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Bioethanol production from lettuce leaves waste by using *Saccharomyces cerevisiae*

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Abstract

An investigation was conducted to examine the potential for producing bioethanol from lettuce leaves waste through fermentation utilizing Saccharomyces cerevisiae. In this study three different of pre-treatments methods were applied to the raw materials using H_2SO_4 (2%), HCl (5%) and NaOH (1%), respectively. The goal of these pretreatments was to break down the cellulose, hemicellulose as well as lignin to a mono-sugar that considered a simpler form which can be consumed directly through the fermentation process by *S. cerevisiae* to produce bioethanol. The results indicate that the NaOH pre-treatment yielded the highest sugar content, followed by the HCL, H_2SO_4 pre-treatment, while the untreated samples had the lowest sugar content. The concentrations of ethanol were reported every two days through the fermentation period by using ethanol sensor and the highest ethanol was measured for the samples pre-treated with HCL, followed by samples without pre-treatment, samples pre-treated with H_2SO_4, and finally, the NaOH-pre-treated samples, which had the lowest ethanol concentration, as measured by an ethanol sensor every two days during the fermentation period. The concentrations of ethanol were also measured by using the High-Performance Liquid Chromatography (HPLC) technique after the first and second distillation, the distillation process was carried out in order to enhance the purity of the bioethanol.

Keywords: Renewable energy, bioethanol, fermentation, lettuce, *Saccharomyces cerevisiae.*

إنتاج الوقود الحيوي من فضلات أوراق الخس بأستخدام خميرة العجين

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الخلاصة

تم إجراء بحث لفحص إمكانية إنتاج الإيثانول الحيوي من مخلفات أوراق الخس من خلال التخمير باستخدام خميرة العجين الجافة. في هذه الدراسة تم استخدام ثلاث أنواع مختلفة من طرق المعاملة للمادة الأولية تضمنت استخدام حامض الكبريتيك (2%) وحامض الهيدروكلوريك (5%) وهيدروكسيد الصوديوم على

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التوالي. ان الهدف من هذه المعاملات هو تحطيم مركبات السليلوز والهيميسليلوز واللكنين الى سكريات أحادية والتي تعد الشكل الابسط الذي تستطيع الخمائر استهلاكه وتحويله الى ايثانول حيوي. بينت النتائج ان أفضل طريقة للمعاملة كانت استخدام هيدروكسيد الصوديوم حيث أعطت اعلى كمية من السكر وبعدها كانت المعاملة بحامض الهيدروكلوريك وحامض الكبريتيك في حين أعطت العينات التي بدون معاملة اقل كمية من السكر. تم قياس الايثانول المنتج خلال كل يومين من التجربة باستخدام حساس الايثانول حيث وجد ان اعلى قيمة سجلت للعينات المعاملة بحامض الكبريتيك والعينات بدون معاملة واقل منها للعينات التي تم معاملتها بحامض الهيدروكلوريك واقلها للعينات المعاملة بهيدروكسيد الصوديوم. وتم أيضا قياس الايثانول المنتج باستخدام تقنية الكروماتوغرافيا السائل عالية الدقة بعد عملية التقطير الأولى والثانية التي أجريت لزيادة نقاوة الايثانول المنتج.

1. Introduction

The modern world has an insatiable demand for energy, which has led to a significant reliance on fossil fuels to meet this need. However, this has resulted in the release of large amounts of greenhouse gases, particularly CO2, which is widely regarded as the primary contributor to global warming and climate change [1]. That's coupled with increasing concerns about depleting the fossil fuel sources in the future which motivates the researchers toward finding an alternative, renewable, eco-friendly and biobased fuel, that can be used to meet the required energy for daily life, industry [2]. One of these promising alternatives is bioethanol which can offer a clean and sustainable alternative for the fossil fuel and it can be formed by utilized the lignocellulose material through fermentation process [3]. The lignocellulose considered a promising feedstock for producing bioethanol, is gaining increasing attention from researchers, industry as well as from the energy production sector, as producing bioethanol through the fermentation of lignocellulosic materials can minimize the dependence on the fossil fuel furthermore minimize the pollution which resulted from consuming of traditional fossil fuel [4]. The production of bioethanol from lignocellulose is consider as the second generation of bioethanol since it used feed stock, and the importance of this method that it does not compete with human importance corps such as sugar can and corn which were used in the production of the first generation of bioethanol [5].

