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Bioethanol Production by Immobilized *Saccharomyces cerevisiae* using Different Lignocellulosic Materials

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Abstract

Ethanol from biomass is an attractive and sustainable energy source for transportation fuel to substitute gasoline. Second generation ethanol production utilizes cheaper and non-food feed stocks like lignocelluloses or municipal solid waste. This, could make ethanol more competitive to fossil fuels.

The aim of the present study is the production of low cost ethanol using the agro wastes like tomato waste and wheat straw and make a comparison between the efficiency of free and immobilized yeast cells in calcium alginate beads with microwave-assisted acidic pretreatment of the lignocellulosic materials.

We have investigated the efficiency of immobilization technique for bioethanol production using *Saccharomyces cerevisiae* isolate which isolated from yogurt. This isolate was identified according to morphological and biochemical characterization tests. Microwave-assisted acidic pretreatment was performed for both wheat straw and tomato waste and show high improvement (45%) in glucose sugar amount compared with conventional mode of heating of dilute 7% HCl or 5% H₂SO₄ hydrolysis. Calcium alginate was used as immobilization matrix for *S. cerevisiae*. The best calcium alginate concentration was 3% and 4 % for reference and isolated yeast respectively. The immobilization technique gave higher ethanol yield compared with free system for tomato waste but lower yield with wheat straw. The maximum amount of ethanol (641mg/g) produced by free cells when used pretreated straw with microwave-assisted 5% H₂SO₄ hydrolysis and (543.5 mg/g) for tomato waste using immobilized cells with microwave-assisted 7% HCl hydrolysis.

Keywords:

Bioethanol,
Saccharomyces cerevisiae,
Immobilization, Microwave,
Wheat straw,
Tomato waste.

1. Introduction:

Nowadays there is intense interest in expanding the use of renewable energy sources and therefore the production of biofuels worldwide. Among the proposed biofuels, bioethanol and biodiesel are the most commonly used with numerous applications

(Koutinas et al., 2016). Demand for ethanol will increase with the reduction of crude oil resource (Sun et al., 2005; Limayem et al., 2012). Ethanol from biomass is an attractive and sustainable energy source for transportation fuel to substitute gasoline

(Wu et al., 2007). Bioethanol is the most widely used liquid biofuel. The use of ethanol fuel can reduce the toxic exhaust emissions and greenhouse gases from vehicles (Ariyajaroenwong et al., 2012). Current ethanol production (so called first generation) using crops such as sugarcane and corn had been established, whereas second generation ethanol production utilizes cheaper and non-food feed stocks like lignocelluloses or municipal solid waste, still investigated which could make ethanol more competitive to fossil fuels (Thangavelu et al., 2016). The demand for bioethanol is expected to increase dramatically until 2020 (Awolu et al., 2011). Fermentation technology produces nearly 80% ethanol as clean fuel where *Saccharomyces cerevisiae* is considered to be of most important yeast strain because of its biotechnological application in the field of fermentation technology (Sreenath et al., 1996; Neelakandan et al., 2009). Bioethanol conversion from lignocellulose holds great potential due to the widespread availability, abundance, and relatively low cost of cellulosic materials (Kostov et al., 2010). Immobilized cell technology has been suggested as an effective mean for improving ethanol production (Yu et al., 2007). The most significant advantages of immobilized yeast cell systems are the ability to operate with high productivity at dilution rates exceeding the maximum specific growth rate. The procedure of immobilization in alginate beads is not only inexpensive but also easy to carry out and provides extremely mild conditions, so there is a higher potential for industrial application. Calcium alginate beads are one of the most commonly used supports for the immobilization of cells (Rosevear et al., 1984; Goksungur et al., 2001). They offer several advantages as a support, such as good biocompatibility, low cost, easy availability, and ease of preparation. However, there are some disadvantages associated with their use, such as gel degradation, severe mass transfer limitations, low mechanical strength (causing cells to be released from the support), and large pore size (Bangrak et al., 2011). Production of ethanol from wheat straw, one of the most abundant agricultural wastes, has been extensively studied. Among many pretreatment methods microwave-assisted chemical pretreatment has proven one of the most efficient pretreatment methods (Zhu et al., 2006). Microwave is found to be an alternative method for conventional heating. Microwave irradiation could be easily combined with chemical reaction and can accelerate the chemical reaction rate (Binod et al., 2010; Binod et

al., 2012; Chen et al., 2012). The aim of this study was bioethanol production by immobilized *S. cerevisiae* using different lignocellulosic materials with acid-microwave assistance

