



ISSN: 0067-2904

## Bioethanol Production from Olive Solid Residues by Using *Rhodotorula Minuta*

Sundus Khudair Al-Rubaie<sup>\*1</sup>, Hind Suhail Abdulhay<sup>2</sup>

<sup>1</sup>Ministry of Science and Technology, Baghdad, Iraq

<sup>2</sup>Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Received: 27/11/2020

Accepted: 26/3/2021

### Abstract

Bioethanol is an attractive fuel with higher potential for energy security and environmental safety. Olive solid residues were used as a raw material for the production of bioethanol through the use of different preliminary treatments. Separate treatments with cellulose, hydrochloric acid (HCl 5%), sulfuric acid (H<sub>2</sub>SO<sub>4</sub> 2%), and liquid ammonia NH<sub>4</sub>OH (20%) were used to convert cellulose and hemicellulose into monosaccharides. The production of ethanol was observed during the fermentation process using *R. minuta* under anaerobic conditions. After 3 days of fermentation, lowest concentrations of ethanol of 0.233, 0.249, 0.261, and 0.275 g/l were produced from olive waste powder sample as a result of separate pretreatment with cellulase, hydrochloric acid (HCl 5%), sulfuric acid (H<sub>2</sub>SO<sub>4</sub> 2%), and liquid ammonia NH<sub>4</sub>OH (20%), respectively, whereas the untreated sample showed ethanol yield of 0.264 g/l. The highest ethanol concentrations for the same samples were 0.510, 0.564, 0.737, and 0.696 g/l, respectively, whereas that for the untreated samples was 0.445 g/l. The highest concentration of ethanol produced (0.737 g/l) was achieved after 3 days of fermentation of olive solid waste pretreated with H<sub>2</sub>SO<sub>4</sub> 2% at 30 °C and pH 5. The average yield of ethanol resulted from these saccharification and fermentation processes following the pretreatment of olive solid waste was 0.59 g/10 g dry olive solid residues.

**Keywords:** ethanol, yeast, *Rhodotorula minuta*, fermentation.

## إنتاج الإيثانول الحيوي من بقايا الزيتون الصلبة باستخدام *Rhodotorula minuta* في بغداد

### العراق

سندس خضير الربيعي<sup>1</sup> . هند سهيل عبد الحي<sup>\*2</sup>

<sup>1</sup>وزارة العلوم و التكنولوجيا، بغداد، العراق

<sup>2</sup>قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق

### الخلاصة

يُعد الإيثانول الحيوي وقوداً جذاباً يتمتع بإمكانيات عالية لأمن الطاقة والسلامة البيئية. تم استخدام بقايا الزيتون الصلبة كمادة خام لإنتاج الإيثانول الحيوي من خلال استخدام المعاملات الأولية، تستخدم هذه المعاملات لتحويل السليلوز والهيميسليلوز إلى سكريات أحادية ولوحظ إنتاج الإيثانول أثناء عملية التخمير باستخدام *R. minuta* تحت ظروف لاهوائية. كان أقل تركيز من الإيثانول الناتج هو من عينة من مسحوق مخلفات الزيتون المعاملة بإنزيم السليلوليز (cellulase)، حامض الهيدروكلوريك (HCl)، حامض الكبريتيك (H<sub>2</sub>SO<sub>4</sub>)، بدون معاملة مسبقة وعينة معاملة بالأمونيا السائلة (NH<sub>4</sub>OH) كان 0.233 و 0.249 و

\*Email: olakhudhair@yahoo.com

0.261 و 0.264 و 0.275 غم / لتر على التوالي بعد 3 أيام من التخمير بينما كان أعلى تركيز إيثانول لنفس العينات 0.510 و 0.564 و 0.737 و 0.445 و 0.696 غم / لتر على التوالي بعد 3 أيام من التخمير. كان أعلى تركيز للإيثانول الناتج هو 0.737 غم / لتر بعد 3 أيام من تخمير بقايا الزيتون الصلبة المعاملة مسبقاً بحامض الكبريتيك المخفف عند 30 درجة مئوية و pH 5. باستخدام عملية التسكر والتخمير بعد المعاملة المسبقة لنفايات الزيتون الصلبة. بلغ متوسط إنتاج الإيثانول 0.59 غم / 10 غم من بقايا الزيتون الجافة الصلبة

