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# Bioethanol production from corn and barley wastes by *Aspergillus flavus*

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## Abstract

Bioethanol has been identified as the most used biofuel worldwide since it significantly contributes to the reduction of crude oil consumption, and environmental pollution. In this work we concentrated on studying the ability of fungi to produce biofuel from plants residues, for that reason the fungus *Aspergillus flavus* was isolated from soil and screened for its ability to degrade sugars and bioethanol production. The results showed its ability to degrade the simple sugar glucose in the primary test and produce 4.795 g/l bioethanol. The results showed its ability to degrade corn, and barley wastes and produce bioethanol, as the highest concentration of bioethanol was 6.363 g/l with corn waste, and 5.570 g/l with barley waste after 96h of incubation. Then, the optimal pH and nitrogen source were applied to the fungus *A. flavus* to know their effect on its ability to produce bioethanol in liquid media containing corn or barley wastes. The results showed that the application of optimum conditions improved the production of bioethanol, where the highest bioethanol production was 8.041 g/l after 96h with corn, and 7.018 g/l with barley wastes after 144h of incubation.

Keywords: Agricultural wastes; *Aspergillus flavus*; Bioethanol production

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## Introduction

The increasing in energy sources demands and shortage of fossil fuel production and led to an increase in the costs of the petroleum fuels that jeopardize the economic progress of nations (Arumugam *et al*., 2020). Fuel crisis accelerated global warming which is another major problem that the world faces today, the increasing $CO_2$ concentrations and other greenhouse gases in the atmosphere are primarily due to the combustion of fossil fuels. In view of the rising world population, depleting fossil fuels resources, and global warming, the utmost challenge for the international community today is to attain sustainable economic development, as well as environmental security. The growing concerns of significant global climate change, and national energy security have triggered the quest for renewable sources of energy (Das and Vanasi,2019).

One of the solutions to this problem is the use of alternative energy sources such as bioethanol which is one of the most used liquid fuels as an alternative to fossil fuel. Bioethanol fermentation is by far the largest scale microbial process, its production from

renewable biomass has received considerable attention in recent years (Zabed *et al.*, 2016).

Lignoellulosic materials were composed of cellulose, lignin, hemicellulose, lipids, proteins, and inorganic materials. The enzymes secreted by fungi play an important role in the decomposition process of lignocellulosic waste. There are many fungal enzymes play an important role in the bioethanol production process, such as lignase and cellulase enzymes (Aranda-Martinez *et al*., 2017). Cellulases enzyme are known as responsible for catalyzing depolymerization reactions of cellulose, one of the most abundant organic compounds on earth with around 15-60 % of the cell wall components, into simple sugar components glucose (Cunha *et al*., 2020).

The lignin is a large molecule and has difficult decomposition properties. Lignin constitutes about 15% to 25% of the content of lignocellulos and it is highly complex due to its high molecular weight although this compound is difficult to decompose. Some fungi, especially white rot fungi, where able to secrete a group of enzymes that can analyze this compound using ligninase and laccases and manganese peroxidase (Moreno *et al.*, 2015; Ali and Al-Dossary, 2019).

The production of bioethanol from lignocellulosic materials involves three steps, pre-treatment, hydrolysis, and fermentation. the pretreatment of the material to decrease the particle size and increase the contact surface area of the plant biomass with enzyme during saccharification. It is usually done to remove lignin or reduce its concentration. This will enhance the hydrolysis of cellulose and hemicellulose either by chemical or enzymes to yield fermentable sugars, that are converted to bioethanol during fermentation. The fermentation of the sugars obtained from the hydrolysis to ethanol is the final step of the process (Mushin, 2014; Adeyemo *et al*., 2021).

This study aimed to recycle agricultural wastes of corn and barley by the fungus *Aspergillus flavus* and produce bioethanol.

## Materials and Methods

### Fungal isolation

Seven soil samples were collected from different agricultural areas in Basra governorate. Fungi were isolated from seven soil samples by using the dilution method (Wicklow and Wittingham, 1974), by using two different types of media Potato Carrot Agar (PCA) and Malt Extract Agar (MEA). The isolation media preparation was done depending to the direction of the manufacturing company (Hi media, India). The isolated fungi identified according to the following references Raper and Fennell (1973), de Hoog and Guarro (1995), Watanabe (2002), Guarro *et al*. (2012).