2. Material and Methods:

2.1 Yeast species and culture media

Local *S. cerevisiae* was isolated from traditional yogurt. The yogurt sample exposed to air for 24 h then plated on yeast peptone glucose agar media (YPG) and incubated at 30°C for 72 h. After incubation, the colonies were plated on YPG medium supplemented with 30 mg/ml chloramphenicol and incubated at the same conditions (Thais et al., 2006). Commercial yeast species of *S. cerevisiae* (Hismaya, Turkey) was used as a reference strain.

2.2 Collection and preparation of straw and tomato waste

Fresh tomato fruit waste was obtained from Hamada factory from the city of Gaza in Palestine and air-dried until the humidity concentration become stable. Raw wheat straw was obtained from local farmers in Gaza. Before any pretreatment it was cut to about 1–2 cm length and washed thoroughly with tap water until the washings become clean and colorless and then air dried for further treatment (Thias et al., 2006).

2.3 Inoculum preparation

The isolated yeast and commercial strain were inoculated in YPG broth and incubated at 30°C for 24 h with constant shaking at 110 rpm. After incubation, a suspension was prepared and adjusted to an optical density of 0.1 at 660 nm. An aliquot of 50 ml was inoculated into broth media used for carbon source assimilation, and ethanol tolerance test. The inoculum size of 10 ml suspension was used that produced an absorbance between 0.1 and 0.2 at OD 660 nm (Thais et al., 2006; Shuvashish et al., 2010).

2.4 Carbohydrate source assimilation test

Yeast fermentation broth medium was used for identification of yeast isolates based on the utilization of various carbohydrates (Glucose, Maltose, Xylose, sucrose and Lactose). In this test, 50 ml of YP media, each containing a specific carbohydrate was inoculated with yeast isolate. Incubated at 30°C for 96 hr with

constant shaking at 150 rpm. Samples were taken after 24, 48, 72, and 96 hr. Yeast growth was measured as turbidity using a spectrophotometer at a wavelength of 660 nm as described by (Walker et al., 2006).

2.5 Tolerance to ethanol

Tolerance of yeast to ethanol was tested in comparison with the reference strain *S. cerevisiae*. 10 ml of 24 hr old culture was inoculated in 100 ml YPG broth and subjected to increasing concentration of ethanol (5, 8, 10, 13 and 15 % v/v) in the YPG broth and incubated at 30°C for 4 days with constant shaking at 150 rpm. After incubation, the population was estimated by spectrophotometer at a wavelength of 660 nm at different time intervals (Thais et al., 2006 and Mansour, 2014). The growth curves were constructed to find out the best growth at specific ethanol concentration (Chandrasena et al., 2006).

2.6 Pretreatment method

The size of the straw was mechanically reduced using a grinder and a fine powder of 40 mesh size obtained (Thais et al., 2006).

2.6.1 Hydrolysis process

In this process, acids and microwave were used to catalyze conversion complex polysaccharides in lignocellulosic materials to simple sugars. The pretreated samples of straw and tomato wastes were subjected to hydrolysis by diluted H₂SO₄ or HCl. Around 10 g of powder, wheat straw or tomato fruit waste are placed in a round bottom flask (100 ml) and completed 100 ml with dilute H₂SO₄ or HCl. Hydrolysis was performed at various concentration of H₂SO₄ ranging from 3%, 5%, and 7%. This process was proceeded at 90°C temperature in shaker water bath for various periods of time (1, 2, 3, 4, and 5 hours). Then the reaction mixture was neutralized with 4M NaOH solution (Thias et al., 2006).

2.6.2 Microwave-acid treatment

Microwave treatment was carried out using a domestic microwave instrument. The microwave instrument was operated at 2450 MHz and 450W. For acid pretreatment, 50 ml of 7% HCl or 5% H₂SO₄ was mixed with 5 g of lignocellulosic material in 250 ml Erlenmeyer flask for 5 min, and shaking well. The

mixture then exposed to microwave for 5 min. The resulted mixture was passed to shaking water bath at 90°C temperature for 3 hr. At the end, the reaction mixture was neutralized with 4M NaOH solution. All experiments were carried out in duplicate, and the given numbers are the mean values (Binod et al., 2012).