The production of bioethanol can be carried out by microorganisms through the alcoholic fermentation of sugar found in the biomass [6]. *S. cerevisiae* is considered as the most common microorganism used for the production of bioethanol since that it considers as a non-pathogenic microorganism, available and has a simple requirement which allow the grow the yeast on a simple media [7, 8]. Numerous studies have explored the potential of producing bioethanol by using *S. cerevisiae* from different biomasses such as plant juice, sugarcane and plant waste [9-11]. However, these feed stocks are considered as an important and costly crop, while the present study use the organic waste as feed stock.

This study was conducted to investigate the ability of *S. cerevisiae* to produce bioethanol from organic wastes (lettuce leaves) through fermentation process, as well as testing the ability of different pre-treatment methods (acidic and alkaline pre-treatment) for enhancing the breakdown of cellulose and hemicellulose materials and increase the release of reduce sugar that can be used for ethanol production. The importance of this study is that it serves two significant purposes which are the removing the organic waste and at the same time use theses wastes for the production of bioethanol which is an important chemical that is used widely.

2. Materials and Methods

2.1. Collection and Processing of Samples

Lettuce leaves waste was collected from AL-Rasheed grand vegetables market in Baghdad/Iraq. The collected samples were rinsed with tap water to remove any impurities, and subsequently, its were dried in open air. Following drying, the samples were grinded by an electrical mixer (Gosonic, China) to obtain a fine powder. The powder was kept in polyethylene bags in the refrigerator (Concord, Lebanon) at 4ºC until it was used [12].

2.2 Experimental Work

2.2.1 pre-treatment of the lettuce leaves

The study focused on the use of lettuce leaves in two distinct scenarios: with and without pre-treatment. The pre-treatment was used to break down the complex cellulosic, hemicellulose and lignin compounds to reduce sugar that can be used by *S. cerevisiae*, acidic and alkaline pre-treatment was used and compared with the samples without pre-treatment as follow:

2.2.1.1 Without pre-treatment

Fifty gram of dried lettuce leaves powder were weighted and then soaked in 1000 ml of distilled water in a conical flask, after that it was used directly in the fermentation process [11].

2.2.1.2 The pre-treatment processes

2.2.1.2.1 Pre-treatment of lettuce leaves by sulfuric acid (H_2SO_4)

Fifty grams of dried lettuce leaves powder was weighted, and then 500 mL of diluted sulfuric acid (2%) (J.T. Baker) was added and mixed thoroughly in the flask, after that the flask was placed on a hot plate stirrer (Labinco, Netherland) at 130°C with 500 rpm for 60 minutes [13].

2.2.1.2.2 Pre-treatment of lettuce leaves by hydrochloric acid (HCL)

Fifty grams of dried lettuce leaves powder was weighted, and then 500 mL of diluted hydrochloric acid (5%) (J.T. Baker) was added and mixed thoroughly in the flask, after that the flask was placed on a hot plate stirrer (Labinco, Netherland) at 130°C for 120 minutes and 500 rpm [14].

2.2.1.2.3 Pre-treatment of lettuce leaves by sodium hydroxide (NaOH)

Fifty grams of dried lettuce leaves powder was weighted, and then 500 mL of diluted sodium hydroxide (1%) (SDFCL) in a flask, then it was left at room temperature for 180 minutes [15].

After pre-treatment, the samples were filtered to remove any remaining impurities. The volume of each sample was completed to 1000 mL with distilled water. The pH value was set at 5, then the four samples (with and without pre-treatment) were ready to undergo the fermentation process [16].