### Production of bioethanol from the glucose containing medium

The fungus *Aspergillus flavus* was selected to study its ability to produce bioethanol from glucose sugar according to Asachi *et al*. (2011), this was done by using 250 ml conical flasks each one containing 100 ml from fermentation medium (Mendel's medium ).It was prepared by adding (g/l): 5 yeast extract, 7.5 $(NH_4)_2SO_4$, 3.5 $K_2HPO_4$, 1 $CaCl_2.2H_2O$, 0.75 $MgSO_4.7H_2O$, and 40 glucose, the pH was adjusted to 5, and then autoclaved at 121 °C for 20min. Then 5 mm disk taken from the fungus culture was added to each flask, control flasks without any fungus inoculum were made. All flasks were made in duplicate and incubated at 25°c for 48 hours

on the semi aerobics. After incubation, detection of the bioethanol was made using Jone's reagent**,** and the absorbance was measured at 580 nm wavelength with UV/ Visible spectrophotometer.

**Production of bioethanol from plant wastes**

**Ability of *Aspergillus flavus* to produce bioethanol from corn and barley wastes.**

The ability of the fungus *Aspergillus flavus* to produce bioethanol from corn and barley wastes was tested according to Im *et al*. (2016) in the fermentation broth medium which contain g/l: 10 g/l yeast extract, 10 g/l $KH_2PO_4$, 2 g/l $(NH_4)_2SO_4$, and 0.5 g/l $MgSO_4 \cdot 7H_2O$ , the pH of medium was adjusted to 6, then 250 ml conical flask was used and 100 ml of the liquid medium was added to each flask, then 2 g of corn or barley wastes powder were added to each flask separately, all flasks sets were made in duplicate and sterilized by autoclaving at 121°C for 20min. After sterilization, a 5 mm disk taken from the fungus culture was added to each flask, control flasks without any fungus inoculum were made all flasks were incubated at 25°c for 6 days. The determination of the residual sugars and bioethanol were made every 48 hours of incubation by Dinitrosalicylic acid (DNSA) reagents Jone's reagent for the bioethanol. Then the absorbance was measured UV/Visible spectrophotometer at 540 nm wavelength for the residual sugars and at 580 nm wavelength for the bioethanol.

**Effect of pH on the fungus growth and bioethanol production**

The effect of pH on consumption of sugars and bioethanol production was tested. This was done by carrying out the fermentation at three different pH values 6, 5.5 and 6.5 according to Sarabana, *et al*. (2015). The fermentation procedure was made as previously**,** and sets were made for each pH value. Then the flasks were incubated at 25°C for 6 days. The residual sugars and bioethanol **were** detected every 48 hours as previously.

**Effect of nitrogen sources on the fungus growth and bioethanol production**

Yeast extract and sodium nitrate were used to study their effect on the consumption of sugars and bioethanol production. The fermentation procedure was made as previously and sets were made for each nitrogen source, these flasks were autoclaved at 121°C for 20 minutes. Then the flasks were incubated at 25°C for 6 days. The residual sugars and bioethanol were detected every 48 hours as previously.

**Production of bioethanol under optimal conditions**

According to previous experiments, the ability of the fungus to consume sugars and produce bioethanol was tested under optimal conditions for its growth.

**Statistical analysis**

Minitab version 16 software was used to analyze the results using one-way analysis of variance. The relative least significant difference values were calculated to determine significant differences between the fungal processes. A completely randomized design was used.

R**esults and discussion**

**Fungal isolates:**

Sixteen fungal species were isolated from seven soil samples (Table 1). 76.4% of the isolated fungi belonged to the anamorphic fungi with thirteen species. Followed by the zygomycota and ascomycota with 11.7 % and two species for each group. The appearance of the anamorphic fungi in high percentage may be due to the ability of these fungi to produce a

large number of reproductive units, and the secretion of different enzymes also, they possess a great ability to tolerate the stress in the environment, all of these features and others made them one of the largest group of fungi in the environment (Al-sadoon *et al*., 2014; Tian *et al*.,2017; Altaee and Al-Dossary, 2021).

**Table 1: The isolated fungi with their percentage of occurrence**

| No. | Fungal species | %of occurrence |
|---|---|---|
| **1** | *Aspergillus clavatus* | 14.2 |
| **2** | *A. candidus* | 14.2 |
| **3** | *A. flavus* | 42.8 |
| **4** | *A. fumigatus* | 71.4 |
| **5** | *A. niger* | 100 |
| **6** | *A. terreus* | 71.4 |
| **7** | *A. versicolor* | 71.4 |
| **8** | *Aspergillus* sp. | 14.2 |
| **9** | *Curvularia* sp. | 28.5 |
| **10** | *Emericella* sp. | 14.2 |
| **11** | *Microascous* sp. | 14.2 |
| **12** | *Mucor* sp. | 14.2 |
| **13** | *Penicillium* sp.1 | 100 |
| **14** | *Penicillium* sp.2 | 28.5 |
| **15** | *Phoma* sp. | 42.8 |
| **16** | *Rhizopus* sp. | 14.2 |
| **17** | *Trichoderma* sp. | 14.2 |

