ISSN: 0067-2904

Bioethanol Production from Organic Wastes by Using the Newly Isolated Bacteria *Klebsiella oxytoca* (SAR)

Sama A. Mustafa [1]*, Ahmed J. Mohammed [2]
[1]*Environmental Health Department, College of Environmental Science, Al-Qassim Green University, Babylon, Iraq*
[2] *Department of Biology, College of science, University of Baghdad, Iraq*

Received: 25/1/2024 Accepted: 1/5/2024 Published: 30/4/2025

Abstract

This study was carried out to study the ability of producing bioethanol from latus leaves as an organic waste rich with cellulose. The study results show that the pre-treatments (alkali and acidic pre-treatments) increase the amount of the sugar released in the media that is accessible for the bacteria to use in the fermentation. The newly recorded bacteria *Klebsiella oxytoca* (SAR) was able to ferment the latus leaves and produce bioethanol. The amount of ethanol produced by the bacteria was increased as the experiment period increased, until the seventh day when it began to decrease. In order to increase the purity of the acquired bioethanol, the resulted ethanol was distilled to reduce the water content in the solution, while the dehydration process did not have any effect on bioethanol purity.

Keywords: Fossil fuel, biofuel, fermentation, Organic waste, *Klebsiella*.

انتاج الايثانول الحيوي من الفضلات العضوية باستخدام بكتريا *klebsiella oxytoca (SAR)* المعزولة جديدا

سما احمد مصطفى[1]*, احمد جاسم محمد[2]
[1] قسم الصحة البيئية, كلية علوم البيئة, جامعة القاسم الخضراء, بابل, العراق
[2] قسم علوم الحياة, كلية العلوم, جامعة بغداد, بغداد, العراق

الخلاصة

تم اجراء هذه الدراسة لدراسة إمكانية انتاج الايثانول الحيوي من أوراق الخس كفضلات عضوية غنية بالسليلوز. بينت نتائج الدراسة ان عملية المعالجة المسبقة (القاعدية والحامضية) قد أدت الى زيادة كمية السكر المتحرر في الوسط والذي يتم استخدامه من قبل البكتريا في عملية التخمر. ان بكتريا الكلبسيلا *Klebsiella oxytoca* (SAR) المسجلة جديداً كانت قادرة على انتاج الايثانول الحيوي من أوراق الخس. ان الايثانول المنتج من قبل البكتريا يبدا بالازدياد كلما ازداد وقت التجربة وصولا الى اليوم السابع حيث يبدأ بالانخفاض من

*Email: sama@environ.uoqasim.edu.iq

اجل زيادة نقاوة الوقود الحيول المتحصل عليه، تم تقطير الايثانول المنتج لإزالة الماء من المحلول، بينما لم تؤثر عملية التجفيف على نقاوة الايثانول المنتج.

1. Introduction

There is an ever-growing demand for fossil fuel by humans in different life aspects, which puts a huge pressure on this finite resource [1,2]. This was accompanied by concerns on the increase in greenhouse gases (GHG) emission [3,4]. These concerns motivated many researchers for attempting different studies to find alternative, cheap, and eco-friendly resources of fuels that will play an important role in minimizing the dependence on fossil fuel and reduce global warming and the associated negative environmental impacts [5]. Biofuel is one of these alternative sources which is a locally available, sustainable, and reliable resource obtained from a renewable source [6]. Biofuels can provide many advantages, such as minimizing GHG emissions and global warming, reducing petroleum price fluctuations, as well as reducing the dependence on fossil fuel [7]. Bioethanol represents an attractive biofuel which can be defined as an alternative and renewable energy source that is produced from organic biomass [8]. The process of producing bioethanol contains two main steps, which are the hydrolysis of cellulosic and hemicellulose compounds and the fermentation of the resulted reduced sugar to produce bioethanol [9]. This study was conducted to investigate the ability of using the newly isolated endogenous bacteria *Klebsiella oxytoca* (SAR) for producing bioethanol from organic wastes (latus leaves) through a fermentation process with different pre-treatment methods that enhance bio-degradation of cellulose for ethanol production.