2.6.3 Estimation of Glucose

The amount of glucose was estimated by using glucose assay kit (Chemelex, S.A., Spain) that depend on enzymatic oxidation of glucose by glucose oxidase (GOD) to gluconic acid. The formed hydrogen peroxide (H₂O₂) is detected by a chromogenic oxygen acceptor, phenol-aminphenazone (AP) in the presence of peroxidase (POD).

2.7 Fermentation process

Anaerobic batch fermentation in 250 mL conical flask of broth media consisting of pretreated and hydrolyzed lignocellulosic substrate was carried out in order to convert the released sugars into ethanol. The pH of the solution was brought to 4.5. The hydrolyzed materials were completely sterilized by autoclaving (120°C, 15 psi pressure and 30 min). After the substrate was prepared, 10 mL of the yeast inoculum was added to each flask. The fermentation was continued for 5 days and samples were taken from each flask every day for analysis (Mohit et al., 2011).

2.8 Estimation of ethanol

The ethanol was estimated colorimetrically by using a potassium dichromate method (Lou et al., 2002; Sudarshan et al., 2011).

2.9 Immobilization of *S. cerevisiae* in calcium alginate and fermentation conditions

The calcium alginate gel-entrapping method was used as immobilization matrix. The spherical gels were readily obtained by adding different concentration sodium alginate solution to 2 % calcium chloride solution using a syringe. Sodium alginate solution added drop wise to form granules (Senthilraja et al., 2011).

2.9.1 Calcium alginate beads

The (no. of grams) % (by mass) Na-alginate solution was prepared by dissolving (2, 3, and 4) grams of sodium alginate powder into 100 ml of distilled water. Polymer/cell suspension was formed by mixing 100 ml of Na-alginate solution with 60 ml of thick yeast suspension at room temperature. Spherical micro beads were formed by extrusion of Na alginate/yeast cell suspension through a syringe with (0.8 X 38 mm) needle. The cell suspension was forced out of the tip of the needle at constant flow rate to the gelling bath, which was 2 % CaCl₂ solution. In this way, the yeast cells were entrapped in a gel matrix of Ca-alginate. The immobilized particles were rather uniform in size with the mean diameter of 0.2 mm. The beads were hardened in the solution for 1hr (Duarte et al., 2013). After gelling, the micro beads were washed twice with distilled water to remove the un-reacted material. The micro beads with cells were stored in a physiological solution at 4°C until use (Neelakandan et al., 2009).

3. Results

3.1 Characterization and identification of the isolated *S. cerevisiae* species

We observed colonies that were 2-3 mm in diameter, slightly convex, of smooth creamy consistency, white to cream in color and having a sweet smell that is typical of *S. cerevisiae* yeast. Microphotograph of yeast cells under the light microscope showing oval shape and budding characters.

3.2 Biochemical characterization

3.2.1 Carbohydrate source assimilation test

The isolated yeast from yogurt was able to utilize various sugars such as glucose, maltose, sucrose, but not lactose and xylose, and this matched the results of the reference *S. cerevisiae* as shown in Table1.

Table 1: The OD of isolated yeast at 660 nm for various carbon sources along 6 days.

		Time(Hr)					
		0	24	48	72	144	
OD 660	(Isolated strain)	Glucose	0.1	0.946	1.753	2.105	2.66
		Sucrose	0.062	1.832	2.161	2.356	2.418
		Maltose	0.103	1.584	1.907	2.238	2.352
		Xylose	0.111	0.093	0.084	0.084	0.084
		Lactose	0.168	0.138	0.193	0.193	0.193
	(Reference strain)	Glucose	0.1	0.781	1.721	2.211	2.55
		Sucrose	0.111	1.73	2.1	2.236	2.419
		Maltose	0.29	1.576	1.863	2.112	2.203
		Xylose	0.085	0.139	0.126	0.172	0.172
		Lactose	0.317	0.316	0.316	0.316	0.316

3.3 Ethanol tolerance test

The growth of the isolated yeast in different ethanol concentration showed that the maximum ethanol tolerance was at 10% concentration and gradually decreased at higher concentrations but, the reference *S. cerevisiae* strain showed maximum ethanol tolerance at 8% concentration and totally decreased at higher concentrations.