**Production of bioethanol from glucose containing medium:**

Preliminary test was conducted for the fungus *Aspergillus flavus* to find out their ability to produce bioethanol in the medium glucose containing medium. The results have shown that the fungus *A. flavus* had a good ability to produce bioethanol with a concentration of 4.795 g/l after 48 hours of incubation. The current study agrees with Abou El- Khair *et al*. (2014) where they found that the fungus *Mucor indicus* was able to produce 4.37 g/l from bioethanol in the medium glucose containing medium.

**Preliminary test of the ability of *Aspergillus flavus* to produce bioethanol from corn and barley wastes.**

The fungus *Aspergillus flavus* was tested to study its ability to degrade corn and barley wastes and find out its ability to produce bioethanol for 6 days of incubation. The results have showed that the concentrations of residual sugars were slightly decreased to 9.822 mg/g after 48h, and reached the lowest concentration 4.542 mg/g after 144 h of incubation compared with the control 10.8 mg/g. While with barley wastes, the results have showed a slight decrease in the concentrations of residual sugars through the first 48 hours of incubation, which reached 2.425 mg/g compared with the control 3.702 mg/g. After 96 hours of incubation, a slight increase was observed in the concentrations of the residual sugars, which reached 3.621 mg/g, and after 144 hours it decreases again to reach 2.731 mg/g (Table 2). The residual sugars in the fermentation media were observed to decrease with time, this could be attributed to the utilization of the sugar as carbon source for the growth of the microorganisms, and subsequent ethanol production, the slight increase in residual sugars concentrations represents the release of more reducing sugars by the action of extracellular enzymes more than which were consumed by the fungus (Ado *et al.*, 2010).

Our study is consistent with Rasmussen *et al*. (2010) and Abood *et al*. (2021).

**Table 2: Concentrations of residual sugars in the preliminary test** by ***Aspergillus flavus*.**

| **Fungus** | **Plant wastes** | **Concentrations of residual sugars (mg/g)** | | | |
|---|---|---|---|---|---|
| | | **Control** | **After 48h** | **After 96h** | **After 144h** |
| *Aspergillus flavus* | Corn wastes | 10.8 | 9.822 | 7.471 | 4.542 |
| | Barley wastes | 3.702 | 2.425 | 3.621 | 2.731 |

As for bioethanol production by *A. flavus* with corn wastes, the concentrations were increased to 2.28 g/l after 48h of incubation and increased to reached 6.363 g/l after 96h. After that it decreased slightly to reached 6.311 g/l. Whereas for barley

wastes the concentrations were increased to 4.049 g/l after 48h of incubation and reached the highest concentration 5.570 g/l after 96h then the concentration was decreased to 5.526 g/l after 144h (Table 3). The decrease in the bioethanol concentrations may be due to the evaporation of bioethanol from the fermentation medium or the oxidation of bioethanol to acetic acid. Another possible explanation is reduction in nutrient of the medium, formation of other metabolites and decrease in the pH of the medium, all these reasons not favorable for bioethanol synthesis (Betiku *et al*., 2015; Kumoro et al., 2021).

The current study agrees with Braide *et al*. (2016) where they found that maximum production of bioethanol reached after three days of incubation.

**Table 3: Concentrations of bioethanol produced in the preliminary test** by ***Aspergillus flavus*.**

| **Fungus** | **Plant wastes** | **Concentrations of bioethanol (g/l)** | | |
|---|---|---|---|---|
| | | **After 48h** | **After 96h** | **After 144h** |
| *Aspergillus flavus* | Corn wastes | 2.28 | 6.363 | 6.311 |
| | Barley wastes | 4.049 | 5.570 | 5.526 |

**Factors effecting the production of bioethanol from corn or barley wastes by *Aspergillus flavus***

**Effect of pH**

The pH 6.5 was the best for the fungus to consume sugars with corn wastes. The highest decrease in residual sugars concentrations was 3.278 mg/g after 144 h of incubation compared with control 10.903 mg/g (Fig. 1). While the pH 5.5 was the best for the fungus to consume sugars, when using barley waste the highest decrease in residual sugars concentrations was after 96h of incubation and reached 2.160 mg/g compared with the control 2.980 mg/g (Fig. 2).

