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Continuous Ethanol Production from Molasses via Immobilized *Saccharomyces cerevisiae* on Different Carriers on Pilot Scale

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IMMOBILIZATION of yeast cells for continuous ethanol production has been extensively studied worldwide during the past few years because it showed significant advantages compared to the production by free cell system. Using renewable substrates such as Egyptian cane and beet molasses becomes necessary to reduce food crops for bioethanol production. This study investigates the immobilization of commercial strain of *Saccharomyces cerevisiae* on different carriers (sugarcane bagasse, rice straw, wheat straw, and Na-alginate) for continuous ethanol production on a pilot scale. The results demonstrated that sugarcane bagasse was the best carrier for yeast cell immobilization. This substrate produced 66.30g/ L ethanol, 2.76g/ L/ hr as ethanol productivity, and 81% fermentation efficiency from the theoretical value, using a mixture of Egyptian cane and beet molasses with 16% initial sugar at 30°C and pH 4.5. Also, the production process is retained until 18 days continuously. From this study, it was clear that lignocellulosic materials provide suitable, cheap, and renewable carriers for immobilization of yeast cells used in the continuous ethanol production process.

Keywords: Beet molasses, Cane molasses, Ethanol production, Immobilization, *Saccharomyces cerevisiae*.

Introduction

As the demand for ethanol is showing a progressive rise over the years, there is an imminent requirement of high yielding production strains and economically viable process realization for the production of ethanol (Kishore et al., 2011). One such process is yeast cell immobilization which facilitates faster fermentation rates by providing higher cell densities per fermentation volume unit.

The fermentation by immobilized cell has additional technical and economical advantages as compare to free cell system or traditional method, for example high fermentation rate, easy manner of preparation and handling higher substrate utilization longer operating life time, reduces the cost required for inoculum development, ease of separation to facilitate their recycle and simple harvest from the product, enhanced bioreactor productivity, reduces the cost of bioprocessing by eliminating long and expensive processes of cell recovery and cell recycle, less inhibition by product, not reduce the desired biocatalytic activity of cell, shield against high shear harm, make favorable microenvironment to cell, reduce the possibility of contamination and has high tolerance to alcohol (Krisch & Szajani, 1997). Bead formed by this process are fully active, flexible and hard to withstand mild agitation (Lee et al., 2011).

Immobilization can describe as the imprisonment of all types of biocatalysts including enzymes, cellular organelles, or cells in a distinct phase that allows exchange with, however is separated from the majority section or the external environment. And also, immobilization is the technique used for the physical or chemical

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fixation of cells, organelles, enzymes, proteins onto or into a solid support, or maintained by a membrane, so their stability is enhanced and it modify their continuous use (Lee et al., 2011). Immobilization encompasses a big selection of application in several industries like biotechnology, pharmaceutical, environmental, food and biosensor industries.

Fermentation systems operated in continuous mode provide variety of benefits compared to other fermentation processes, generally resulting in enhanced volumetric productivity and, consequently, smaller bioreactor volumes and lower investment and operational prices (Brethauer & Wyman, 2010). These continuous processes can benefit by whole cell immobilization techniques in order to retain high cell densities inside the bioreactors (Kierstan & Bucke, 1977).

The development of a fermentation medium based on industrial substrates is economically desirable (Pereira et al., 2010). and the optimization of industrial medium and fermentation conditions is a simple and effective way to economically produce of bioethanol (Darvishi & Hiligsmann, 2017). In the bioethanol production, the composition of the medium affects the physiological state and, consequently, the fermentation performance of the microorganism employed (Hahn-Hägerdal et al., 2005). Molasses from the sugar beet or sugar cane processing due to the high content of fermentable sugars, which can be used for fermentation, is a very good raw material which is traditionally used for ethanol production (Krajnc & Glavic, 2009; Zohri et al., 2012, 2014).