## Introduction

The era of renewable energy has begun and motivated countries and societies to search for sources of energy other than those dependent on fossil fuels, including solar and wind energy. In addition, the topic of biofuel production from organic materials, especially organic waste that may pollute the environment, has gained a remarkable amount of research interest worldwide [1]. Bioethanol fuel is becoming one of the best alternatives for countries that import crude oil in large amounts. Ethanol is flammable liquid; its vapor concentrations have flammability in the range of 3.3–19.0% (v/v) in air. The flammability is not a serious problem in the industrial environment if there is sufficient ventilation, which also prevents ethanol from causing severe problems as an industrial poison [2]. Bioethanol, in many ways, is superior to petroleum fuel for spark-igniting engines. It has a higher octane number than that of the conventional petroleum. Also, it is safe for storage and has a good thermal efficiency [3]. Many natural resources, including olive solid waste, date syrup wastes, and banana peels are used as raw materials for the production of bioethanol [4-6]. Since ancient times, the olive oil industry was established in Iraq, in particular olive mills. As for the olive solid waste, it is currently used in the manufacture of soap, charcoal, or even as fuel. Studying the use of olive solid residues for the production of ethanol is an important step in the field of waste recovery [7]. Olive waste is currently supplying global production of ethanol with 2,616,000 barrels per day, according to 2018 statistics. The advantage of producing ethanol from olive waste is that it contributes to the protection of the global environment from the increased levels of pollution [8]. The present study aims at the achievement of three main goals; (a) investigating the ability of olive oil waste to produce bioethanol; (b) evaluating the ability of *Rhodotorula minuta* yeast isolated from dairy products to ferment sugar to ethanol. For this purpose, different pretreatments were applied to find the most effective one in hydrolyzing cellulose, hemicellulose, and lignin into monomeric sugars, which can be then converted to ethanol; (c) using olive solid waste as an alternative biofuel to reduce the phenomenon of environmental pollution in a way that does not affect food security.

## MATERIALS AND METHODS

### Raw materials

Before starting to grind and squeeze the olives, they were washed with water to remove impurities and dust that may be stuck to them, in addition to isolating the olive leaves from the specimens. Olive solid waste was produced through the grinding of olive (*Olea europaea*). Gauze cloth was used to separate olive oil from olive waste by the manual squeezing method. As a result, the mixture was composed of a liquid part, which contained crude olive oil and other liquids (Figure 1a), and olive solid residues (Figure 1b).



(a) Olive oil and other liquid

(b) Olive solid waste

**Figure 1-**The separated olive oil (a) from olive waste (b) as a result of the manual squeezing process.

The ligninocellulosic waste was dried in the oven, cut to small pieces, and then crushed by a grinding miller to powder form in order to increase its susceptibility to chemical pretreatment. The resulting powder was sieved with a fine 50 mesh sieve and stored in a clean container at room temperature for further use [9].

#### **Pretreatment of olive solid waste**

Raw material powder was treated with four separated pretreatments; first, pretreatment with  $H_2SO_4$  (2%) at a temperature of  $130^\circ C$  and at 500 rpm for one hour [10]; second, pretreatment with HCl (5%) at a temperature of  $130^\circ C$  and at 500 rpm for two hours [11]; third, pretreatment with  $NH_4OH$  (20%) at a temperature of  $70^\circ C$  and at 600 rpm for 12 hours [12]; fourth, pretreatment with cellulase enzyme in a shaker incubator at  $50^\circ C$  at 150 rpm for three days [13]. In addition, a fifth sample was left without treatment.

#### **The fermentation stage**

The liquid produced from the previous processes was autoclaved at a temperature of  $121^\circ C$  for a period of 20 minutes for sterilization from any bacteria or other microorganisms. Equipment was designed to complete and facilitate this method of fermentation, separation and disposal of carbon dioxide, as shown in Figure 2 [6].



**Figure 2-**Fermentation unit.

### Isolation of *Rhodotorula minuta* yeast from dairy products

*Rhodotorula minuta* is a genus of unicellular pigmented yeasts, which is part of the Basidiomycota division. It can be easily recognized by the distinct orange/ red colonies which have smooth and glossy shape when grown on SDA (Sabouraud's Dextrose Agar). This distinctive color is the result of the dyes that the yeast creates to block out specific wavelengths of light (620-750 nm) that would otherwise damage the cells. This genus can be isolated from natural environments, soil, water, milk, fruit juice and air samples [14]. *R. minuta* is part of the division Basidiomycota, family Sporidiobolaceae.