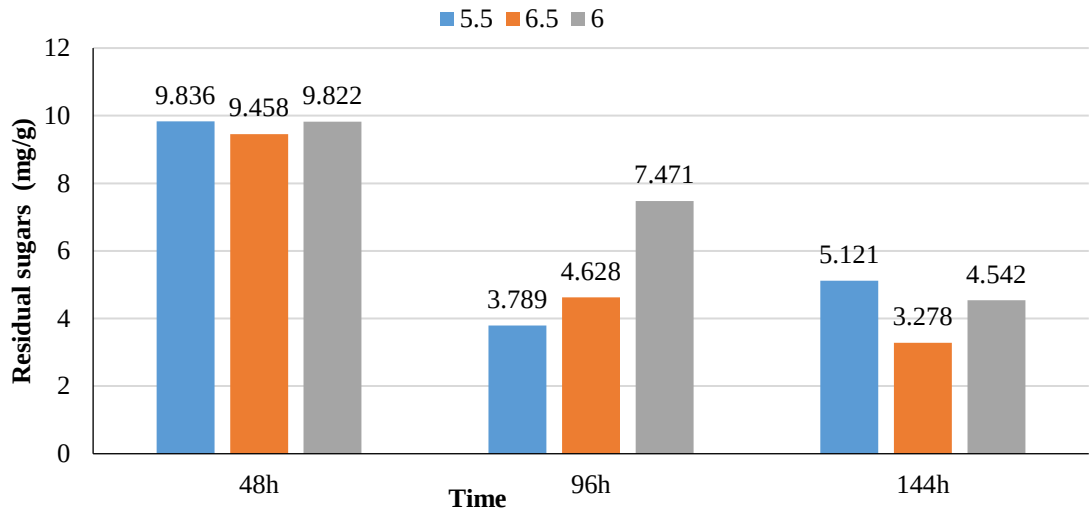


**Fig. 1: The effect of pH on sugars concentrations in corn wastes**

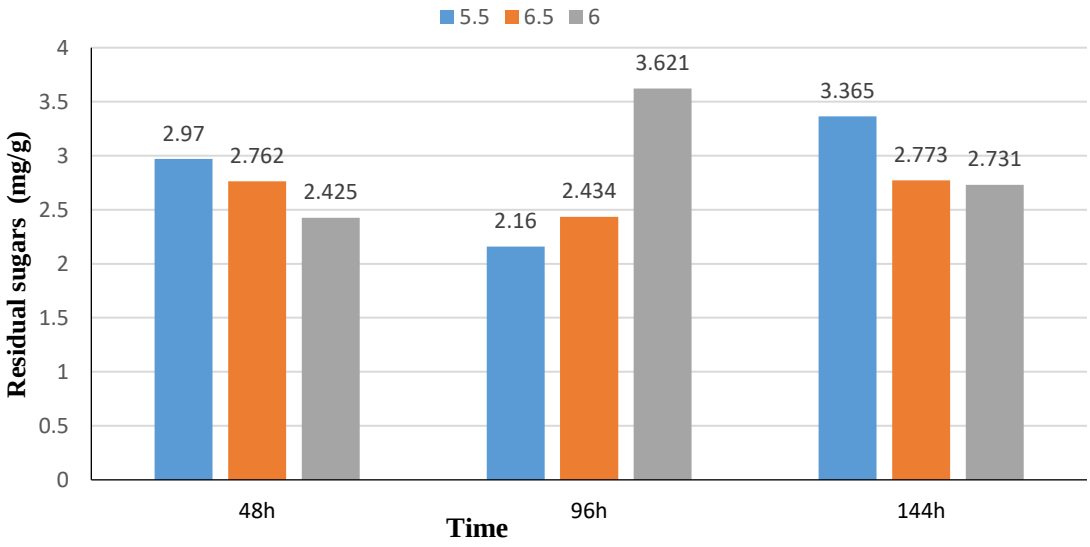


**Fig. 2: The effect of pH on sugars concentrations in barley wastes**

The highest bioethanol production with corn wastes at pH 5.5**,** and 6.5 were 7.01 g/l and 5.201 g/l respectively after 144h of incubation, and at pH 6 was 6.363 g/l after 96h. (Fig. 3). While the highest bioethanol production with barley wastes at the pH 5.5 and 6.5 were 5.964 g/l and 4.279 g/l respectively after 144h and at the pH 6 was 5.571 g/l after 96h of incubation (Fig. 4).

The pH of the culture medium has a significant effect on the growth and metabolic activities of the microorganisms, thus any change in the pH of the medium affects many biological functions such as enzymatic processes and transportations of various components across the cytoplasmic membrane (Betiku *et al*., 2015; Udegbunam et al., 2018; Al-Dossary *et al*., 2020).