2. Materials and Methods

2.1 Isolation and Identification of Bacterial Species

The organic waste (latus leaves) was collected from AL-Rasheed grand vegetables market in Baghdad/Iraq. The leaves were cut to pieces and kept in sterilized peptone water for 24 hours to enhance the growth of fermenting microorganisms [10]. Then, 1 mL of peptone water that contains the microorganism was used to prepare serial dilutions. From each dilution, 1 mL was kept in nutrient broth for 24 hours to promote the growth of the bacteria and then 1 mL of the nutrient broth was cultured in sterilized UTI medium. The final step was repeated until obtaining a pure culture which was sent for identification by using the 16S rRNA technique [11] at Wahaj AL-DNA Laboratory in Baghdad. The bacteria used in this study was identified as *Klebsiella oxytoca* (SAR) and was recorded for the first time in Iraq at the NCBI. The identified bacterial species was used in the following experiments.

2.2 Bacterial Optimization

For the newly recorded *Klebsiella oxytoca* (SAR), the optimal conditions for growth were tested according to Babu *et al.* [12] and was found to involve incubation at 35°C for 7 days at pH value of 7.

2.3 Samples Collection and Processing

Latus leaves that were collected from AL-Rasheed grand vegetables market in Baghdad/Iraq were cleaned and dried in the sun then grinded by an electrical grinder to obtain a fine powder. After that, the powder was kept in polyethylene bags in the refrigerator at 4ºC [13].

2.4 Experimental Work

In the experimental work, the first step was to prepare the latus leaves for fermentation by the bacteria. Two experiments were made, i.e. with and without pre-treatment of the cellulosic content of the latus leaves, as follows:

2.4.1. Without pre-treatment

Fifty gram of dried latus leaves were soaked in 1 litre of distilled water and then used directly in the fermentation process.

2.4.2. Pre-treatment processes

A. Pre-treatment with sulfuric acid (H_2SO_4)

Fifty grams of dried latus leaves were pre-treated with 0.5 L of diluted H_2SO_4 (2 %) and placed on a hot plate stirrer for 60 minutes at 130 °C with 500 rpm [12].

B. Pre-treatment with Hydrochloric acid (HCL)

Fifty grams of dried latus leaves were pre-treated with 0.5 L of diluted HCL (5%) then placed on a hot plate stirrer for two hours at 130 °C and 500 rpm [14].

C. Pre-treatment with Sodium hydroxide (NaOH)

Fifty grams of dried latus leaves were pre-treated with 500 ml of diluted NaOH (1%) then placed on a hot plate stirrer at room temperature for 3 hours [15].

For A, B, and C, after pre-treatment, the samples were filtered and the volume of each was completed to 1 litre with distilled water. The pH value was set at 8, then the samples were ready for fermentation.

2.5. Fermentation Process

The samples with and without pre-treatment were autoclaved, then the bacterial inoculum was added to each sample in sterilized conditions. After that, the samples were incubated for seven days. Both ethanol concentration and sugar content were measured every two days [16].

2.6. Distillation Process

In order to obtain the produced ethanol, the fermented solution was distilled by passing through a condenser column depending on the variation between water and ethanol boiling point [17]. During this process, the fermented solution was set on a digital heating mantle and ethanol vapor was condensed and collected. Then, a second distillation was carried out to increase ethanol purity.

2.7. Dehydration Process

This process was performed to maximize ethanol purity, through which 5 gm of calcium oxide (CaO) was added to ethanol that resulted from the second distillation and mixed gently for 10 minutes. After that, the mixture was distilled again [18].

2.8. 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. 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 by using a spectrophotometer [19].

2.9. Ethanol Measurement

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

1-Daily measuring ethanol vapor by using a Lab Quest 3 ethanol sensor.

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

3. Results and Discussion

The bacterial species was recorded for the first time in Iraq and identified by using the 16S rRNA test. At first, DNA extraction and electrophoresis were achieved, then the purity of the

sample was tested using the nanodrop technique (Nabi /Korea) under the 260-280 column. Sample purity is considered good when it ranges from 1.8 to 2.0 [20]. The prepared sample showed a good purity, as shown in Table 1.