2.2.2 Fermentation Process

The samples with pre-treatment and without pre-treatment, were subjected to autoclaving (LabTech, Korea). Under sterile conditions, activated *S. cerevisiae* inoculum was added to each sample in sterilized conditions. The pH of prepared substrate fermentation was then adjusted to 5 [17]. The fermenters were kept airtight to ensure that the fermentation condition was anaerobic, after that, the samples were incubated for seven days. At two-day intervals, both sugar content and ethanol concentration were measured every two days.

2.2.4 Distillation Process

At the end of the fermentation process the samples were distilled to sperate the ethanol from the solution by passing the solution through a condenser column depending on the variation between water and ethanol boiling point [18]. The fermented solution was then heated using a digital heating mantle, causing the ethanol to vaporize, which was subsequently condensed and collected. Then, a second distillation was carried out to increase ethanol purity.

2.3 Measuring Sugar Content

The phenol-sulfuric acid method was used in this study to measure sugar content of each sample. At first, 10 mL of each sample was collected and centrifuged (Hettich, Germany). Then, 1 mL of phenol reagent (5%) with 5 ml of concentrated H_2SO_4 was added to 1 mL of the supernatant of each sample and mixed well. After that, the mixture was kept in a water bath for 30 minutes at 30°C, then sample concentration was measured using a spectrophotometer (CARY 100 Conc /Australia) at λmax 490 nm [19].

2.4 Ethanol Measurement

The ethanol concentration of each sample was measured by using two methods, as follows:

1-Daily measuring ethanol vapor using a Lab Quest 3 ethanol sensor (Vernier, Germany) [20].

2-After each distillation process, the concentration ethanol was measured using high performance liquid chromatography (HPLC) by direct injection (Sykam, Germany) technique [21].

3. Results and Discussion

The initial step involved measuring the amount of sugar present in the samples, as depicted in Table 1.

Table 1: The amount of sugar in each sample through the experiment period

Days	Amount of sugar (mg/ml)			
	Without pretreatment	Pretreatment with H_2SO_4	Pretreatment with HCl	Pretreatment with NaOH
Day zero	5.085	6.292	7.732	12.795
Day1	5.205	5.415	4.755	11.535
Day 3	4.23	4.05	1.965	10.29
Day 5	3.187	1.897	1.86	9.592
Day 7	2.7	1.79	1.83	9.097

As demonstrated in the Table 1, the amount of sugar released from the sample differ significantly with change in the pre-treatment, and the lowest was without pre-treatment and a little bit higher when treated with H_2SO_4 followed by the treatment with HCl and much higher when treated with NaOH, since that NaOH is the most convenient method for the pre-treatment through the breakdown of inner bond and the C-C bond which could enhance the breakdown of the cellulose and the hemicellulose and lignin to mono sugar that can be consumed by the *S. cerevisiae* with less inhibitors than other methods [15]. These study's results are similar to the findings of Johannes and Xuan [22], who reported that alkali pre-treatment (NaOH in specific) was much higher than the results of acidic pre-treatment. Additionally, the results of the Wang *et al.* [23] showed that the pre-treatment with NaOH was highly effective in the removal of lignin from both wheat straw and corn straw.

Even though that the NaOH pre-treatment give the highest amount of sugar but the consume of the sugar was low due to the fact the optimum pH value for the *S. cerevisiae* is in the acidic condition while the NaOH pre-treatment shift the media to the alkaline state which slow the growth the of the yeast. The present study results also agree with the findings of Wong and Sanggari [24] who reported that the optimum pH value for the *S. cerevisiae* growth was between 4 to 6.

On the other hand, the ethanol vaper concentration was measured daily by using the ethanol sensor as shown in figure 1. It must be explained here that the readings of the ethanol sensor are depend on predicting the ethanol value in the vapor above the solution during fermentation period, so it's an estimated value that are used as indicator for the beginning and ending of fermentation process, while the exact concentrations of the ethanol were determine by using HPLC technique which measure the amount of ethanol that actually exist in the solution.

