 4.8. Sugar content after hydrolysis was measured using the blood glucose meter, described in the experimental part. The exact concentration of produced sugar was determined from a pre-prepared calibration curve.

The effects of several variables such as enzyme concentrations, hydrolysis time, pre-hydrolysis agents, consistency and pressure on the rate of hydrolysis were evaluated to determine optimum cellulose hydrolysis conditions.

The percent of conversion to glucose was calculated using the following equation:

$$\% \text{ conversion to glucose} = (\text{concentration of glucose (mg/dl)} * V \text{ (dl)}) / (\text{oven dry weight of OISW (g)} * \% \text{ cellulose in OISW}).$$

V represents of the volume reaction solution in dl, % cellulose present in the OISW was determined to be about 45% [13].

**Hydrolysis of OISW pre-activated with water**

Samples of OISW activated with water were subjected to enzymatic hydrolysis over a time period of 8.0 hr. Enzymes dose was 3.0 mg cellulase and 4.0 mg  $\beta$ -glucosidase per gram OISW. Results are shown in Fig. 2. In Fig. 1, three curves are shown which represent different volumes of water used in prehydrolysis. As shown in Fig. 2, a sharp increase in the rate of hydrolysis represented by percent of glucose was shown in the first 6 hr period of hydrolysis, then it almost ceased. The sharp increase in the hydrolysis of OISW sample treated with four times its weight water started immediately. All samples showed an almost equal percentage of conversion to sugar, as shown in Fig.1. So, the amount of water used in the pre-activation stage showed no effect on the amount of glucose produced, which indicated that the crystalline region was not affected by water treatment.

**Hydrolysis OISW pretreated with Sodium Hydroxide**

Samples of OISW were pre-hydrolyzed with aqueous solutions of NaOH at various concentrations: 1%, 2.5%, 5%, 7.5%, and 10%. The samples of OISW were hydrolyzed as above using 15.0 mg cellulose and 20.0 mg  $\beta$ -glucosidase per one gram of OISW at 45 °C for 2, 4, 6 and 8 hr period. The results are summarized in Fig. 2. Results show that the OISW sample pre-hydrolyzed with 10% NaOH showed the highest percent of conversion. Samples of OISW pre-hydrolyzed with 1% NaOH showed a glucose concentration of 363 mg/dl, while the one pre-hydrolyzed with 10% NaOH showed a glucose concentration of about 512 mg/dl after 8 h. This concentration of glucose represents about 53% and 85.0 % of conversion into glucose, respectively.

As shown in Fig. 3, in case of a hydrolysis time of 6 h NaOH solution with 5% concentration could be used.

**Hydrolysis of calcium Hydroxide-treated OISW samples**

Five solution of  $\text{Ca(OH)}_2$  with different concentration: 1%, 2.5%, 5%, 7.5%, 10% were used

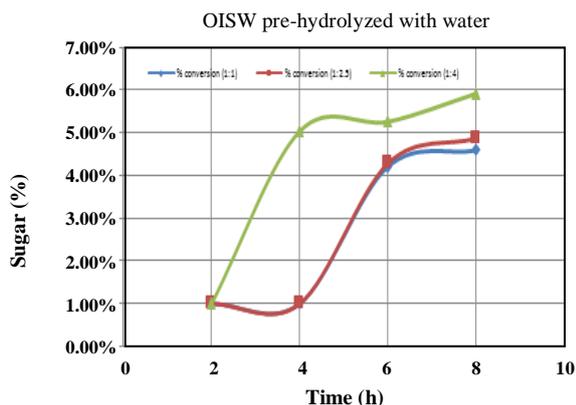


Fig. 2: The percent conversion of OISW pretreated with water at three different ratios.

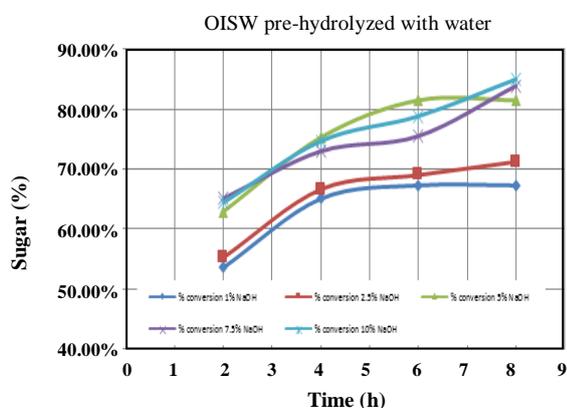


Fig. 3: The percent of glucose obtained from the hydrolysis of OISW samples pre-treated with NaOH.