Table 1: Nucleic acid concentration and sample purity of *Klebsiella oxytoca* (SAR).

Sample number	*Bacteria*	*Nucleic acid conc. (ng/ µl)*	*260/280 purity*
1	1(Klebsiella VTI)	63.2	1.878

The results of PCR test are shown in Figure2 and Table 2.

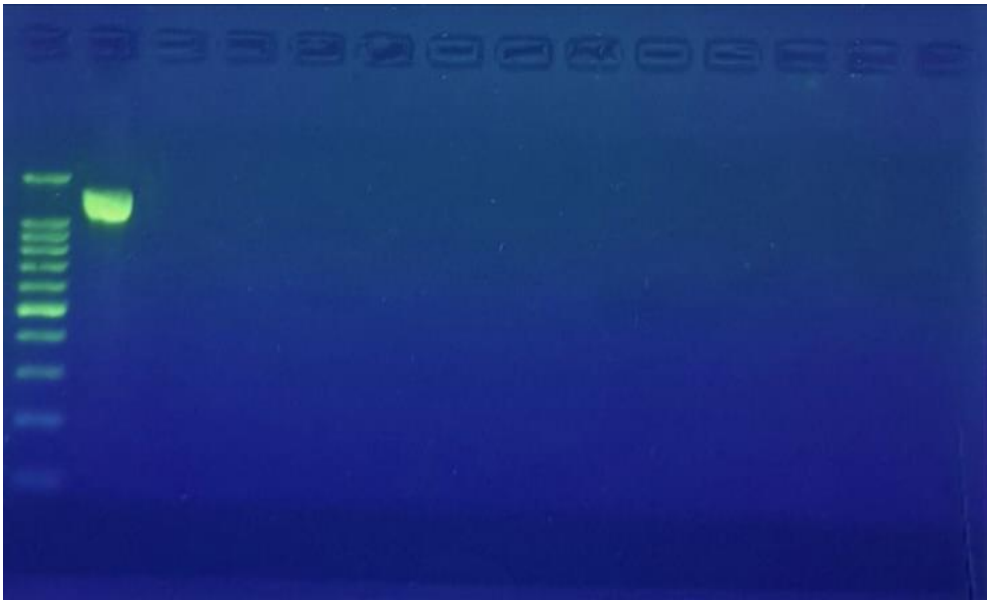

Figure 1: PCR product with a band size of 1250 bp. The product was subjected to electrophoresis on 2% agarose at 5 volt/cm^2 using 1x TBE buffer for 1hr. N: DNA ladder (100).

Table 2: PCR results of *Klebsiella oxytoca* (SAR)

Sample number	*bacteria*	*DNA Result*	*PCR Result 16srRNA 1250 bp*
1	1(Klebsiella VTI)	+	+

Finally, sequencing was carried out by Macrogen company in Korea and the findings were recorded at the NCBI. The bacterial species was used in the experiments that followed. It is well known that 16S rRNA gene sequencing is currently considered as method for biochemical identification of unidentified bacteria [21].

This study was conducted to find an alternative, more sustainable, and cheaper energy source in comparison with fossil fuel. During this study, the newly recorded sub-strain *Klebsiella oxytoca* (SAR) was used to produce bioethanol from organic waste (latus leaves). The contents of latus leaves is illustrated in Figure1.