Yeasts were isolated from four types of dairy products purchased from Baghdad local markets. A swab was taken from each layer of the dairy product (surface, middle, and bottom) and cultured on SDA medium, then incubated for 5 days at 28°C. During this period, three colonies with morphological appearance were selected for further purification. The initial diagnosis of yeast was based on the distinct orange/ red coloration and smooth and glossy shape of the colonies, as stated before [14].

### Culturing of *Rhodotorula minuta* yeast on agar media

SDA medium was used for cultivating and maintaining the yeast *R. minuta* at 28 °C for 72 h. After that, the yeast was grown on the agar medium and stored at 4°C. Figure 3a shows *R. minuta* culture after 72 h incubation, while Figure 3b shows this genus under microscope. The diagnosis was confirmed by using vitek 2 compact system (Table 1).



**Figure 3-**The *Rhodotorula minuta* yeast (a) Cultured on SDA medium, (b) Under light microscope (×40).

**Table 1-**The diagnosis of yeast by vitek 2 compact system with the probability that the organism is *Rhodotorula minuta* (87%)

Identification Information				Analysis Time: 17.78 hours				Status: Final									
Selected Organism				87% Probability <b><i>Rhodotorula minuta</i></b>													
				Bionumber: 6504104063223561													
ID Analysis Messages																	
<b>Biochemical Details</b>																	
3	LysA	-	4	IMLT a	+	5	LeuA	+	7	ARG	+	10	ERY a	-	12	GLYL a	+
13	TyrA	-	14	BNA G	-	15	ARBa	-	18	AMY a	-	19	dGA La	-	20	GENa	+
21	dGLU a	+	23	LACa	-	24	MAdG a	-	26	dCE La	-	27	GG T	-	28	dMA La	-
29	dRAF a	-	30	NAG Al	-	32	dMNE a	+	33	dME La	-	34	dML Za	-	38	ISBEa	-
39	IRHA a	-	40	XLTa	+	42	dSOR a	+	44	SAC a	+	45	URE	+	46	AGL U	-

47	dTUR <sub>a</sub>	-	48	dTRE <sub>a</sub>	+	49	NO3a	-	51	IAR <sub>Aa</sub>	(-)	52	dGA <sub>Ta</sub>	+	53	ESC	-
54	IGLT <sub>a</sub>	+	55	dXYL <sub>a</sub>	+	56	LATa	-	58	ACE <sub>a</sub>	+	59	CIT <sub>a</sub>	-	60	GRTa <sub>s</sub>	+
61	IPRO <sub>a</sub>	-	62	2KGa	+	63	NAGa	+	64	dGN <sub>Ta</sub>	+						

### Preparation of yeast culture in broth

SD broth was used for preparing the culture of *R. minuta*. Two slants of the yeast culture were inoculated into a 100 ml borosilicate glass reagent bottle with screw cap, containing 80 ml of SD broth medium. Then, the culture was incubated at 28°C for 72 h; these active cultures were used as inoculums for ethanol production [15].

### Fermentation of raw materials

Activated *R. minuta* with inoculum size of  $5.55 \times 10^5$  cells / ml was added to olive solid residue for each experiment (with pretreatment and without treatment) under aseptic conditions in order to prevent any contamination [16]. Distilled water (900 ml) was added to each mixture and then incubated at 30°C for a period of three days. During that, ethanol concentration was determined daily by using ethanol sensor (Vernier / USA) [17].

## RESULTS AND DISCUSSION

### Influence of different pretreatment methods

The effects of different pretreatments on olive solid residues were studied. Large amounts of monosaccharides were obtained at the end of the process, as shown in Table 2. Pretreatment of the olive solid residues sample with NH<sub>4</sub>OH (20%) resulted in the highest concentration of sugar (8.25 mg/ml), obtained on the second day, while pretreatment with HCl (5%) produced the lowest sugar concentration of 2.2 mg/ml after 24 h. The results show the effect of acid concentration, temperature and residence time on the biomass structure for the recovery of monosaccharides [18]. Also, the results suggest a strong alkaline effect and selective removal of lignin, without losing reducing sugar and carbohydrates, enhancing porosity and surface area of biomass and, therefore, improving enzymatic hydrolysis [19]. The results also suggest that these pretreatments enhanced the activity of cellulase in catalyzing the process of cellulolysis, which involves the decomposition of cellulose and some related polysaccharides. Cellulases breaks down cellulose molecules into monosaccharides, such as beta-glucose, or short polysaccharides and oligosaccharides [20].