Our results were consistent with Gawande and Patil (2018) who found that the best pH value for the fungus of the consumption sugars and production of bioethanol for the fungus *Aspergillus niger* was at pH 5.5.

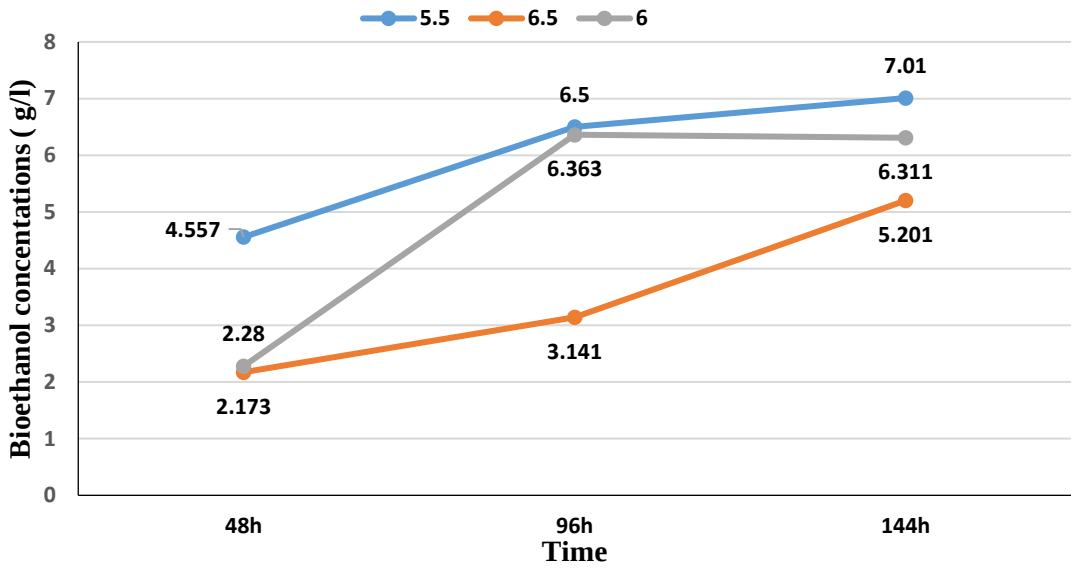


**Fig. 3: The effect of pH on bioethanol concentrations in corn wastes**

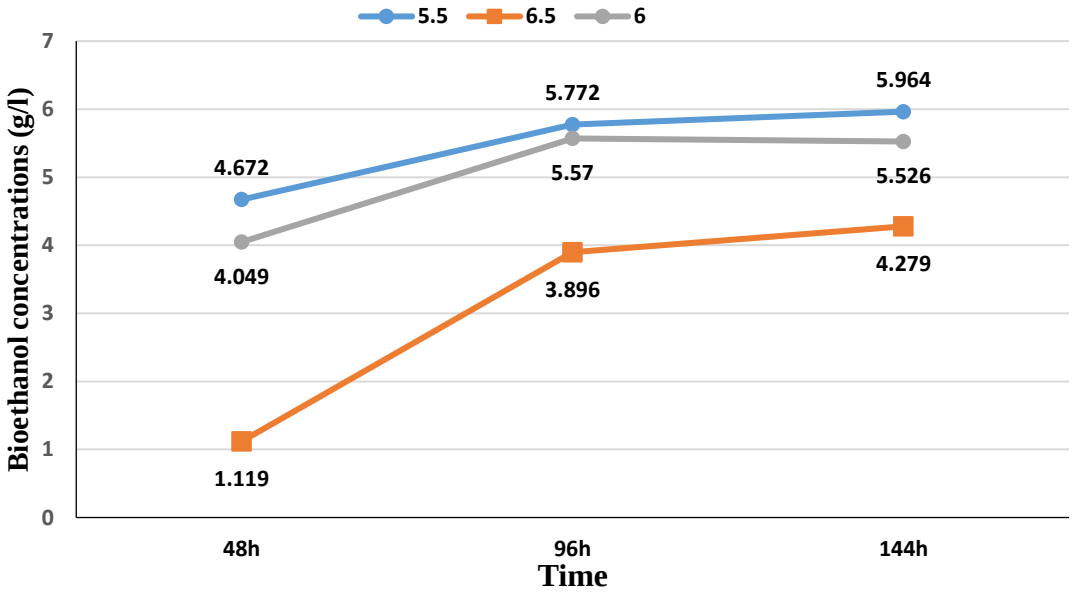


**Fig. 4: The effect of pH on bioethanol concentrations in barley wastes**

**Effect of nitrogen source**

Two nitrogen sources, yeast extract and sodium nitrate were taken to optimize the nitrogen source for maximum bioethanol production by the fungus *Aspergillus flavus*. the yeast extract was the best for the fungus to consume corn sugars, in which the highest decrease in residual sugars concentrations with corn wastes were 4.542 mg/l after 144h of incubation compared with control 10.8 (Table.2) mg/g, while with sodium nitrate, the higher decrease was 5.369 mg/g after 48h compared with control 10.8 mg/g (Fig. 5). In barley waste sodium nitrate was the best for the fungus, in which the highest