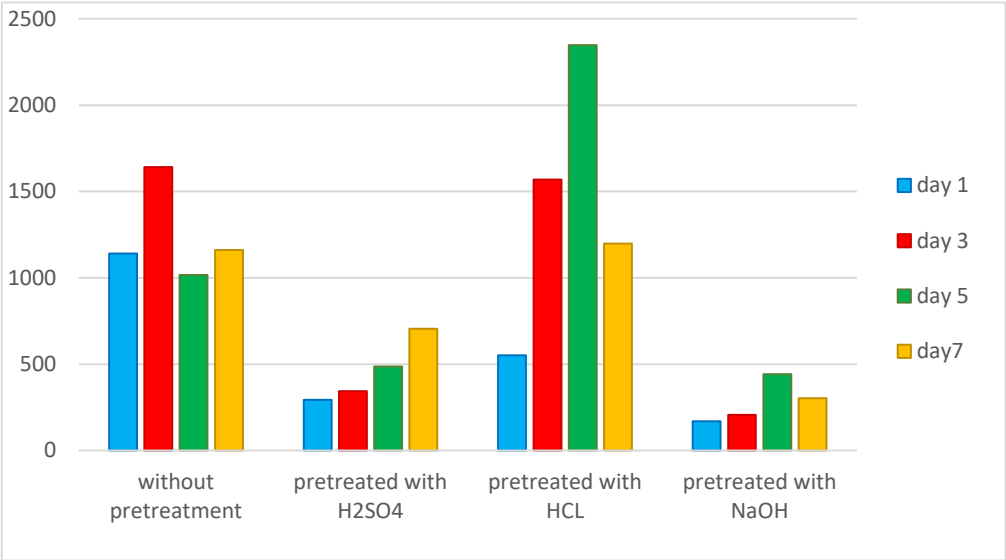

Figure 1: The ethanol concentration measured by using the ethanol sensor through the study period

As illustrated in Table 2, the highest ethanol concentration was measured in the samples that were pre-treated with HCl at day 5. These study results are in agreement with the results of Hidayati [25] who found that the production of bioethanol from breadfruit using *S. cerevisiae* was increase when the cellulosic compounds were pre-treated with HCL. As well as, the findings of Hyder and Mahmoode [26] who found that the HCL pre-treatment give a higher ethanol production than the pre-treatment with H_2SO_4, while the results disagree with the results of Johannes and Xuan [22] who used both H_2SO_4 and NaOH for the pre-treatment of Perennial grasses for bioethanol production but they found that the bioethanol produced by the samples pre-treated with NaOH give a higher ethanol amount than the samples pre-treated with H_2SO_4.

The ethanol concentrations were also measured in the end of the experiment after the first and the second distillation process as shown in table 3. The measurements obtained by the HPLC are shown in Figures (2-5) which represent the actual ethanol readings of the samples in HPLC.

Table 3: The ethanol concentration measured by the HPLC technique after the 1st and 2nd distillation processes.

Distillation process	Ethanol concentrations (ppm)			
	Without pre-treatment	Pre-treatment with H_2SO_4	Pre-treatment with HCl	Pre-treatment with NaOH
1St distillation	38.85	60.06	51.81	48.95
2nd distillation	38.29	60.08	54.19	44.30

For the mode of action of both acidic and alkaline pre-treatment, it can be explained by the fact that the Acidic pre-treatment acts on degrading the hemicellulose as well as breakdown of the cellulose crystallization by affecting the bonds (covalent and hydrogen bonds) and van der Waals forces that keep the cellulose, lignin and hemicellulose intact. On the other hand, alkaline pre-treatment affects the cell wall integrity through the dissolution of the polysaccharides and causes swelling, which subsequently removes the lignin and that leads to the increase of both porosity and surface area of the biomass and a decrease the polymerization degree [22].