**Table 2-**The concentrations of monosaccharides (mg/ml) yielded from olive fruit residues during 48 h of different pretreatments.

Time (h) after treatment	Treatment type and sugar yield (mg/ml)			
	Sulfuric acid (2%)	Hydrochloric acid (5%)	Liquid ammonia (20%)	Cellulase
0	1.39 mg/ml	0.9 mg/ml	2.29 mg/ml	2.67 mg/ml
12	1.70 mg/ml	1.2 mg/ml	2.11 mg/ml	2.89 mg/ml
24	2.91 mg/ml	2.2 mg/ml	2.42 mg/ml	3.11 mg/ml
36	2.32 mg/ml	0.8 mg/ml	3.29 mg/ml	2.65 mg/ml
48	2.05 mg/ml	1.5 mg/ml	8.25 mg/ml	2.59 mg/ml

The process of hydrolyzing jeft (the word is used in some Arab country, which is olive waste dried and compact to billet shape) into its monomers was conducted. A combination of two enzymes, namely cellulase and  $\beta$ -glucosidase, were used in the hydrolysis process to convert glucose to ethanol [21]. The highest yield of 85.02% glucose was obtained using cellulase concentration of 15 mg/g jeft, while  $\beta$ -glucosidase concentration of 20 mg/ml jeft produced 512 mg/dl glucose after 8 h. Saccharification of cellulosic waste with different treatment methods was studied earlier [9]. The pretreatment of cellulosic waste with 1% of HCl and

H<sub>2</sub>SO<sub>4</sub> produced 21 and 15.8 g, respectively, of reducing sugar/ 100 g of cellulosic waste. In comparison, hydrolysis with *Streptomyces* sp. B 167 enzymes resulted in a significantly higher amount of reducing sugar yield (25 g/ 100 g cellulosic waste).

### The fermentation stage

Different pretreatments were applied to olive solid waste to increase sugar content. Several fermentation experiments were conducted to determine the suitable number of cells for inoculating the fermentation medium that contains monosaccharides. Based on the haemocytometer method [22], four different initial yeast cell numbers ( $5.55 \times 10^3$ ,  $5.55 \times 10^5$ ,  $5.55 \times 10^7$  cells/ml) were used in the fermentation medium. Based on the fermentation experiments, it was found that the best inoculum size was  $5.55 \times 10^5$  cells/ml, which increased ethanol yield by about +0.2 g/l each 24 h, while the inoculum size of  $5.55 \times 10^7$  cells/ml increased ethanol yield by about +0.1 g/l. Therefore, the inoculum size of  $5.55 \times 10^5$  cells/ml was used in all the following experiments of ethanol production (Table 3).

**Table 3-**Initial yeast cell numbers used in the fermentations experiments related to *Rhodotaurula Minuta* to find the best initial cell numbers for ethanol production

Ethanol production g/l	Yeast cell number (cells/ ml)			
	$5.55 \times 10^3$	$5.55 \times 10^5$	$5.55 \times 10^7$	$5.55 \times 10^9$
24 h	-	-	≈+0.2 g/l	≈+0.1 g/l
48 h	-	-	≈+0.2 g/l	≈+0.2 g/l
72 h	-	-	≈+0.2 g/l	≈+0.1g/l

High ethanol production depends on high cellulose and hemicellulose concentrations. Increases in cellulose and hemicellulose content are best affected by removal of non-cellulose components by pretreatment. The presence of lignin is a key limiting factor for the saccharification of lignocellulosic feedstocks [23].

The mean concentrations of ethanol produced from olive solid waste samples, without pretreatment, were 0.264, 0.301, 0.445, and 0.373 g/l, respectively, after each day of the four days incubation period, while they were 0.261, 0.310, 0.737, and 0.100 g/l, respectively, during pretreatment with H<sub>2</sub>SO<sub>4</sub> (2%). The statistical comparison made between the days of pretreatment of samples with H<sub>2</sub>SO<sub>4</sub> showed significant differences in the yield gained in these days at  $p \leq 0.05$ , as listed in Table 3. Also, the mean concentrations of ethanol produced from olive solid waste samples pretreated with cellulase were 0.233, 0.399, 0.510 and 0.361 g/l, respectively, after each day of the four days incubation period, while they were 0.275, 0.528, 0.696 and 0.446 g/l, respectively, for samples pretreated with NH<sub>4</sub>OH. The statistical comparison made between the days of pretreatment of samples with cellulase and NH<sub>4</sub>OH showed significant differences in the yield of ethanol ( $p \leq 0.05$ ), as listed in Table 3.