Among many microorganisms that have been exploited for ethanol production, *Saccharomyces cerevisiae* still remains as the prime species (Bai et al., 2008) which keeps the distillation cost low as it gives a high ethanol yield, a high productivity and can withstand high ethanol concentration (Kasavi et al., 2012). So, in this study immobilization of a commercial *Saccharomyces cerevisiae* yeast strain on three different lignocellulosic materials (sugar can bagasse, rice straw and wheat straw) in addition to Na-alginate was examined for ethanol production from Egyptian cane and beet molasses.

Materials and Methods

Yeast strain and inoculum preparation

Yeast strain used in this study was *Saccharomyces cerevisiae* (with the commercial name : EL-Tayeeb) which kindly obtained from EL-Hawamdia Chemical Factories, Egyptian Sugar and Integrated Industries Company (ESIIC), Giza, Egypt.

Inoculum was prepared by transferring one loopfull of 48hrs culture grown on a slant of yeast malt peptone glucose agar medium (YMPGA) which composed of 3g yeast extract, 3g malt extract, 5g peptone, 10g glucose and 20g agar dissolved in one liter of water (Wickerham, 1951) to 250mL Erlenmeyer flask containing 50mL sterilized YMPG broth. After incubation on a rotary shaker (150rpm) at 30ºC for 48hrs, the inoculum was propagated on molasses medium with 10% sugar concentration at 30ºC for 48hrs on rotatory shaker at 150rpm, immobilized on the carrier and transferred at 20% volume to the prepared fermentation molasses medium. The initial concentration of inoculum was maintained at 2×10^7 cells/mL in every case.

Preparation of fermented molasses medium

A mixture of Egyptian cane and beet molasses (1:1) from Abo-Qorqas sugar factory, EL- Minia, Egypt, was collected and used as a fermentation medium in this study. The physic- chemical analysis of cane and beet molasses in Abo- Qorqas sugar factory was achieved by Ibrahim (2016) and recorded in Table 1.

TABLE 1. Physico-chemical analysis of sugar cane and beet molasses.

The chemical analysis	Cane molasses	Beet molasses
Brix°	86.45	93.8
pH	5.1	8.1
Ash % g molasses	12.1	10.62
Total sugar % g	55.13	57.32
Non fermentable sugar % g	4.79	2.0
Fermentable sugar % g	50.34	55.32
Total reduced sugar % g	24.9	1.82
Nitrogen % g	0.61	1.3
Protein % g	3.81	8.12
CaO % g	1.58	2.0
Phosphate % g (as P_2O_5)	0.3	0.1

Mixture of Egyptian cane and beet molasses (1:1) was used with 16% sugar concentration which prepared by addition of water to the crude molasses mixture and supplemented by 1.5g/ L urea and 0.25g/ L magnesium sulphate. The treatment of molasses was achieved by heating the diluted molasses at 90°C for 30min after adjusting pH at 4.5 by H_3PO_4.

Immobilization of yeast inoculum

Three different lignocellolusic materials were tested as carriers for yeast immobilization: Sugar cane bagasse, rice straw and wheat straw. Fresh sugar cane bagasse was provided from Abo-Qurqas sugar factory, El-Minia, Egypt, while each of rice straw and wheat straw were provided from Agriculture research Center, Assiut, Egypt. The carrier materials were prepared by washing with distilled water until it become clean, cut into 1-2cm and then air dried. For pretreatment each one of carriers was diluted by distilled water (1:3, w/v), steamed at 100°C for 30min then incubated for 24hrs at room temperature. Afterwards, they were autoclaved at 121°C for 15 min. The immobilization performed by mixing equal weight of tested yeast strain with sterilized solid carriers suspension then it placed on a rotary shaker (120rpm) and kept at 30°C for 24hrs.