3.4 Pretreatment of wheat straw and tomato waste

3.4.1 Effect of different concentrations of acid on wheat straw

Effect of different concentrations of HCl and H₂SO₄ on the hydrolysis of wheat straw were tested with different time intervals of 1 to 5hrs by estimating the concentration of glucose as shown in Figures 1 and 2, respectively. The maximum amount of glucose was observed at 5% of H₂SO₄ with wheat straw at 3 hrs of hydrolysis process.

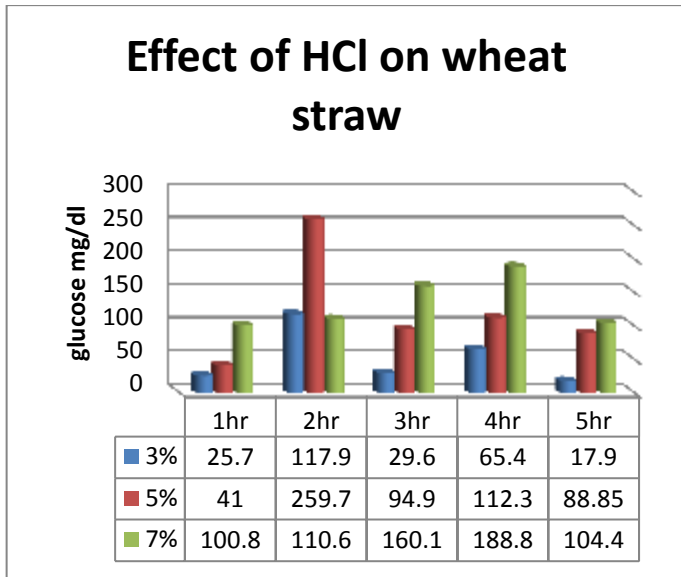


Figure 1: Effect of different concentrations of HCl on hydrolysis of wheat straw at different time intervals.

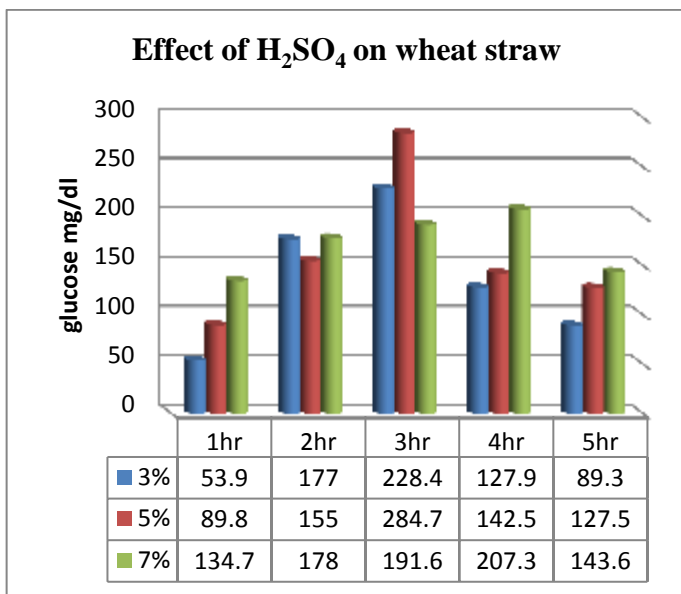


Figure 2: Effect of different concentrations of H₂SO₄ on hydrolysis of wheat straw at different time intervals.

3.4.2 Effect of different concentrations of acid on tomato wastes

Effect of different concentrations of HCl and H₂SO₄ on hydrolysis of tomato wastes were tested with different time intervals of 1 to 5hrs by estimating the concentration of reducing sugars as shown in Figures 3 and 4, respectively. The maximum amount of reducing

sugars was observed at 7% HCl concentration with tomato waste at 3 hr of hydrolysis process.