decrease in residual sugars with yeast extract was 2.425 mg/g after 48h compared with control 3.702 mg/g and as for the concentrations of residual sugars with sodium nitrate the highest decrease in sugars concentration was 1.335 mg/g after 48h compared with control 3.621 mg/g (Fig. 6).

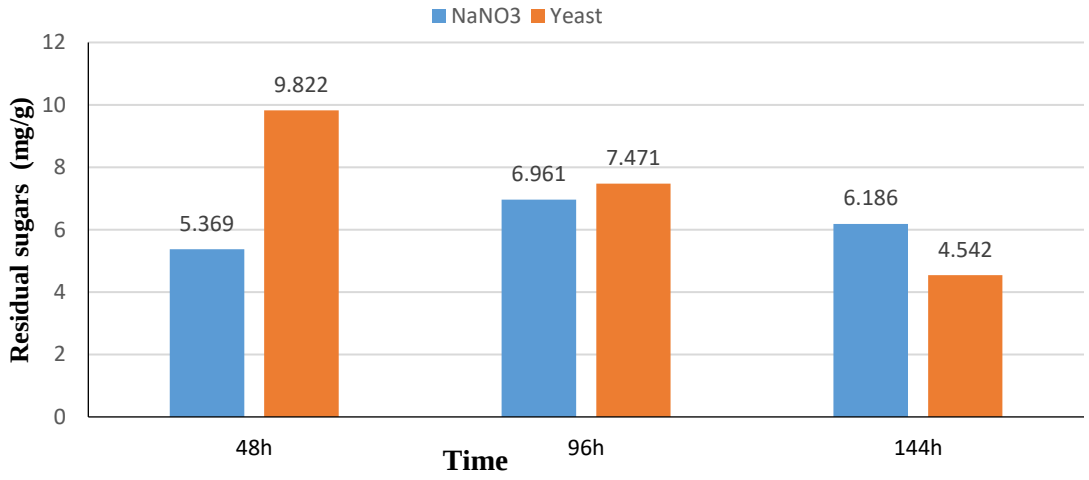


**Fig. 5: The effect of nitrogen source on sugars concentrations in corn wastes**

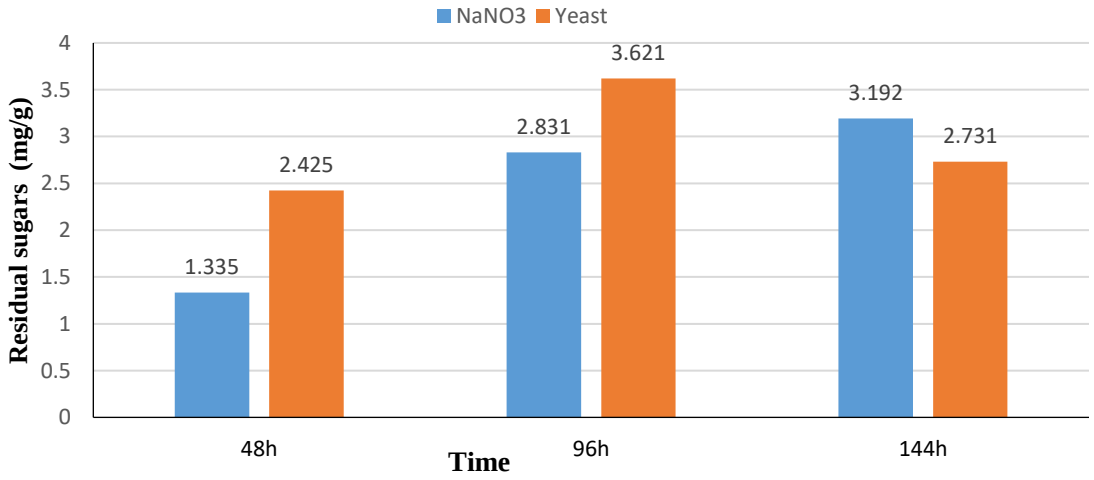


**Fig. 6: The effect of nitrogen source on sugars concentrations in barley wastes**

The results showed that sodium nitrate is the best nitrogen source for bioethanol production, where the highest concentration of bioethanol with corn wastes was 7.1 g/l after 144h of incubation and when using yeast extract, at the same time the results showed that the highest concentration of bioethanol was 6.363g/l after 96h (Fig. 7), but in barley wastes, the highest concentration of bioethanol with sodium nitrate was 5.957 g/l after 96h of incubation , and with yeast extract was 5.571 g/l after 96h of incubation (Fig. 8).