Alginate beads also used during this study for yeast cell immobilization which prepared as following: Prepared yeast inoculum were mixed with an equal volume (1:1, v/v) of 5% (w/v) Na-alginate solution (Roukas, 1996). Each 10l aliquot of alginate-cell suspension (unless otherwise stated) was added drop wise to 100l of 2% $CaCl_2$. Alginate drops solidified upon contact with $CaCl_2$, forming beads and the beads were allowed to solidify for 30min and then were cleaned with sterile saline solution (0.85% NaCl) to exclude excess calcium ions and cells. The average diameter of the beads was 5.01mm (Goksungur & Zorlu, 2001).

Continuous technique

This experiment was achieved at a set of three fermentors (75 liters each with working volume of 60 liters) attached directly and works as a battery (Fig. 1). This system consists of prepared molasses feeding tank, fermentation battery vessels (a, b & c) and fermented mash discharge tank (Fig. 1). A total of 50L of prepared molasses was first feeding to each of the three vessels (a, b & c) and inoculated with 10L of immobilized yeast inoculum for each one. Aerobic fermentation for the first 3hrs was employed and continues fermentation under anaerobic condition for 21hrs. After the first 24hrs, continuous feeding of new prepared molasses with discharge of fermented molasses at rate of 7.5L/ hr. The fermentation process was achieved at 30°C and 4.5 initial pH. The analytical data represented the average of the three ± standard deviation.

Analytical methods

Ethanol content was estimated by bichromate method (Zohri & Mostafa, 2000). Volumetric ethanol productivity (V. E. P. g/L/h) and ethanol yield from theoretical value ($Y_{E\ of\ TH}$) were calculated according to Siqueira et. al. (2008). Fermentation efficiency (F. eff %) expressed as g sugar utilized/100 g initial sugar (Roukas, 1996). Ethanol concentration over the consumed sugar by gram/gram [$Y_{E/CS}$ (g/g)] and ethanol concentration over the initial sugar by gram/ gram [$Y_{E/IS}$ (g/g)] as fermentation kinetics were calculated according to (Chanda & Chakrabarti, 1996). Total initial and total residual sugars (TIS and TRS) were determined using the 3, 5- dinitrosalicylic acid (DNS) method (Miller, 1959) after neutralization with 1N NaOH. Total consumed sugar (TCS) was calculated by deduct TRS from TIS. pH value measured by Microprocessor pH-mv meter pH526.

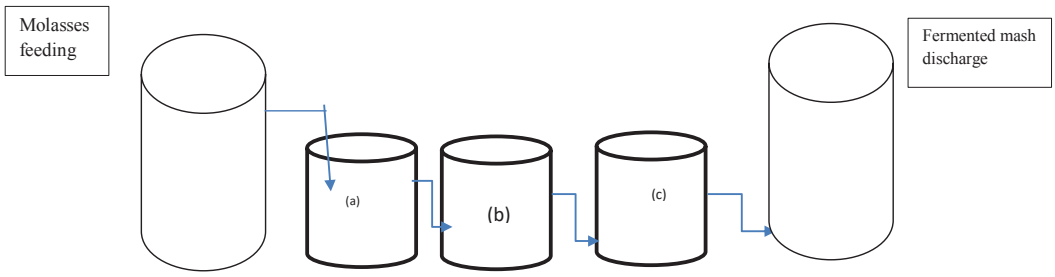

Fig. 1. Schematic diagram of continuous fermentation system.

Results and Discussion

Bioethanol has been identified as the mostly used biofuel worldwide since it significantly contributes to the reduction of crude oil consumption and environmental pollution. It can be produced from various types of feedstocks such as sucrose, starch, lignocellulosic and algal biomass through fermentation process by microorganisms (Azhar et al., 2017). Younis (2020) mention that it should be focused on addressing the global oil shortage by replacing biofuel production from edible crops with renewable natural source of perennial non-edible wild plants.

The cell immobilization and ethanol productivity of immobilized yeast cells depends on the surface characteristics of the support, such as pore size, water content, hydrophilic properties and magnetism (Fujii et al., 1999). And also, the process of cell adhesion to solid supports by biosorption is believed to occur due to electrostatic or van der Waals interactions between the cell membrane and the support. These adhesion forces are affected by variations in the medium composition and component concentrations, because they can strongly influence the surface energy of the immobilization support (Yu et al., 2007).