The mean ethanol concentrations produced from olive solid waste samples pretreated with HCl 5% were 0.249, 0.435, 0.564 and 0.412 g/l, respectively, after each day of the four days incubation period. The statistical comparison revealed significant differences between these values at  $p \leq 0.05$  (Table 4).

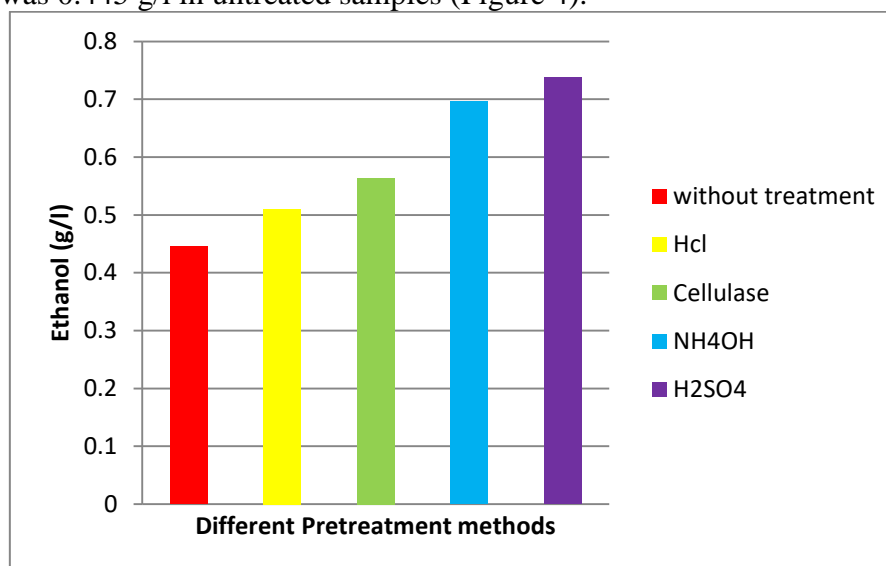
**Table 4-**Ethanol production by olive solid waste samples according to the days of pretreatment and among different chemical pretreatments

Days after treatment	Ethanol concentration (g/l)					LSD p $\leq$ 0.05
	Without pretreatment	H <sub>2</sub> SO <sub>4</sub> (2%)	HCl (5%)	NH <sub>4</sub> OH (20%)	Cellulase Enzyme	
1 st day	0.264 aA ± 0.076	0.261 abA ± 0.113	0.249 aA ± 0.066	0.275 aA ± 0.043	0.233 aA ± 0.019	0.089
2 nd day	0.301 bA ± 0.116	0.310 b A ± 0.100	0.435 bBC ± 0.191	0.528 bcB ± 0.126	0.399 bc CA ± 0.115	0.119
3 rd day	0.445 cA ± 0.182	0.737 cB ± 0.246	0.564 cC ± 0.058	0.696 cD ± 0.117	0.510 b E ± 0.129	0.034
4 th day	0.373 dA ± 0.108	0.100 aB ± 0.047	0.412 bC ± 0.065	0.446 bcC ± 0.182	0.361 bcA ± 0.084	0.035
<b>LSD P<math>\leq</math>0.05</b>	0.019	0.031	0.049	0.136	0.100	

\*Small letters indicate comparison within column. Means with the same letter are not significantly different (p $\leq$  0.05) using LSD test.

\*Capitals letters indicate comparison within row. Means with the same letter are not significantly different (p $\leq$  0.05) using LSD test.

After 3 days of fermentation of olive waste, significant differences were observed in ethanol yield among all pretreatments (p $\leq$  0.05). The higher production of ethanol, after three days, was 0.737 g/l in olive waste samples pretreated with H<sub>2</sub>SO<sub>4</sub> 2%, while the lowest production was 0.445 g/l in untreated samples (Figure 4).