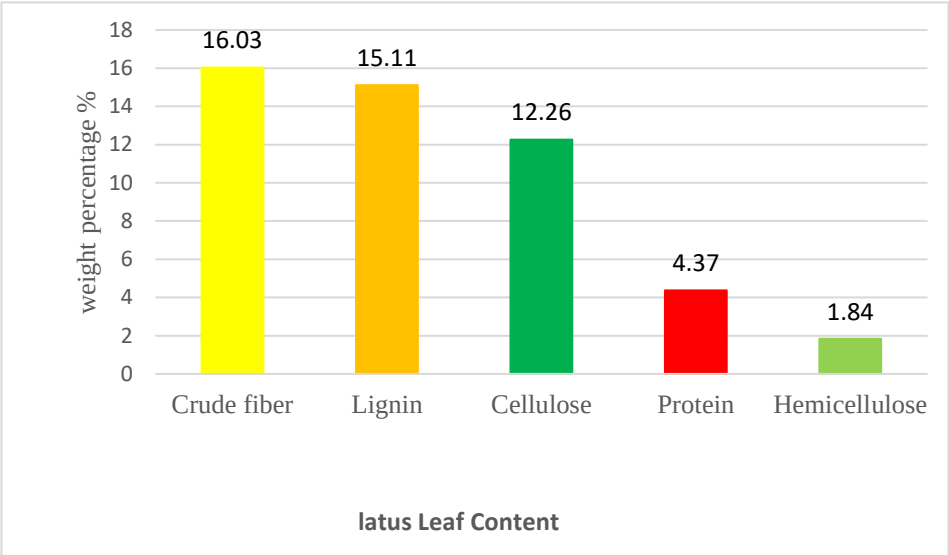

Figure 2: The most important contents of latus leaves.

As shown in Figure 1, high levels of cellulose, hemicellulose, and lignin were observed. The breakdown of these complex compounds to monosaccharides that can be utilized by bacteria to produce ethanol requires fermentation with different pre-treatment processes [22]. In this study, three types of pre-treatment represented by H_2SO_4, HCl, and NaOH were used to discover their effects on sugar concentration that can be available for fermentation by bacteria, as shown in Figure2.

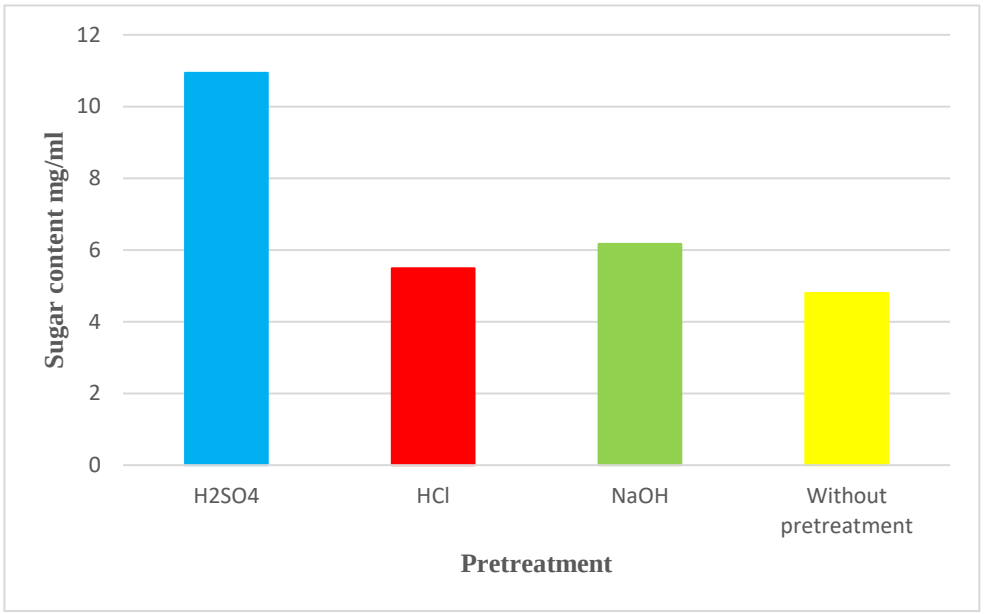

Figure 3: Sugar content provided after each pre-treatment.

The results show that the highest sugar content was recorded when the latus leaves were pre-treated with H_2SO_4, followed by NaOH, and HCL, whereas the lowest was without pre-treatment. This can be easily explained by the fact that the pre-treatment increased the release of monosaccharides resulting from the breakdown of cellulose and hemicellulose [23]. The results of this study are in agreement with the findings of Babu *et al.* [12] who found that the pre-treatment process was important in reducing the crystallinity of cellulose as well as removing lignin and hemicellulose and increasing the porosity of the lignocellulosic materials.