Continuous ethanol production from mixed molasses with 16% sugar concentration for 18 days by free cells of *Saccharomyces cerevisiae* and immobilized cells with different carriers (sugar cane bagasse, rice straw, wheat straw and Na-alginate) was investigated. It was cleared that ethanol concentration by using immobilized yeast cells on different carriers was better than that which produced by free cells on the long term. Where the peak ethanol concentration by free cells was (65g/L) after 48hrs (Fig. 2) then it showed an earlier termination of the process (after 10 days) comparing by immobilized yeast cells which continue the production of ethanol until 18 days (Table 2). This might be due to the high ethanol tolerance of immobilized comparing to free cells (Norton & D'Amore, 1994) discussed this phenomenon and suggested that can be attributed to cell encapsulation by a protective layer of gel material or to modified fatty acid concentration in cell membranes due to oxygen diffusion limitations. They also reported the partial removal of substrate inhibition by cell immobilization. Similar high tolerance was recorded by Dale et al., 1994) for immobilized *Kluyveromyces marxianus* yeast cells. This result is harmony with several studies as in case of Singh et al. (2009) who found that the maximum ethanol production was in cases of immobilized yeast cells on jute stick, bagasse, coconut coir and calcium alginate comparing to free cells. On the other hand, using immobilization technology prevents the removal of yeast cells with discharge of fermented mash as in case of using free cells technique.

TABLE 2. Some fermentation kinetic parameters of continuous ethanol production by immobilized *S. cerevisiae* on different substrates.

Immobilization substrate	$Y_{E/CS}$ (g/g)	$Y_{E/IS}$ (g/g)	E.L (g/L)	V.E.P (g/L/H)	Y_{Et} % of theo.	Fermentation period for ethanol level > 60 g/L (days)
Cane sugar bagasse	0.440	0.414	66.3	2.76	81.52	18
Wheat straw	0.438	0.410	65.52	2.73	80.29	17
Rice straw	0.437	0.405	64.74	2.70	79.33	16
Alginate	0.424	0.392	62.78	2.61	76.93	16
Free cells	0.431	0.406	65	2.71	79.65	10

[$Y_{E/CS}$ (g/g)]: Ethanol concentration over the consumed sugar by gram/gram.
[$Y_{E/IS}$ (g/g)]: Ethanol concentration over the initial sugar by gram/gram.
E. C: Ethanol concentration g/L.

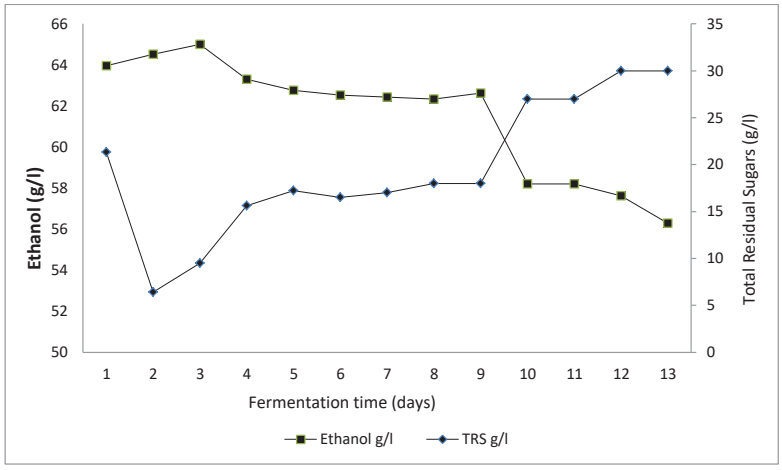

Fig. 2. Ethanol concentration (g/L) and total residual sugar (g/L) during ethanol production by 20% inoculum size of *S. cerevisiae* free cells in continuous fermentation culture using a mixture of Egyptian cane and beet molasses with 16% initial sugar at 30°C and pH 4.5.