**Figure 4-**Ethanol production by olive fruit waste after three days using different pretreatment methods

The amount of ethanol produced was decreased in the fourth day. It has been reported that the fermentative activity of non-Saccharomyces yeasts is manifested in the presence of small amounts of oxygen, which lead to an increase in cell biomass and a decrease in ethanol yield

[24]. Although ethanol is a final product of anaerobic fermentation of sugars by yeast, it is toxic to yeast cells and induces stress responses, such as the expression of heat shock proteins and the accumulation of trehalose [25]. A 6% concentration of ethanol was reported to decrease the growth rate of the cells by 50% [26].

A similar study [27] studied the use of rice cake to produce ethanol using a collection of yeasts, including *Rhodotorula minuta*. Rice cake was mixed with raw starch digesting enzymes of *Aspergillus niger*, *Lactobacillus fermentum*, *R. minuta*, *Rhodotorula mucilagnosa*, *Candida krusei*, and *Kodamara ohmeri* into different fermenting chambers. *R. minuta* showed the highest efficiency of ethanol production (52.06%) at temperature of 30°C and pH of 2.58. Similar results were recorded by another study [13] which applied separated hydrolysis and fermentation processes on hydrolysate that undergoes detoxification. *Issatchenkia orientalis* was the most efficient in producing of ethanol when supplemented with glucose. Using simultaneous saccharification and fermentation processes following pretreatment of olive mill solid wastes (OMSW), the average ethanol yield was 3 g/100 g dry OMSW. The results are comparable to those obtained elsewhere [28], in which one gram of extracted olive pomace was used as substrate for bioethanol production. Enzymatic hydrolysis of extracted olive pomace pretreated with dilute acid hydrolysis was investigated. The enzymatic hydrolysis and bioconversion were firstly carried out by separate hydrolysis, fermentation, and presaccharification processes, followed by simultaneous saccharification and fermentation. The latter process showed a relatively higher bioethanol fermentation yield (0.46 gg<sup>-1</sup>) when compared to the separate hydrolysis and fermentation processes.

Fermentation experiments showed that there are many factors that could affect ethanol production from olive waste. These factors include sugar content produced during pretreatment, type of yeast used in fermentation process, pH value during the simultaneous saccharification and fermentation processes, and temperature.

### Conclusions and Recommendations

The different pretreatments caused different levels of efficiency in converting the lignocellulosic material in the treated olive waste into monosaccharides. Pretreatment of raw material with H<sub>2</sub>SO<sub>4</sub> for 24 h and NH<sub>4</sub>OH for 48 h demonstrated the highest sugar conversion rates in relation to contact time. The average ethanol yield was 0.59 g/10 g dry olive solid waste. The yeast *R. minuta* has a promising future for bioethanol production.

### References

- [1] S. Jose and T. Bhaskar (Eds.). *Biomass and biofuels: Advanced biorefineries for sustainable production and distribution*, London. New York: CRC Press, 2015.
- [2] P. Worsfold, A. Tonshend, C. Poole, "Encyclopedia of analytical science", second edition, *Journal of Chemical Education*, vol. 82, no. 9, p. 1313, 2005.
- [3] L.R. Lynd, "Large scale fuel ethanol from lignocelluloses", *Applied Biochemistry and Biotechnology*, vol. 24, no. 1, pp. 695-719, 1990.
- [4] U.A. Mansour, Production of bioethanol from olive solid waste "JEFT". M.Sc.Thesis, Biological science, islamic university, Gaza.
- [5] A.D. Aljoborey and H.S. Abdulhay, "Production of bioethanol from reed (*Phragmites australis*)", *International Journal of Sciences: Basic and Applied Research*, vol. 15, no. 2, pp. 145-150, 2014.
- [6] Y.M. Yousif and H.S. Abdulhay, "Bioethanol production from banana peels using different pretreatments", *Asian journal of biological and life sciences*, vol. 6, no. 3, pp. 408-412, 2017.
- [7] M. S. Abdyl-Kadir and A. J. Mohammed, "Olive (the green gold) in the Quran and Sunna and its development in Iraq on light of some countries experiments", *Iraq journal of market research and consumer protection*, vol. 3, no. 5, pp. 93-130, 2011.
- [8] NS Energy, Top five countries for biofuel production across the globe, 2019, <https://www.ns-businesshub.com/>.
- [9] N.H. Hayder and B. Mahmood, "Biofuel production from cellulosic wastes by local isolates of *Streptomyces* sp", *Iraqi journal of science*, vol. 55, no. 4B, pp.1844-1858, 2014.



