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(Original Article)

Identification and Molecular Characterization of High Ethanol Producing Microorganisms Isolated from Different Natural Sources in Assiut Governorate

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Abstract

Ethanol is an important bioproducts used in the field of clean energy. It is produced through the fermentation of sugars by various microorganisms.

Yeast and bacteria are found in terrestrial and aquatic environments and can be isolated from natural substances such as soil, fruits, and vegetables. Yeasts, particularly *Saccharomyces cerevisiae*, are the primary organisms used in ethanol production due to their efficiency and adaptability. Bacteria such as *Bacillus subtilis* also play an important role in this process, offering an alternative or supplementary method of fermentation. The choice of microorganism depends on some factors like substrate availability, desired ethanol yield, and process conditions.

This study aims to isolate and identify several yeasts and bacterial isolates, evaluate the production ethanol, molecularly characterize the producing isolates, and to improve some isolates to produce more Ethanol. Fifty different isolates were collected from contaminated fruit and vegetables. Three isolates were identified as *S. cerevisiae* by PCR specific DNA primers, while 47 isolates were bacterial isolates. All the yeast and bacterial isolates were screened for bioethanol productivity. The bacterial isolate LS-6 showed the highest bioethanol productivity (50%), which was identified by 16S rRNA gene sequencing as *B. subtilis.* Yeast isolate (CJ-12) and bacterial isolate (LS-6) were subjected to UV- mutagenesis to improve the bioethanol productivity, all obtained mutants from the two yeast and bacteria isolates showed higher productivity compared to the wild-type.

Keywords: *Bioethanol, B. subtilis, Molecular identification, Mutagenesis, S. cerevisiae*

Introduction

Bioethanol is one of the most promising renewable biofuels. It is used in pharmaceuticals, cosmetics, and industrial products. Its output is growing year after year (Cardona and Sánchez, 2007). With increased oil prices and global environmental concerns, bioethanol production has recently gained traction (Bai

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et al., 2008). Products ranging from explosives to perfume contain ethanol. The most widespread use of ethanol is in the car fuel industry.

Although bioethanol has long been widely utilized in daily life in the form of antiseptics, and disinfectants, newer trends have seen a growth in its use as a renewable and ecologically friendly energy source, such as an additive or supplement to gasoline (Baras *et al.,* 2002).

Bioethanol has various advantages over gasoline, including larger flammability limitations, faster flame speeds, a higher-octane number (108), and greater evaporation temperatures (Balat *et al.,* 2008). Bioethanol is less hazardous, biodegradable, and emits fewer airborne pollutants than petroleum fuel (John *et al.,* 2011). In addition, its production is growing annually. It is utilized in industrial products, pharmaceuticals, and cosmetics (Cardona and Sánchez, 2007).

Both yeasts and bacteria, including *S. cerevisiae* and *B. subtilis* (Singh, 2014), play significant roles in ethanol production, but their involvement and impact differ depending on the specific production method.

The *S. cerevisiae* is the preferred yeast for industrial ethanol production, making it the most widely used yeast in biotechnology. *S. cerevisiae* is ideal industrial yeast due to its rapid growth, efficient glucose anaerobic metabolism, high ethanol productivity, high yield, and tolerate environmental stress factors like high ethanol concentration, low pH, and low oxygen levels (Dmytruk, *et al.,* 2017).

A wide range of bacteria may create ethanol from polysaccharides. However, an optimal microbe employed for ethanol production must have quick fermentative potential, better flocculating ability, acceptable osmo-tolerance, enhanced ethanol tolerance, and strong thermotolerance (Brooks, 2008).

According to Hahn-Hägerdal *et al.* (2006), Sadik and Halema (2014), bacteria have a few benefits over yeasts. In the industrial setting, bacteria are favored over fungal strains due to their superior yield, tolerance, shorter generation times, reduced biomass generation, better utilization of substrates, and less complicated downstream processing steps (Yang *et al*., 2016).

Promon (2015) mentioned that B. subtilis increased the alcohol production rate from the fermentation of cellulosic materials. The cellulolytic activity of this cellulose degrading bacteria converts cellulose into smaller sugars which will be easier to be fermented by yeast.

Any tiny increase in the generation of ethanol by enhanced thermo tolerant yeasts could have a substantial economic impact due to the size of the fermentation ethanol business; induced mutagenesis using chemical and physical mutagens appears to be a straightforward and sensible strategy for yeast strain improvement. A significant amount of improvement in yeasts has been achieved through selection after mutagen therapy (Demchenko and Kobrina, 1979).

The aim of this study was to isolate and molecularly identify different isolates of yeasts and bacteria in addition to characterizing their ethanol production and enhance the production by mutagenesis.

Materials and Methods

1-Samples collection

Different samples were collected from different natural sources such as fruits, vegetables, and soils in sterile bags and were immediately transported to the laboratory and kept at 4°C to be used to isolate different microorganism (Mamun-Or-Rashid *et al*., 2022)

2-Isolation of different microorganisms

The yeasts were isolated using YPD medium, which contains peptone (20g), yeast extract (10g), dextrose (20g), and agar (20 g/L). Chloramphenicol (0.01 g/L) is added to the medium to prevent the growth of bacteria (Tesfaw *et al.,* 2021) After sample collection, 1 g of the sample were diluted with 9 ml of sterile water, then 0.1 ml of the suspension were plated on Yeast Extract Peptone Dextrose Agar (YEPD) and incubated for 24 to 48 hours at 37°C. Isolates were then refined and cultured in liquid media and incubated at 30 for 48 hours in a shaker, and then glycerol stocks were prepared and stored at -80°C.