In case of immobilization of yeast cells on sugarcane bagasse the ethanol production level reached 66.30g/ L after 96hrs (Fig. 3) compared to 64.74, 65.52 and 62.70g/ L ethanol after 72hrs by using immobilized yeast cells on rice straw, wheat straw and Na-alginate, respectively. These ethanol production levels were slower than that comparing by free cells which reached 65g/ L ethanol after 48hrs. This can be explained by the diffusion barrier. This barrier restricts some of the elements necessary for living cells and slows removal of secondary metabolites (Kourkoutas et al., 2004).

As shown in Table 2 the sugarcane bagasse was the most ideal carrier for ethanol production where the range of ethanol production was 60.06–66.30g/ L and the production process continue until 18 days followed by wheat straw (Fig. 4) which was 60.86– 65.52g/ L until 17 days and then rice straw (Fig. 5) was 60.60– 64.74g/ L until 16 days. These results may be depending on the content of lignin in the carrier. Whereas, sugarcane bagasse has 23- 32% of its dry weight as lignin and this is higher than lignin content of wheat straw (17– 91% of dry weight) and rice straw (12–14% of dry weight**)** (Kuhad et al., 1997; Reddy & Yang , 2005; Li et al., 2010). Escobar et al. (2012) mention in his research that lignin is the substance that gives stability and rigidity to lignocellulosic material. So, the low value of it may cause a structural weakness of material, it could affect the continuous operation for long periods, reducing their potential for use in immobilization processes on an industrial level.

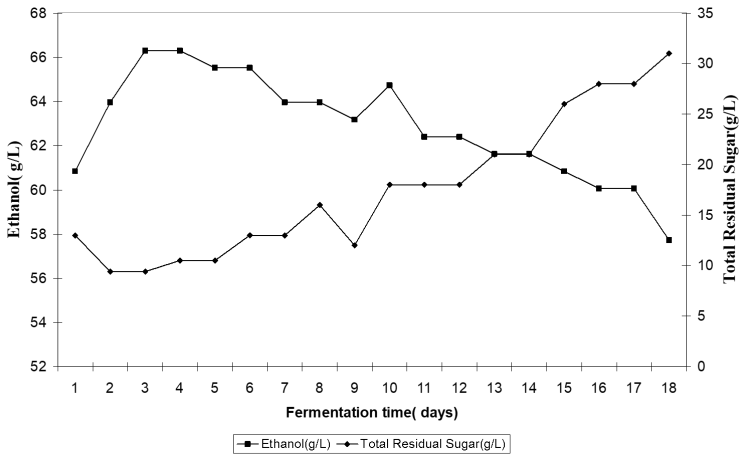

Fig. 3. Ethanol concentration (g/L) and total residual sugar (g/L) during ethanol production by 20% inoculum size of *S. cerevisiae* immobilized on sugar cane bagasse in continuous fermentation culture using a mixture of Egyptian cane and beet molasses with 16% initial sugar at 30°C and pH 4.5.