in the pre-hydrolysis of OISW. The treatment was performed in buffered solutions as above, using 15.0 mg of cellulase and 20.0 mg  $\beta$ -glucosidase per one gram of OISW at a temperature of 45 °C for 2, 4, 6 and 8 hr time periods. The results are summarized in Fig. 4 show that the hydrolysis ceased after about 6.0 hr, except for sample pre-treated with 7.5%  $\text{Ca}(\text{OH})_2$ , after 8 hr the hydrolysis continued at a low rate. However for the OISW sample treated with 10% calcium hydroxide showed the highest concentration of glucose: 363.0 mg/dL after 8 h of hydrolysis, which represented a conversion of about 75%. A sample of OISW pre-hydrolyzed with 10% NaOH showed 10% more conversion, indicating that NaOH is more effective in increasing the accessibility within the cellulose structure.

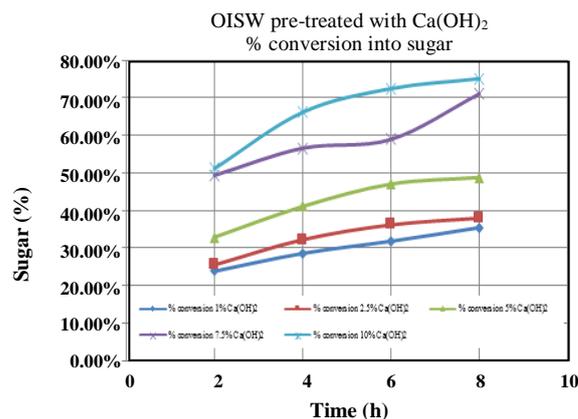


Fig. 4: The percent of glucose obtained from the hydrolysis of OISW samples pre-treated with various concentrations of  $\text{Ca}(\text{OH})_2$  solution.

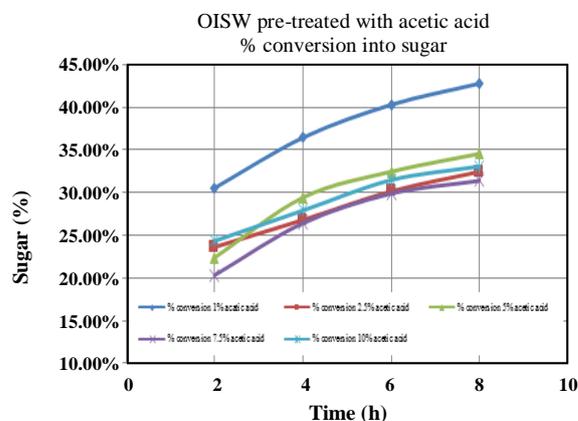


Fig. 5: The percent conversion of OISW pre-hydrolyzed with acetic acid.

#### Hydrolysis of OISW pre-hydrolyzed with acetic acid

The prehydrolysis was carried out at various concentrations of acetic acid in water to determine the optimum concentrations for prehydrolysis. The hydrolysis was again conducted using 15.0 mg cellulase and 20.0 mg  $\beta$ -glucosidase per one gram of OISW at 45 °C for 2, 4, 6 and 8 h periods. The results are shown in Fig. 5. As shown in Fig. 4, pre-hydrolysis with acetic acid showed a slight improvement in the rate of hydrolysis when compared to OISW samples pre-activated with only water. A concentration of 196 mg/dl of glucose was achieved using OISW pre-hydrolyzed with 7.5% acetic acid solution. The glucose concentration represents about 43% conversion.

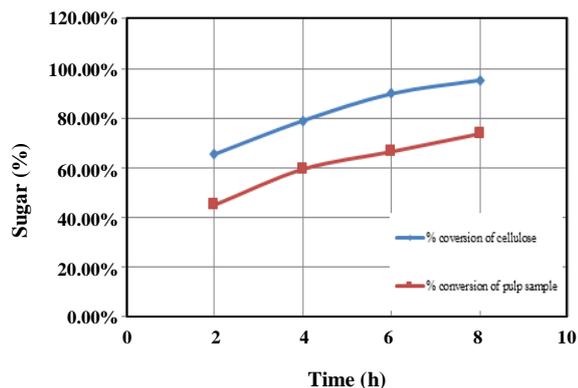


Fig. 6: Hydrolysis results of pulp and cellulose extracted from OISW.