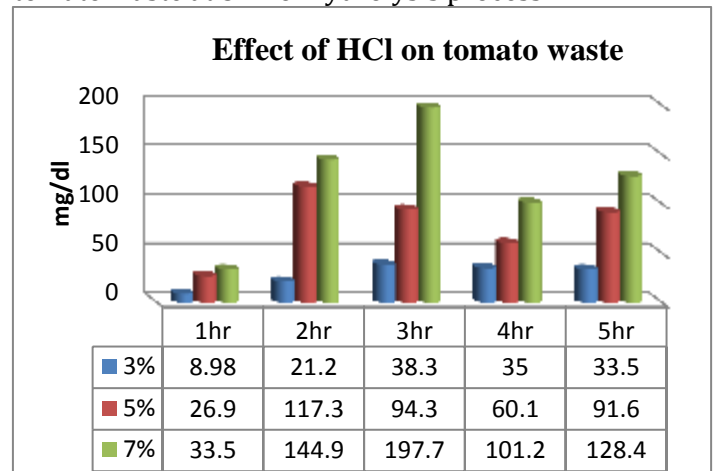


Figure 3: Effect of different concentrations of HCl on hydrolysis of tomato waste at different time intervals.

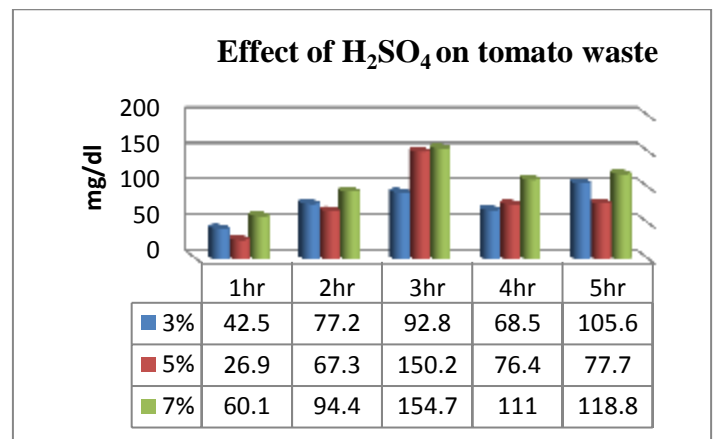


Figure 4: Effect of different concentrations of H₂SO₄ on hydrolysis of tomato waste at different time intervals.

3.5 Immobilization of yeast in calcium alginate beads

3.5.1 Size of beads

After adding the sodium alginate to calcium chloride, we examined the size of beads and we found that the beads size was equal to 2 mm as shown in the Figure 5. The beads kept in media at 4°C until used as shown in Figure 6.



Figure 5: Calcium alginate beads with 2 mm size.



Figure 6: Yeast cells entrapped in sodium alginate beads within media

3.5.2 Sodium alginate concentration

The different concentrations of sodium alginate were tested for 2 batches, the results shown that the second batch was better than the first but the beads start to degrade. Table 2 shows the ethanol yield for the different concentrations of sodium alginate used with isolated and reference yeast strains after 3 days of fermentation.

Table 2: Effect of different concentrations of sodium alginate for first and second batch fermentation

Na-alginate concentration	Type of cell	Ethanol conc. % (v/v)	
		Batch 1	Batch 2
2%	Reference	3.37	5.02
	Yogurt isolate	3.31	4.74
3%	Reference	3.18	6.59
	Yogurt isolate	2.88	4.98
4%	Reference	3.13	6.19
	Yogurt isolate	2.54	5.48

The results showed that the best concentration of sodium alginate used for cell immobilization was 3% and 4%, which gave the highest concentration of ethanol for reference and isolated yeast strains, respectively.

3.7 Glucose concentration (mg /dl)

Table (3) showed high increase in glucose concentration for free system for wheat straw with microwave assisted by 32.1% and 21.6% compared with conventional acid treatment for yogurt isolated yeast and reference strains, respectively. Also for immobilized system by 35.3% and 45.7% compared with acid treatment alone for yogurt isolated yeast and reference strains, respectively.

For free system with microwave assisted the glucose concentration increased by 15.3% and 31.3% compared with acid treatment alone for yogurt isolated yeast and reference strains, respectively.

Table 3: Reducing sugars at zero time illustrating the efficiency of microwave assistance treatment.