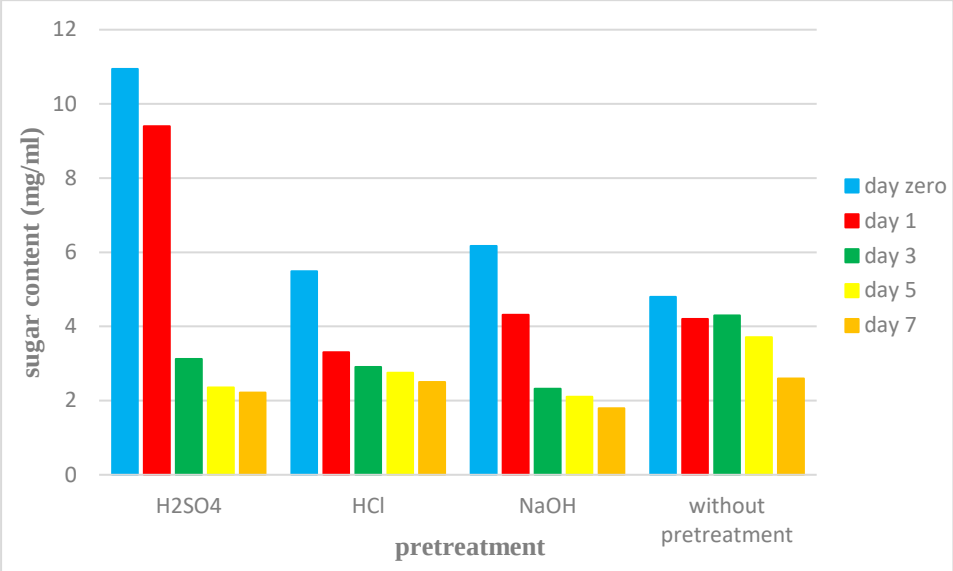

Figure 4: The changes in sugar content throughout the study period.

The sugar present in each sample was consumed by the bacteria to produce ethanol through the saccharification process [24], as shown in Figure3.

Ethanol concentration was measured daily by using an ethanol sensor. The results indicated that the maximum concentration was obtained from the pre-treatment with HCL (7288 ppm), followed by NaOH (6987 ppm) and H_2SO_4 (4852 ppm), while the lowest result was 3140 ppm in the group without pre-treatment, as shown in Table 1. This may be due to the fact that HCL was able to convert cellulose and hemicellulose to simple sugar (monomers) through the breakdown of the polymeric bonds [25]. On the other hand, NaOH pre-treatment acts on the ester bond between the lignin and the carbohydrate, or even between the lignin and other lignin components, which leads to the breakdown of the lignocellulosic materials [26]. These results are similar to the findings of Radillo *et al.* [27] and Iranmahboob *et al.* [28], who found that the pre-treatment alters the lignocellulosic matrix, thus allowing the subsequent hydrolytic degradation of polysaccharides to monosaccharides (saccharification).

Table 1: Ethanol concentration produced by *K. oxytoca* SAR throughout seven days.

Days	Without Pre-treatment (ppm)	Pre-treatment(ppm)		
		HCL	H_2SO_4	NaOH
1	1037	1433	1620	3024
3	3140	5466	3917	4440
5	1614	7288	4852	6987
7	1421	3503	4455	6413

After each experiment, two distillation processes were performed to obtain the resulted ethanol, the concentration of which was measured with the HPLC technique, as appears in Figure4.