The nitrogen source is one of the most important factors that nourish the fungal growth, the nitrogen source is one of the most important factors affecting the metabolites processes of the microorganism, including protein synthesis, DNA production and enzymes (Abdullah *et al.*, 2000; Abbasiliasi *et al.*, 2017).

The current study disagrees with Sarabana *et al.* (2015) who found that the highest concentration of bioethanol produced by the fungus *Phanerochaete chrysosporium* was found when using yeast extract as an organic nitrogen source.

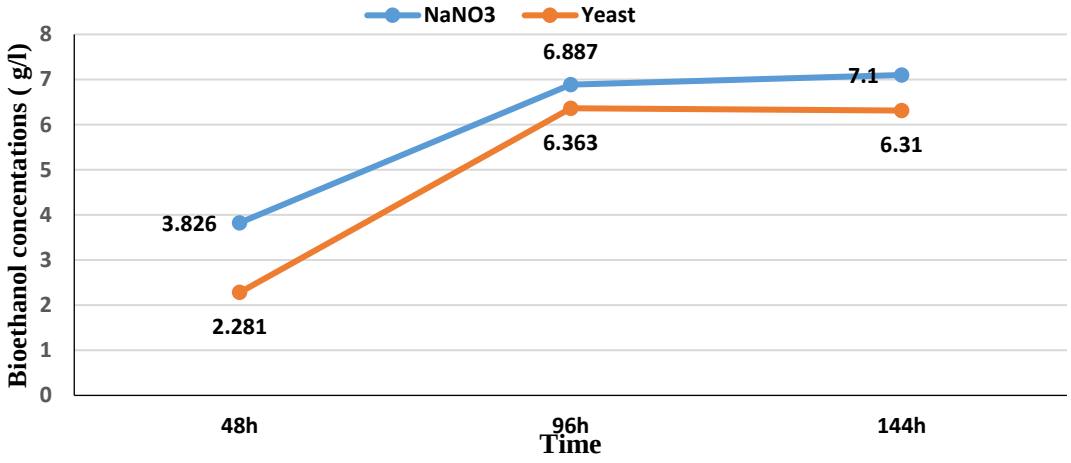


**Fig. 7: The effect of nitrogen source on bioethanol concentrations in corn wastes.**

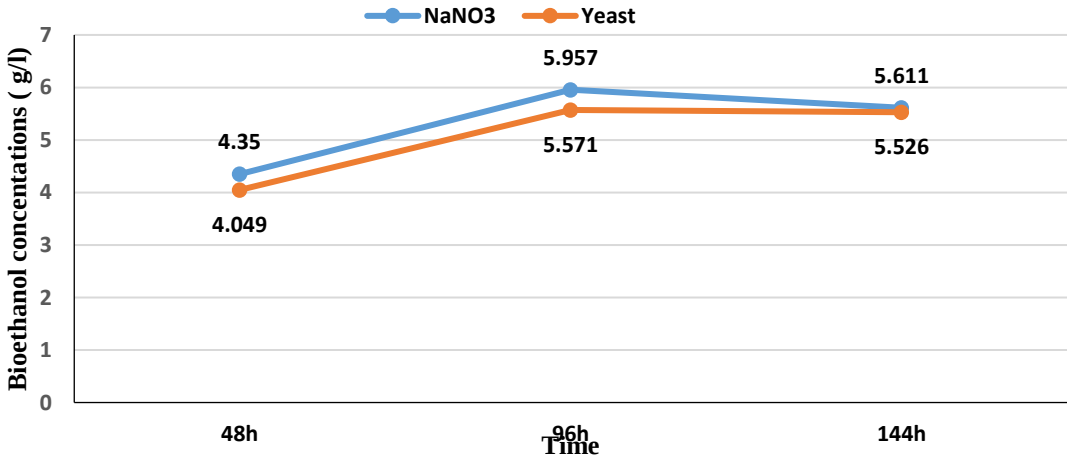


**Fig. 8: The effect of nitrogen source on bioethanol concentrations in barley wastes**

**Production of bioethanol under optimal conditions**

The ability of the fungus to consume sugars and produce bioethanol was tested when applying the optimum conditions selected from the previous experiments. The results showed that the highest decrease in the concentrations of residual sugars with corn wastes was 1.62 mg/g after 144h of incubation compared with control 10.216 mg/g, and with barley wastes was 0.807 mg/g after 144h compared with control 3.947 mg/g (Table 4).