		Without microwave		With microwave	
		Tomato waste	Wheat straw	Tomato waste	Wheat straw
Free	Isolated	66.32	62.54	78.24	92.06
	Reference	49.61	70.81	72.25	90.21
Immobilized	Isolated	55.01	72.35	61.32	111.76
	Reference	47.91	46.32	68.09	85.29

3.8 Estimation of ethanol yield from pretreated lignocellulosic materials

Using potassium dichromate method, we constructed a standard curve for ethanol concentrations. Figure (7) showed that the highest concentration of ethanol was 641.22 mg/g from wheat straw which was produced by free cells with 5% H₂SO₄ microwave assisted hydrolysis.

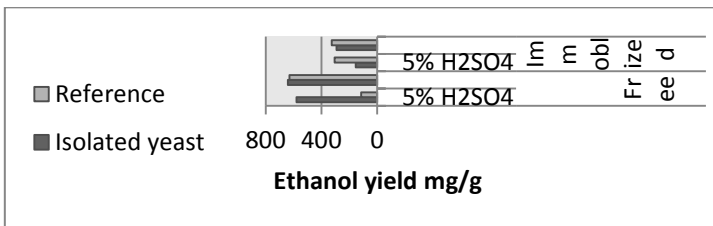


Figure 7: Ethanol yield from pretreated wheat straw mg/g.

As shown in Figure (8) the highest concentration of ethanol was 543.5 mg/g from tomato waste which was produced by immobilized cells with 7% HCl microwave assisted hydrolysis.

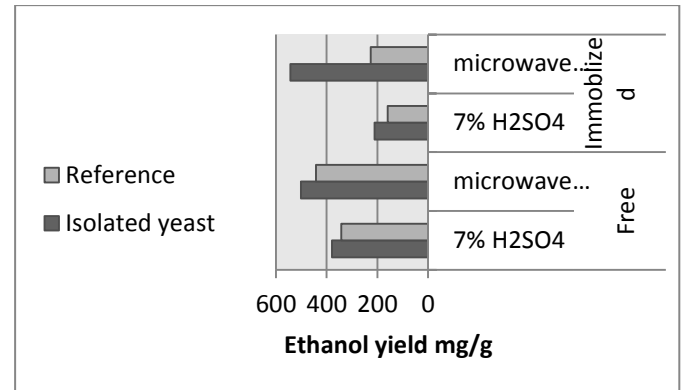


Figure 8: Estimation of ethanol yield from pretreated tomato waste mg/g.

4. Discussion

The biochemical characterization showed that the isolated strain can ferment glucose and many other hexoses (maltose, sucrose, galactose) but not lactose or xylose, which is similar to reference strain and to what reported by previous studies about *S. cerevisiae* (Shahbazi et al.,1998 and Goffeau, 2000). These finding along with the morphological characteristics confirm that the isolated strain was *S. cerevisiae* strain.

For tomato waste, the results indicated that pretreatment with acid (H₂SO₄) was not sufficient. This is similar to the results obtained by (Kumar et al., 2009) who concluded that pretreatment with acid (H₂SO₄) generally reduce the availability of total carbohydrate and reducing sugar for fermentation. For wheat straw (Al-Haj Ibrahim, 2012) reported that among acid pretreatment methods, dilute acid pretreatment using H₂SO₄ is the most-widely used method.

The results showed that the pretreated substrate with microwave assisted 5% H₂SO₄ and 7% HCl at 3 hr had significantly produced higher concentration of glucose yield than the samples treated with chemical pretreatment alone. Microwave heating could convert starch directly to glucose in relatively short time (Sunarti et al., 2012). The yields from microwave- HCl pretreatment were approximately 2.8 times that of chemical pretreatment alone but, the yields from microwave- H₂SO₄ pretreatment were approximately 1.48 times that of chemical pretreatment alone. Several other works are in agreement with our results which indicated that microwave pretreatment in the presence of chemical reagents would be more effective, as

reported by (Zeeman et al.,2009; Chen, et al., 2012; Sunarti et al., 2012 and Xia et al., 2013).

Calcium alginate is the best matrix that can be used as reported by (Beshay, 2003; Adinarayana et al.,2005 and Karagoz et al.,2014). Concentration of CaCl₂ in Ca-alginate preparation can influence the rate of ethanol production via altered matrix permeability for substrate as well as for product (Kongkiattikajorn et al., 2007). However Senac et al., (1991) found no improvement of the productivity after cell immobilization, indicating that the physiology of immobilized cells differ from that of free cells (Lohmeier-Vogel et al.,1995). The difference in behavior between immobilized and free cells is related to several factors. Nutrient limitations and microenvironment surrounding the cells are widely used to explain physiological and morphological changes of cells after immobilization (Holcberg et al., 1981).