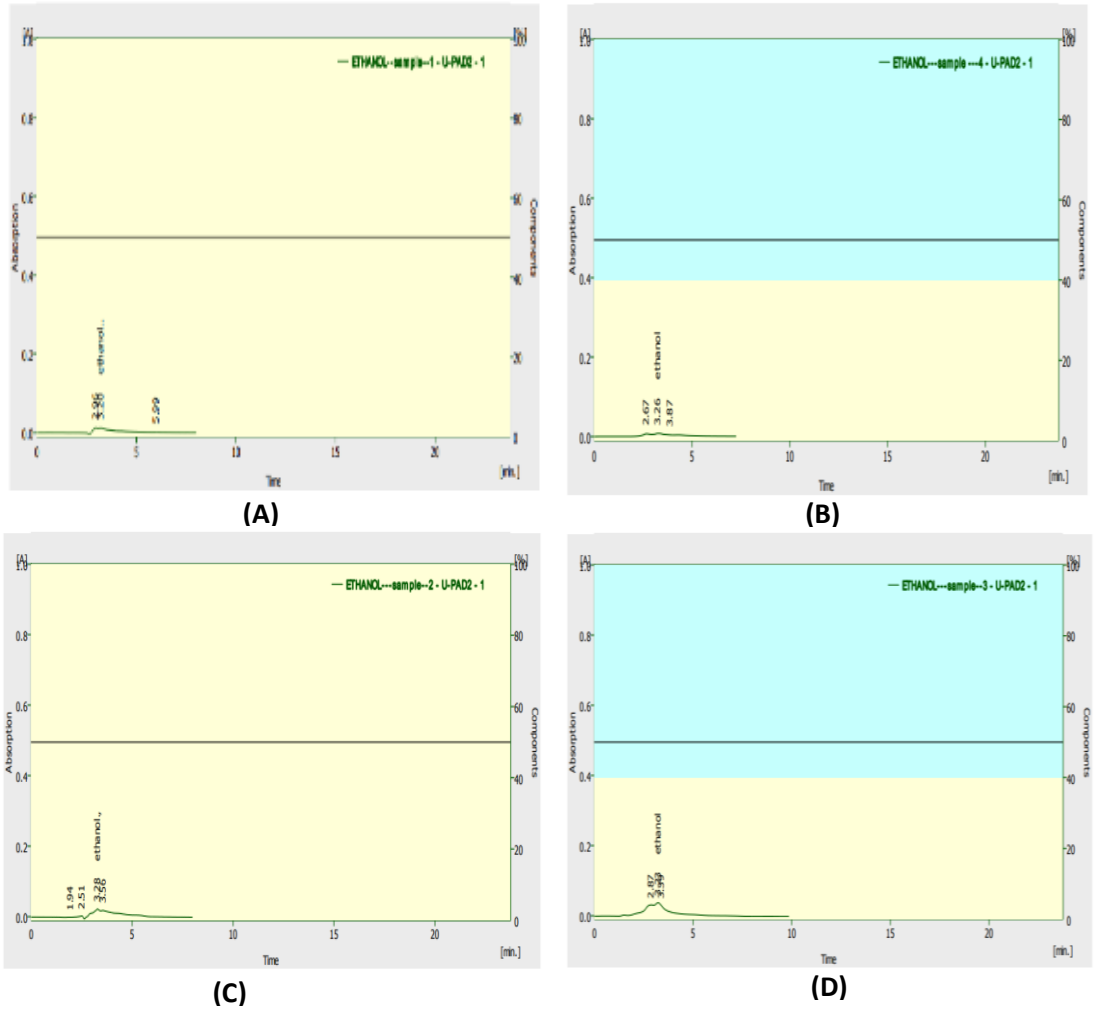

Figure 5: Ethanol concentrations measured by HPLC. (A) 1st distillation without pre-treatment. (B) 1st distillation pre-treated with NaOH. (C) 1st distillation pre-treated with H_2SO_4. (D) 1st distillation pre-treated with HCL.

Ethanol concentrations obtained from the fermentation process are show in Table 2. The results showed that there was no difference between the first and the second distillations in terms of ethanol concentration and purity. The results of this study disagree with the findings of [19], who found an increase in the purity of ethanol resulted from the fermentation process starting from the second distillation step.

Table 2: Ethanol concentrations in the first and second distillations.