#### Hydrolysis of cellulose extracted from OISW

A sample of cellulose (pulp) was extracted using the Kraft pulping process and subjected to hydrolysis with enzymes as is without any further purification. A second sample was bleached using the bleaching sequence DEHP (chlorine dioxide cold caustic extraction, hypochlorite) [11], the sample was called cellulose. Both samples pulp and cellulose were pre-hydrolyzed by treatment with 10% NaOH, as shown in the experimental section. The two samples were hydrolyzed for various periods of time using 15.0 mg cellulase and 20.0 mg  $\beta$ -glucosidase per one gram cellulose or pulp. The results are summarized in Fig. 6. The produced graphs show that the conversion into cellulose reached about 95% in 6 hr of hydrolysis. While the pulp sample showed a conversion of about 77.0%. The results are in agreement with literature observation that, hemicelluloses and lignin act as a barrier preventing the enzymes to reach cellulose. The barrier was removed by pulping and bleaching process making cellulose more accessible [14].

#### Hydrolysis of OISW under a pressure of air

The hydrolysis of OISW was also evaluated under a positive pressure of air. Two samples of OISW pre-hydrolyzed with water and 10% NaOH were used in this study. The hydrolysis was carried out in a reactor vessel under an air pressure of about 10 psi at 45°C. The treatment was performed in a buffer solution using 15.0 mg cellulase and 20.0 mg  $\beta$ -glucosidase per gram OISW for various periods of time. The results are plotted as shown in Fig. 7. As shown in Fig. 6, the concentration of

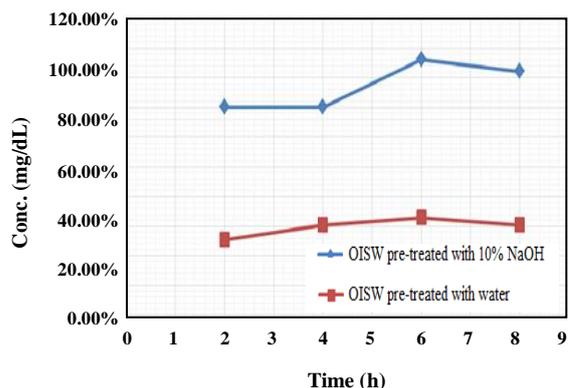


Fig. 7: The percent of glucose obtained from hydrolysis of OISW under an air pressure of 10 psi.

sugar after about 6 hr hydrolysis was 530.0 mg/dl and 260.0 mg/dl from OISW preactivated with 10% NaOH and water, respectively. The concentrations represent 85.02% and 52.2% conversion.

A reported study showed that enzymatic hydrolysis that maximum yield of glucose was about 70% [15].

The results indicate that the hydrolysis could be enhanced and becomes quantitative by running it under apposite pressure of air [15-20]. The results show that purification of OISW could be avoided by carrying out the hydrolysis under a positive pressure of air. Little improvement was obtained by running the hydrolysis under a pressure of 5 psi and 20 psi.

#### CONCLUSIONS

Biomass residues available from the olive industry (OISW) constitute a potential source for production of chemicals, such as bioethanol and furfural, using acid-catalyzed hydrolysis or enzymes. Olive industry solid waste constitutes about 40-45% cellulose. A process for hydrolyzing cellulose present in OISW to sugar was developed. In the process, the OISW went through a pre-hydrolysis stage. This stage is required to break down the hemicellulose to sugars, lignin into water-soluble low MW polymers and monomers. The stage is also required to open up the structure and make it more accessible for reagents. The pre-hydrolyzed OISW was then subjected to enzymatic hydrolysis using a combination of two enzymes: cellulase and  $\beta$ -glucosidase. The pre-hydrolysis as well the hydrolysis stage was performed under various reaction conditions to determine the optimum condition

for the conversion of cellulose material to the glucose sugar. Results showed that running the hydrolysis under a positive pressure of air enhanced the enzymes' activities and a conversion equal to that obtained from pure cellulose. The highest % of glucose was obtained from OISW sample pre-hydrolyzed with 7.5% NaOH and hydrolyzed at 45°C for 6 h and at a pH of 4.8. The enzyme concentration used in the hydrolysis of cellulase was 15.0 mg and  $\beta$ -glucosidase 20.0 mg per gram of OISW. Quantitative hydrolysis (95.3% conversion) was achieved using cellulose extracted from OISW using the kraft method.

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