The process of dehydration was done in order to purify the resulted ethanol and decrease the amount of water presented [27] In general the highest concentration of ethanol was resulted from the samples pre-treated with H_2SO_4 followed by HCl and NaOH and the lowest was in the samples without pre-treatment. That could be due to the fact pre-treatment allows the breakdown of the lignocellulose and cellulosic compounds int sugar monomers that can be converted to bioethanol [28]. The results of this study were in agreement with the findings of Tulahie *et al.* [27] who found that when they treated sawdust with H_2SO_4 and NaOH give the highest bioethanol concentration. And the results of Ochaikul *et al.* [29] who found that the ethanol produce from the water hyacinth after acidic pre-treatment with H_2SO_4 was slightly higher that resulted from pre-treating with NaOH. As well as it agrees with the findings of Shukla *et al.* [30] who found that between the different acidic pre-treatment, the use of sulfuric acid was the best acid pre-treatment from both effectiveness and economic point of view.

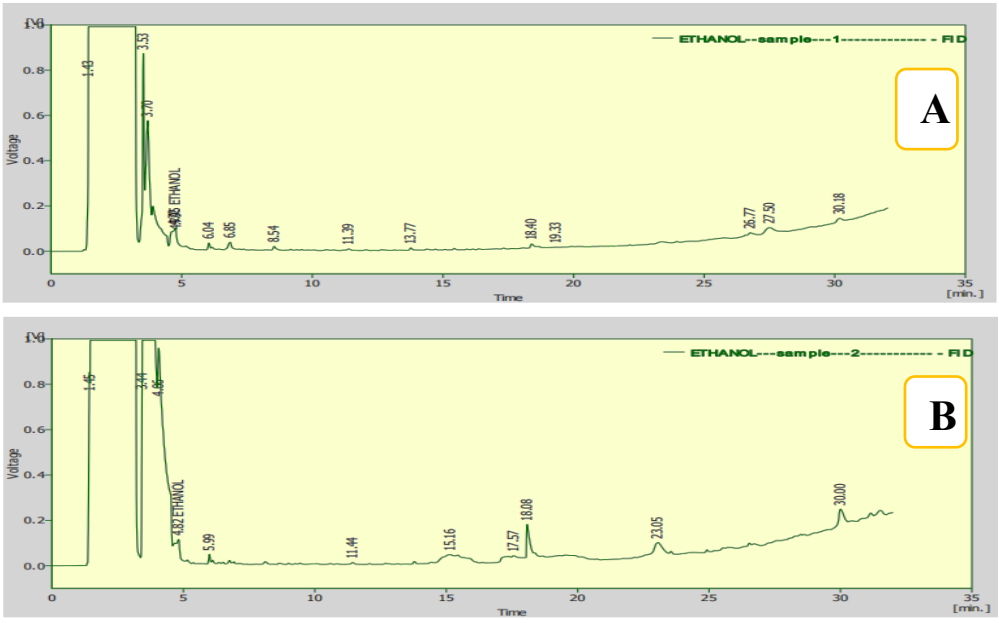

Figure 2: The ethanol measured by the HPLC produced by S. *cerevisiae* from lettuce leaves without pre-treatment (A) after 1st distillation (B) after 2nd distillation