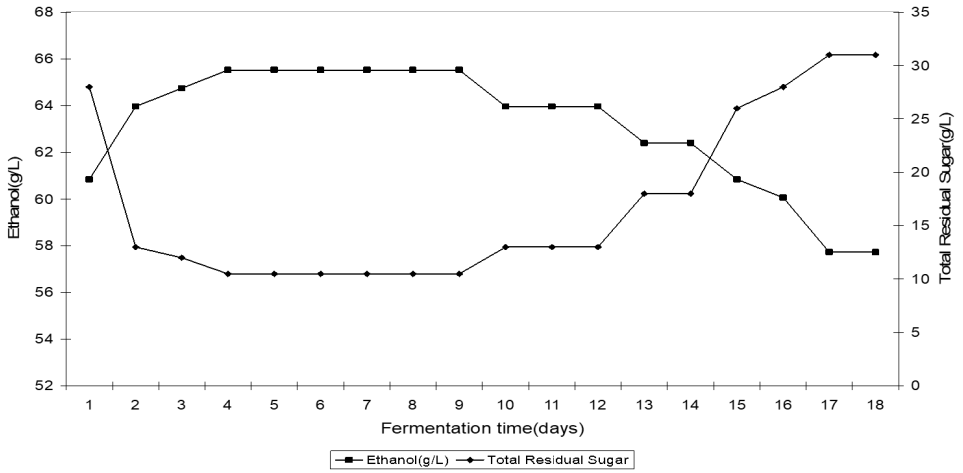

Fig. 4. Ethanol concentration (g/L) and total residual sugar (g/L) during ethanol production by 20% inoculum size of *S. cerevisiae* immobilized on wheat straw in continuous fermentation culture using a mixture of Egyptian cane and beet molasses with 16% initial sugar at 30°C and pH 4.5.

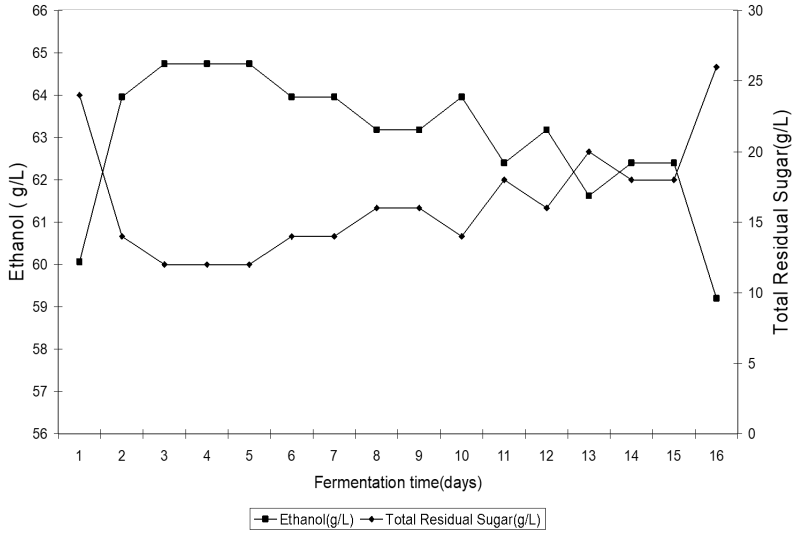

Fig. 5. Ethanol concentration (g/L) and total residual sugar (g/L) during ethanol production by 20% inoculum size of *S. cerevisiae* immobilized on rice straw in continuous fermentation culture using a mixture of Egyptian cane and beet molasses with 16% initial sugar at 30°C and pH 4.5.

Arinbasarova et al. (1982) mention that the degree of cell immobilization depends on the structure and therefore the size of adsorbent pores. Samonin & Elikova (2004) suggested that the nature of adsorbents is also important and organic adsorbents are with chemicals stable and show an excellent form of surface properties and pore structures.

The lowest concentration of ethanol (62.78g/ L) was produced when alginate used as yeast carrier in this investigation (Fig. 6). It well known that alginate present as gel beads in the medium but during fermentation carbon dioxide which produced by yeast cells caused the formation of cracks and capillaries in the alginate hydrogel, thus reducing the diffusion limit and so the transport of nutrients and metabolites is facilitated through the created structures using capillary forces (Poreda et al., 2011). In contrast Reda & Shanawany (2020) revealed that the maximum hyaluronidase enzyme immobilization yield (67.2%) was observed with Ca-alginate followed by agar-agar and Silica gel respectively.