The highest concentration of bioethanol with corn wastes was found after 96h of incubation 8.041 g/l. While, with barley wastes the highest concentration of bioethanol was after 144h 7.018 g/l (Table 5).

The results showed that the application of optimal conditions stimulated the growth of the fungus, and increased their enzymatic activity, and when the growth of the fungus increase its enzymatic activity also increased, the greater in enzymes secretion led to more hydrolysis and more consumption of the plant wastes, and thus increase the production of bioethanol (Rath *et al*., 2014).

**Table 4: The effect of optimal conditions on sugars concentrations in corn and barley wastes.**

| Fungus | Plant wastes | Concentrations of residual sugars (mg/g) | | | |
|---|---|---|---|---|---|
| | | Control | After 48h | After 96h | After 144h |
| *Aspergillus flavus* | Corn wastes | 10.216 | 8.382 | 4.207 | 1.62 |
| | Barley wastes | 3.947 | 1.508 | 0.818 | 0.807 |

**Table 5: The effect of optimal conditions on bioethanol concentrations in corn and barley wastes.**

| Fungus | Plant wastes | Concentrations of bioethanol (g/l) | | |
|---|---|---|---|---|
| | | After 48h | After 96h | After 144h |
| *Aspergillus flavus* | Corn wastes | 4.695 | 8.041 | 5.749 |
| | Barley wastes | 3.946 | 4.864 | 7.018 |

## Conclusion

In this study, we have demonstrated that the fungus Aspergillus flavus is capable of degrading agricultural residues such as corn and barley waste and producing bioethanol in significant concentrations. After 96 hours of incubation, we obtained a bioethanol concentration of 6.363 g/l from corn waste and 5.570 g/l from barley waste. Furthermore, our findings highlight the importance of providing optimal conditions for fungal growth to enhance bioethanol production. We observed that by optimizing the pH and nitrogen source, the bioethanol production increased to 8.041 g/l after 96 hours with corn waste and 7.018 g/l after 144 hours with barley waste. In conclusion, A. flavus proves to be an effective biological tool for addressing agricultural waste and generating bioethanol.

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## انتاج الايثانول الحيوي من مخلفات الذرة والشعير بواسطة *Aspergillus flavus*

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**المستخلص**

يعد الإيثانول الحيوي من أكثر انواع الوقود الحيوي استخداماً في جميع انحاء العالم وذلك لأنه يساهم بشكل كبير في تقليل استهلاك النفط الخام، والتلوث البيئي. ركزت هذه الدراسة على دراسة قابلية الفطريات على انتاج الوقود الحيوي من المخلفات النباتية، لذا تم عزل الفطر *Aspergillus flavus* من التربة وقيست قابليته على تحليل السكريات وإنتاج الإيثانول الحيوي. أظهرت النتائج قابلية الفطر على تحليل سكر الكلوكوز في الاختبار الأول وإنتاج 4.795 غم/لتر من الإيثانول الحيوي. وأظهرت النتائج قابليته على تحليل مخلفات الذرة والشعير وإنتاج الإيثانول الحيوي، إذ كان أعلى تركيز للإيثانول الحيوي 6.363 غم/لتر مع مخلفات الذرة و5.570 غم/لترمع مخلفات الشعير بعد 96 ساعة من الحضن. بعد ذلك اختبر الاس الهيدروجيني والمصدر النيتروجيني الأمثل للفطر *A. flavus* لمعرفة تأثيرهما على قابليته لإنتاج الإيثانول الحيوي في الوسط السائل الذي يحتوي على مخلفات الذرة اوالشعير. أظهرت النتائج ان تطبيق الظروف المثلى أدى إلى تحسين انتاج الإيثانول الحيوي، اذ كان أعلى انتاج للإيثانول الحيوي 8.041 غم/لتر مع مخلفات الذرة بعد 96 ساعة، و7.018 غم/لتر مع مخلفات الشعير بعد 144 ساعة من الحضن.

الكلمات المفتاحية :المخلفات الزراعية؛*Aspergillus flavus* ؛ انتاج الإيثانول الحيوي