In our study the size of particle was approximately 2 mm which is preferred for mass and heat transfer which agree with previous studies (Goksungur et al.,1999; Goksungur et al., 2001and Chen et al., 2015). Smaller beads yielded more ethanol, probably due to an increase in surface-volume ratio. The low amount of ethanol extracted from tomato waste was due to the presence of lycopene and carotenoids in tomato as described by (Muthumani et al., 2014). Ethanol yield using wheat straw was approximately 0.55g/g, which is in consistence with (Chen et al., 2015), indicate that immobilization of the yeast into Ca- alginate exhibited low substrate inhibition and high tolerance to ethanol since there was not declining phase in ethanol production.

Our result is considered relatively acceptable and close to the published data (Badger, 2002; Karimi et al., 2006 and Muthumani et al., 2014).

5. Conclusion and Recommendation

Wheat straw are promising feedstock for bioethanol production but, the tomato waste showed low ethanol yield. Using of immobilization technique is very promising technique, which can improve ethanol production, and reducing production cost. Wheat straw have higher cellulose content than tomato, but its degradation process is difficult and energy consuming. Microwave assisted dilute acid hydrolysis technology can greatly improve the hydrolysis efficiency of wheat straw and tomato waste. The immobilization technique

improves the ethanol yield with tomato waste using calcium alginate beads. The concentration of calcium alginate affects the process, therefore, it should be optimized. The production of ethanol in the second batch was better than the first one. Many factors should be optimized such as type of immobilization, type of matrix and concentration of calcium alginate.

Acknowledgments

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إنتاج الإيثانول الحيوي من بعض المخلفات الزراعية باستخدام خميرة الخبز بنظام التثبيت

كلمات مفتاحية:
الإيثانول الحيوي،
خميرة الخبز،
عملية التثبيت،
الميكرووييف،
قش القمح،
مخلفات البندورة.

إن عملية إنتاج الإيثانول الحيوي باستخدام المخلفات الزراعية يستحوذ على اهتمام العديد من الدول لاستخدامه كبديل للوقود الأحفوري كونه أمن ومتجدد. في الجيل الثاني من إنتاج الإيثانول يتم استخدام مواد رخيصة الثمن مثل المواد السيلولوزية مما يجعلها منافسة لمصادر الوقود الأحفوري في حين يعتبر قصب السكر والذرة من أكثر المواد المستخدمة لإنتاج الإيثانول ولكن كان لابد من التعرف على امكانية استخدام بقايا المخلفات الزراعية الفنية بالسكريات القابلة للتخمر مثل القش ومخلفات صناعة صلصة البندورة لتوافرها في فلسطين ورخص أثمانها. لقد كان هدف هذه الدراسة هو إنتاج كحول رخيص الثمن اعتمادا على مخلفات القش و مصانع البندورة في قطاع غزة مع المقارنة بين التخمر الحر والمثبت لكل منهما باستخدام خميرة محلية معزولة من اللبن المخيض. وقد تم التعرف و تشخيص هذه العزلة بناء على الفحوصات الشكلية و الكيميائية. وقد اعتمدت جميع النتائج على استخدام الميكرووييف مع الحمض المخفف للمعالجة الكيميائية حيث أظهرت النتائج تحسن كبير في كمية السكريات الأحادية عند استخدام الميكرووييف مع الحمض مقارنة مع المعالجة التقليدية بالحمض فقط وذلك بنسبة 45% مقارنة بالطرق التقليدية بتسخين الحمض المخفف من 7% H₂SO₄ أو 5% HCl . و كان أفضل تركيز للكاسيوم أجيئات المستخدم للتثبيت هو 3% للعينة الجاكمة و 4% للعزلة. وخلصت الدراسة إلى أن استخدام التثبيت يحسن من إنتاج الإيثانول عند استخدام مخلفات البندورة مقارنة مع الطريقة الحرة حيث بلغت كمية الإيثانول (543.5 ملغم/جم) في حين كانت أعلى إنتاجية للإيثانول (641 ملغم/جم) عند استخدام القش كمصدر لسكريات التخمر دون تثبيت.