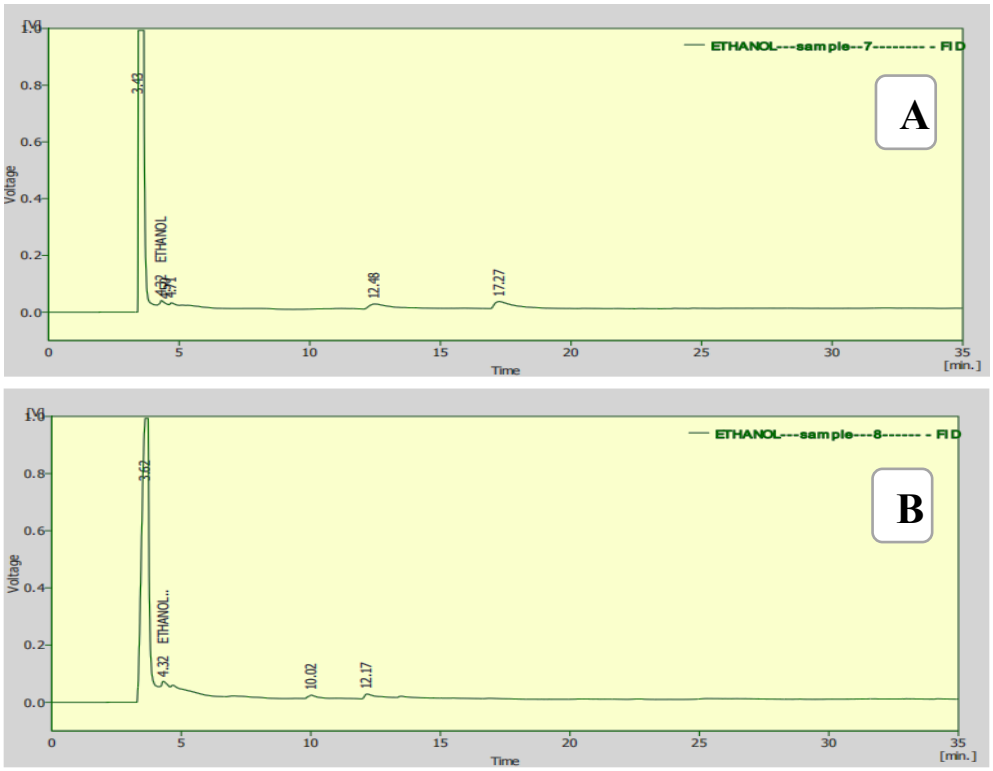

Figure 3: The ethanol measured by the HPLC produced by S. *cerevisiae* from lettuce leaves pre-treated with H_2SO_4 (A) after 1st distillation (B) after 2nd distillation

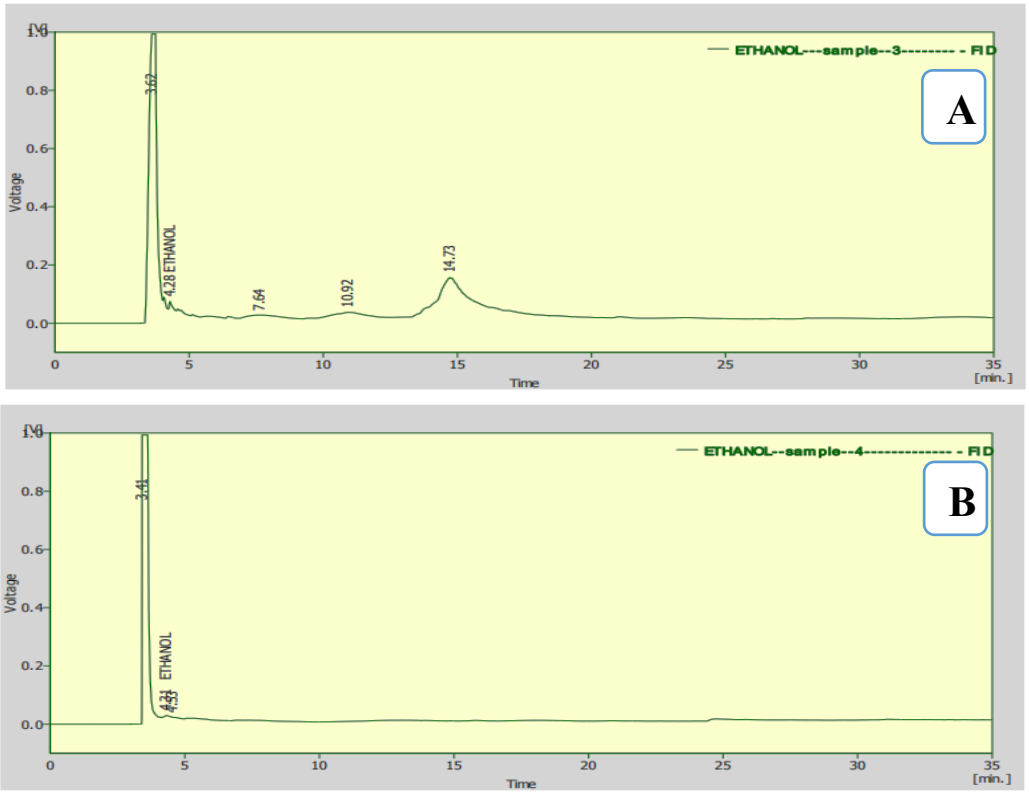

Figure 4: The ethanol measured by the HPLC produced by S. *cerevisiae* from lettuce leaves pre-treated with HCL (A) after 1st distillation (B) after 2nd distillation