- [10] 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. 2299-6788, 2015.
- [11] A. O. H. Shalabi, "Production of bio-ethanol from olive pulp", M.sc. Thesis, department of chemistry, College of science, Al –Najah national university, Palestine. 2011.
- [12] J. K. Ko, J. S. Bak, M. W. Jung, H. J. Lee, I. Choi, T. H. Kim and K. H. Kim, "Ethanol production from rice straw using optimized aqueous-ammonia soaking pretreatment and simultaneous saccharification and fermentation processes", *Bioresource Technology*, vol. 100, no.19, pp. 4374–4380, 2009.
- [13] H. Abu Tayeh, N. Najami, C. Dosoretz, A. Tafesh and H. Azaizeh, "Potential of bioethanol production from olive mill solid wastes". *Bioresource technology*, vol. 152, pp. 24-30, 2014.
- [14] F. Wirth and L.Z. Goldoni, "Epidemiology of *Rhodotorula*: an emerging pathogen", *Interdisciplinary Perspectives on Infectious Diseases*, (Article ID 465717), 7 pages, 2012.
- [15] M.W. Sadik and A.A. Halema, "Production of ethanol from molasses and whey permeate using yeasts and bacterial strains", *Int.J.Curr.Microbiol.App.Sci*, vol. 3, no. 3, pp. 804-818, 2014.
- [16] J. Lu, X.Z. Li, J. Zhao and Y. Qu, "Enzymatic saccharification and ethanol fermentation of reed pretreated with liquid hot water". *BioMed Research International*, (Article ID 276278), 9 pages. 2012.
- [17] Vernier Software and Technology, info@vernier.com, USA, 2014. www.vernier.com.
- [18] J. C. Solarte-Toro, J. M. Romero-García, J. C. Martínez-Patiño, E. Ruiz-Ramos, E. Castro-Galiano and C. A. Cardona-Alzate, "Acid pretreatment of lignocellulosic biomass for energy vectors production: A review focused on operational conditions and techno-economic assessment for bioethanol production", *Renewable and Sustainable Energy Reviews*, vol. 107, pp. 587-601, 2019.
- [19] J. S. Kim, Y. Y. Lee and T. H. Kim, "A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass", *Bioresource technology*, vol. 199, pp. 42-48, 2016.
- [20] Barkalow, D. G. and Whistler, R. L. 2019. Cellulose. Access Science. DOI: <https://doi.org/10.1036/1097-8542.118200>.
- [21] I.J.M. Dagher, Enzymatic Hydrolysis of Olive Industry Solid Waste into Glucose, the Precursor of Bioethanol. M.Sc. Thesis. An-Najah National University. Palestine. 2015.
- [22] P.J. Hansen, Use of a hemacytometer. Department of animal science, University of Florida, 2000.
- [23] T. van der Weijde, A.F. Torres, O. Dolstra, *et al.*, "Impact of Different Lignin Fractions on Saccharification Efficiency in Diverse Species of the Bioenergy Crop Miscanthus". *Bioenerg. Res.*, vol. 9, pp.146–156, 2016.
- [24] M. Ciani, A. Capece, F. Comitini, L. Canonico, G. Siesto, P. Romano, "Yeast interactions in inoculated wine fermentation". *Front. Microbiol.* 7, 555, 2016.
- [25] H. Alexandre, V. Ansanay-Galeote, S. Dequin and B. Blondin, "Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*", *FEBS Lett*, vol. 498, pp. 98–103, 2001.
- [26] S. Kubota, I. Takeo, K. Kume, M. Kanai, A. Shitamukai, M. Mizunuma, T. Miyakawa, H. Shimoi, H. Iefuji and D. Hirata, "Effect of ethanol on cell growth of budding yeast: genes that are important for cell growth in the presence of ethanol", *Bioscience, biotechnology, and biochemistry*, vol. 68, no. 4, pp. 968-972, 2004.
- [27] A. A. Shittu, O. A. Ayodeji and M. S. S. Datsugwai, "Bioethanol production from rice winery cake using lactic acid bacteria and yeasts by the process of simultaneous scarification and fermentation", *International journal of microbiology and biotechnology*, vol. 1, no. 1, pp. 33-39, 2016.
- [28] M.C. Fernandes, I. Torrado, F. Carvalheiro, V. Dores, V. Guerra, P. M. L. Lourenço and L.C. Duarte, "Bioethanol production from extracted olive pomace: dilute acid hydrolysis", *Bioethanol*, vol. 2, no. 1, Corpus ID: 101534793, 2016.