	Without pre-treatment (ppm)	***pre-treatment (ppm)***		
		NaOH	**HCL**	**H_2So_4**
Ethanol Concentration	22.339	12.93	27.65	23.97

2.5. Conclusions

Klebsiella oxytoca (SAR) showed the ability to produce bioethanol from organic waste (latus leave). It was obvious that the pre-treatments (acidic and alkaline) increase the amount of ethanol produced through the breakdown of cellulosic materials to a simpler sugar that can be utilized by the bacteria as a substrate for the fermentation process.

3. Acknowledgements

Deepest thanks to the University of Baghdad/ College of Science/ Biology Department for allowing us to use the ecology laboratory for higher studies for carrying out this work.

4. Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] J. Wang, W. Azam "Natural resource scarcity, fossil fuel energy consumption, and total greenhouse gas emissions in top emitting countries", *Geoscience Frontiers*, vol.15, no. 2, March 2024.

[2] E. F. Khanjer, M. A. Yosif, and M. A. Sultan, "Air Quality Over Baghdad City Using Ground and Aircraft Measurements", *Iraqi Journal of Science*, vol. 56, no. 1C, pp. 839–845, May 2023.

[3] J. L. Holechek, H. M. E. Geli, M. N. Sawalhah and R. Valdez "A Global Assessment: Can Renewable Energy Replace Fossil Fuels By 2050?", *Sustainability*, vol. 14, no.8, 4792, pp. 1-22. April 2022.

[4] S. M. Khazael and M. Al-Bakri, "The Optimum Site Selection for Solar Energy Farms using AHP in GIS Environment, A Case Study of Iraq", *Iraqi Journal of Science*, vol. 62, no. 11, pp. 4571–4587, Dec. 2021.

[5] Z. Liu, J. Wang and J. Nielsen "Yeast synthetic biology advances biofuel production", *Current Opinion in Microbiology*, vol. 65, pp. 33–39. February 2022.

[6] A. Demirbas "Biofuels sources, biofuels policy, biofuels economy and global biofuels projections", *Energ Convers Manage*, vol. 49, pp. 2016–2116. August 2008.

[7] C. Fogel, "Constructing progressive climate change norms: the US in the early 2000s," in The social construction of climate change, power, knowledge, norms, discourses, M. E. Pettenger, Ed. London, Routledge, 2007.pp. 123–147.

[8] R. L. Berevoianu**, "**Bioethanol: Renewable energy source", in Agrarian Economy and Rural Development - Realities and Perspectives for Romania. International Symposium. 12th Edition. Bucharest, 2021. pp. 129-134.

[9] N. H. Hayder and B. Mahmoode, "Biofuel Production from Cellulosic Wastes by Local Isolates of Streptomyces sp", *Iraqi Journal of Science*, vol. 55, no. 4B, pp. 1844–1858, Oct. 2023.

[10] V.L. Gray, C.T. Muller, I.D. Watkins and D. Lloyd, "Peptones from diverse sources: pivotal determinants of bacterial growth dynamics. *Journal of Applied Microbiology*, vol.104, no.2, pp. 554–565, February 2008.

[11] A. K. Al-Muttairi and I. K. Al-Mayaly, "The Potential Efficiency of Bacillus subtilis AIK to Remove Nickel from Aqueous Solutions", *Iraqi Journal of Science*, vol. 64, no. 8, pp. 3859–3866, Aug. 2023.

[12] S. Babu, K.M. Harinikumar, R. K. Singh and A. Pandey, "Optimization of Bioethanol Production from Fruit Wastes using Isolated Microbial Strains", *International Journal of Advanced Biotechnology and Research* (IJBR), vol. 5, no. 4, pp.598-604. October 2014.

[13] K. Satyanagalakshmi, R. Sindhu, P. Binod, K. U. Janu, R. K. Sukumaran and A. Pandey, "Bioethanol production from acid pretreated water hyacinth by separate hydrolysis and fermentation", *Journal of Scientific and Industrial Research*, vol. 70, pp:156-161. March 2011.