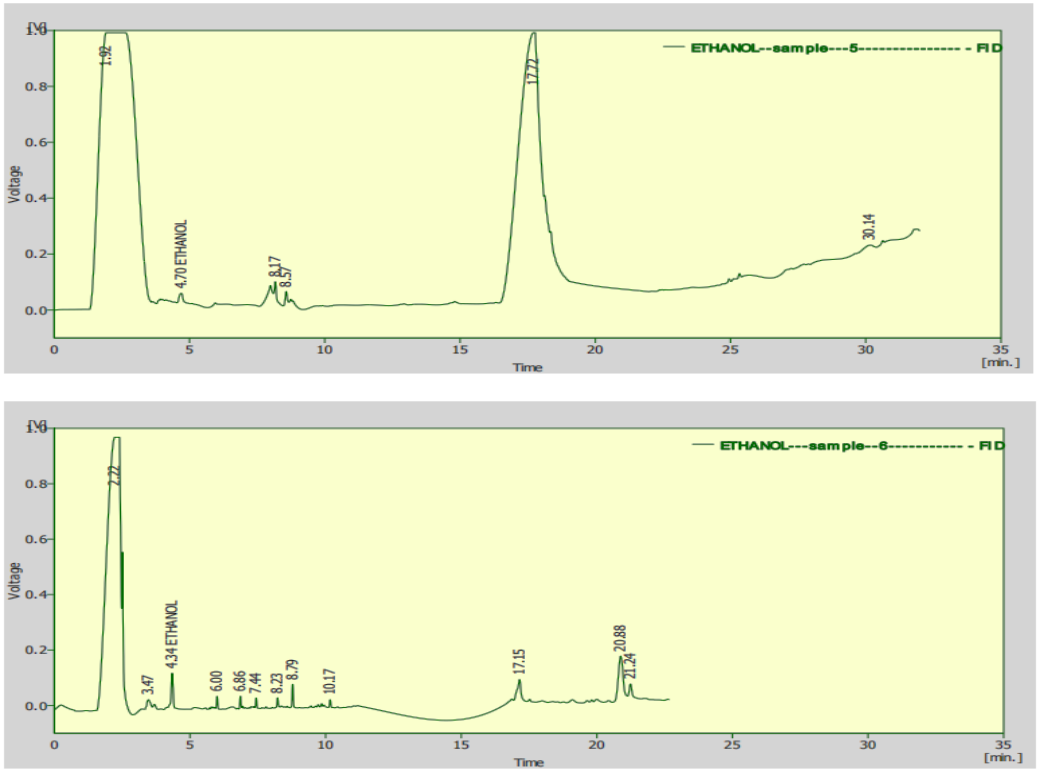

Figure 5: The ethanol measured by the HPLC produced by S. *cerevisiae* from lettuce leaves pre-treated with NaOH (A) after 1st distillation (B) after 2nd distillation

Conclusion

This study shows the ability of the *S. cerevisiae* for production of bioethanol from the organic waste (lettuce leaves), the acidic and alkaline pre-treatment of the raw materials (lettuce leaves) was effective in the breakdown of the cellulose, hemicellulose and lignin to mono-sugars which could be consumed by the *S. cerevisiae* for the production of bioethanol. The application of NaOH pretreatment resulted in the highest yield of reduced sugars, whereas acidic pretreatment yielded the highest ethanol production. It also clear that the distillation process enhances the resulted bioethanol purity.

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6. Statements on compliance with ethical standards and standards of research involving animals

"This article does not contain any studies involving animals performed by any of the authors."

7. Disclosure and conflict of interest

The authors declare that they have no conflicts of interest.

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