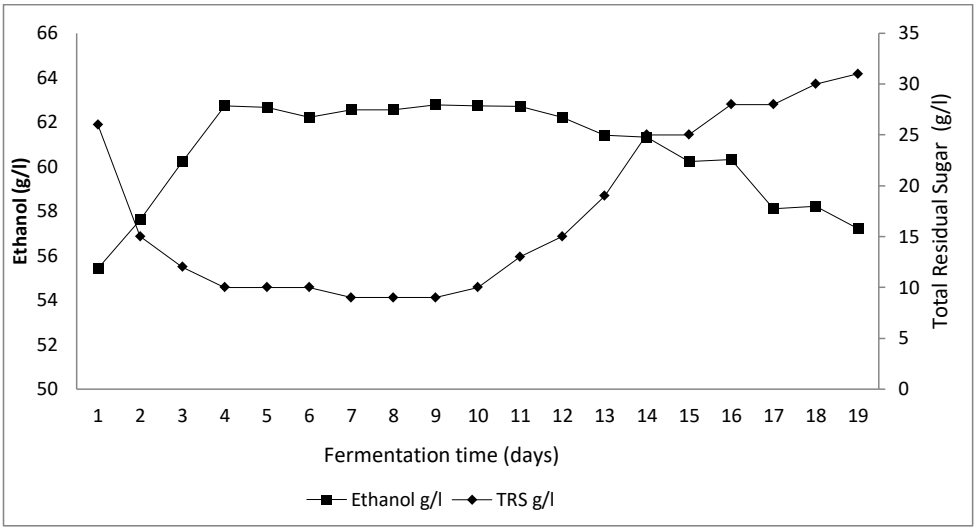

Fig. 6. Ethanol concentration (g/L) and total residual sugar (g/L) during ethanol production by 20% inoculum size of *S. cerevisiae* immobilized on Na-alginate in continuous fermentation culture using a mixture of Egyptian cane and beet molasses with 16% initial sugar at 30°C and pH 4.5.

Vucurovic et al. (2009) observed that the ethanol concentration produced by immobilization of yeast cells of *Saccharomyces cerevisiae* on corn stems is more than that produced by free cells. Also, Tang & Le (2013) compared the productivity of ethanol produced by the immobilized yeast cells of *Saccharomyces cerevisiae* on cork root and that produced by free yeast, and they observed that the productivity of ethanol in case of immobilized cells was more than that for free cells.

Conclusion

This study demonstrated that lignocellulosic materials (sugarcane bagasse, wheat straw and rice straw) could be used as suitable carriers for continuous ethanol production on industrial scale. It offers additional advantages where immobilization using these materials is easy and it is available in large amounts with low cost. Bagasse of sugarcane was the best one comparing to other carriers. Stability of immobilization system was better than free cell system. So, this was attractive option to maximize yield of ethanol production on industrial scale.

Conflict of interest: The authors reported no potential conflict of interest.

Authors contribution: AZ suggested the idea of the paper, follow up the work and also help in writing the paper; MA contributed in practical part and also in writing the paper; OI helped in the practical part in the AboQurqas Distillation Factory, Al-Minia, Egypt.

Ethical approval: Not applicable.

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الانتاج المستمر للكحول الايثيلى من المولاس باستخدام خلايا الخميرة المقيدة على دعامات مختلفة على النطاق التجريبى

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فى الاعوام القليلة الماضية هناك العديد من الدراسات التى تم فيها استخدام تقنية خلايا الخميرة المقيدة للانتاج المستمر للكحول الايثلى وقد اظهرة هذه الدراسات نتائج أفضل من استخدام الخلايا الحرة. ولذلك أهتمت هذه الدراسة بأستخدام تقنية تقييد خلايا الخميرة *Saccharomyces cerevisiae* على دعامات سيليلوزية مختلفة وهى (مصاصة قصب السكر و قش الارز و قش القمح و الجينات الصوديوم) للانتاج المستمر للكحول الايثيلى على النطاق التجريبى. لقد أظهرت الدراسة أن مصاصة قصب السكر تعتبر أفضل الدعامة السيليلوزية لانتاج الكحول الايثيلى حيث انتجت66.30 جرام/لتر باستخدام مخلوط من مولاس القصب و مولاس البنجر بتركيز 16% سكر عند درجة حرارة 30 °C و 4.5 pH .