[14] J. M. Romero-García, C. Martínez-Patiño, E. Ruiz, I. Romero and E. Castro, "Ethanol production from olive stone hydrolysates by xylose fermenting microorganisms", *Bioethanol*, vol. 2, no. 1, pp.51–65. September 2016.

[15] P. Kumar, D. M. Barrett, M. J. Delwiche, and P. Stroeve, "Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production," *Ind. Eng. Chem. Res*. vol. 48, pp. 3713-3729. March. 2009.

[16] S. Krahulec, R. Kratzer, K. Longus and B. Nidetzky, "Comparison of *Scheffersomyces stipitis* strains CBS 5773 and CBS 6054 with regard to their xylose metabolism: implications for xylose fermentation," *Microbiology Open*, vol.1, pp. 64-70, March, 2012.

[17] K. M. R. Cui-Lim, J. A. H. Brensis, F. M. S. Dagalea, M. J. M. Bangco, M. R. Castillo, H. G. Pulga, M. G. M. Cruz, J. L. Erivera, F. M. Chiquito, T. J. L. Abobo1, M. J. Madario and C. I. Ultra, "Extraction of Ethanol from Nypa fruticans (Nipa) Palm Fruit. Asian Journal of Physical and

Chemical Sciences", *Asian Journal of Physical and Chemical Sciences*, vol. 8, no.4, pp. 41-45, December 2020.

[18] Y. M. Y. Al-Ani "Production of bioethanol as alternative fuels from bio renewable resources," MSc. Thesis, Dept. Biology, University of Baghdad, Baghdad, Iraq, 2018.

[19] S. K. Al-Rubaie and H. S. Abdulhay, "Bioethanol Production from Olive Solid Residues by Using Rhodotorula Minuta", *Iraqi Journal of Science*, vol. 63, no. 1, pp. 53–61, Jan. 2022.

[20] P. Desjardins and D. Conklin, "NanoDrop Microvolume Quantitation of Nucleic Acids," *Journal of Visual Experiments* , vol. 45, e2565, doi:10.3791/2565, November 2010.

[21] J. M. Janda and S. L. Abbott, "16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls," *Journal Of Clinical Microbiology*, vol. 45, no. 9, pp. 2761–2764. Sept. 2007.

[22] A. K. Ebraheem, "Production and analysis of bioethanol from dates syrup," M.Sc. thesis, Dept. Eng., University of Babylon, Babylon, Iraq, 2013.

[23] M. Galbe, and G. Zacchi, "Pretreatment of lignocellulosic materials for efficient bioethanol production," *Adv. Biochem. Eng./Biotechnol*., vol. 108, pp. 41-65. January 2007.

[24] K. Ohgren, O. Bengtsson, M. F. Gorwa-Grauslund, M. Galbe, B. Hahn-Hagerdal, and G. Zacchi, "Simultaneous saccharification and cofermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with Saccharomyces cereVisiae TMB3400", *J. Biotechnol*., vol. 126, no. 4, pp. 488-498. December 2006.

[25] D. O. Usino, T. Sar, P. Ylitervo and T. Richards, "Effect of Acid Pretreatment on the Primary Products of Biomass Fast Pyrolysis", *Energies*, vol.16, no. 5, 2377, March 2023

[26] A. A. Modenbach and S. E. Nokes, "Effects of sodium hydroxide pretreatment on structural components of biomass", *Transactions of the ASABE*, vol. 57, no. 4, pp. 1187-1198.augest, 2014.

[27] J. J. V. Radillo, M. A. Ruiz-López, R. R. Macías, L. B. Ramírez, P. M. García-López, F.A. López-Dellamary, "Fermentable Sugars from Lupinus rotundiflorus Biomass by Concentrated Hydrochloric Acid Hydrolysis," "Sugars from lupine biomass", *BioResources*, vol. 6, no. 1, pp. 344-355. February 2011.

[28] J. Iranmahboob, F. Namied, and S. B. Monemi, "Optimizing acid-hydrolysis a critical step for production of ethanol from mixed wood chips," *Biomass Bioenergy*, vol. 22, pp. 401-404. May 2002.