3-Estimation of Bioethanol Productivity

All the isolates were screened for their bioethanol productivity according to Seo *et al.* (2009) using the following media: 10 g of glucose; 5 g of yeast extract; and 10 g of peptone.

The isolates were cultivated and incubated at 37°C for 48 hours. The culture samples underwent a 3-minute centrifugation at 10,000 rpm, and then 750 µl of the culture supernatant was combined with 750 µl of Tri-n-butyl phosphate (TBP) and subjected to a vigorous vortex for 10 minutes. Following phase separation, 750 µl of the solvent phase (upper) was moved to a separate tube, to which 750 µl of the dichromate reagent (dissolve 10 g potassium dichromate in 100ml distilled water and gradually add 5 ml of concentrated sulfuric acid) was added. For ten minutes, the mixture was aggressively vortexed. Following phase separation, the lower phase was transferred. A spectrophotometer was used to detect the optical density at OD595. All the obtained OD was compared to the standard curve to determine ethanol productivity. All the isolates were tested in 3 replicates.

4-DNA Isolation from different isolates

Isolation of DNA was performed according to Saghai-Maroof *et al.,* (1984) with some modification. 1 ml of overnight liquid culture was placed in a 1.5 ml disposable centrifuge tube. The isolates were collected through centrifugation at 10000 rpm for 5 minutes. 700 µl of extraction buffer and add 50 µl of β-mercabtoethanol were added to the sample pellet then the samples were vortexed and incubated at 65 °C for 20 minutes. 500 µl of chloroform: Isoamyl alcohols (24:1) were added and vortexed for 20 second, then centrifuged at 10000 rpm for 10 minutes and the aqueous supernatant was transferred to a new tube. An equal volume of cold ethanol 100% was added then cooled at -20ºC for 30 min. To pellet the DNA, centrifugation at 1300 rpm for 5 min was performed. Washing was done with ethanol 70% followed by centrifugation for 5 min. Finally, the pellets were

kept for drying for 1hr at room temperature and then dissolved in 100 µl of distilled H_2O.

5-Molecular identification of yeasts isolates using PCR

The *S. cerevisiae* isolates were identified by PCR using Saccharomyces-species-specific PCR primers. The SC1/SC2 primers (Table 1) designed by Josepa *et al.* **(**2000) were used to distinguish between yeast and bacteria. PCR was performed in a final volume of 20 µl containing 10 µl of PCR master mix, 0.25 µl of each primer (0.25 mM), 8.5 µl of distilled water, and 1 µl of the DNA template. The thermal cycler was programed as follows: an initial denaturation at 94º C for 10 min, followed by 35 cycles of denaturation at 95ºC for 30 s, primer annealing at 54 º C for 30 s, and a primer extension at 72ºC for 1 min. Finally, the reaction mixture was heated to 72º C for 10 min and subsequently cooled to 4ºC. 10 µl of the amplified mixture was then analyzed by agarose gel electrophoresis which were visualized and photographed by a Gel documentation system.

Table 1. Primer used for molecular identification of *S. cerevisiae* isolates.

Primer	Primer sequence	band amplification size	Reference
SC1-F SC2-R	5′- AGCTGGCAGTATTCCCACAG-3′ 5′- AACGGTGAGAGATTTCTGTGC-3′	170 bp	(Josepa *et al*., 2000)

6-Molecular identification of best bioethanol producing bacterial isolates by 16S rRNA sequencing

To identify molecularly the most ethanol producing bacterial isolate, 16S rRNA gene sequencing (Josepa *et al.,* 2000) was done. The sequencing process was as follows: two universal bacterial primer sets (Pastrik and Maiss, 2000), PS-1 (5'-AGT CGA ACG GCA GGG G-3') and Ps-2 (5'-GGG GAT TTC ACA TCG GTC TTG CA-3') were used. The sequencing was performed at the Sol Gent Company in Daejeon, South Korea. Using BLAST, the acquired sequence was used to find matching sequences in the GenBank database (NCBI).

7-Enhancing bioethanol productivity by mutagenesis

UV light was utilized to generate mutations to enhance bioethanol productivity in different isolates. (Al Makishah and Elfarash, 2022). One mL of yeast cell suspension (about 1x 10^5 cells/mL) was dispersed over YPD plates prepared as stated above.

Different plates were exposed to UV rays (234 nm) at a distance of 5 cm with intervals of 10 s, 15 s, 30 s, 60 s, and 1 min, then incubated at 37°C for 48 hours. Different mutants from plates showed half the number of the colonies compared to the control (LD_{50}), due to the UV exposure, were selected, cultured in YPD broth at 37℃, and transferred to 250 ml conical flasks containing 100 ml of YPD medium. These flasks were incubated at 30℃ and were tested for their ethanol productivity.

Results and Discussion

1-Isolation of different microbial isolates from natural sources and its identification

In the present investigation, fifty different isolates were collected from various natural sources (Table 2). The primary identification of the isolates was carried out based on morphological characteristics (colony morphology, pigment, elevation, edge, and surface appearance) of colonies on solid media. Moreover, microscopic observation (Figure 1) was also used to characterize the isolates.

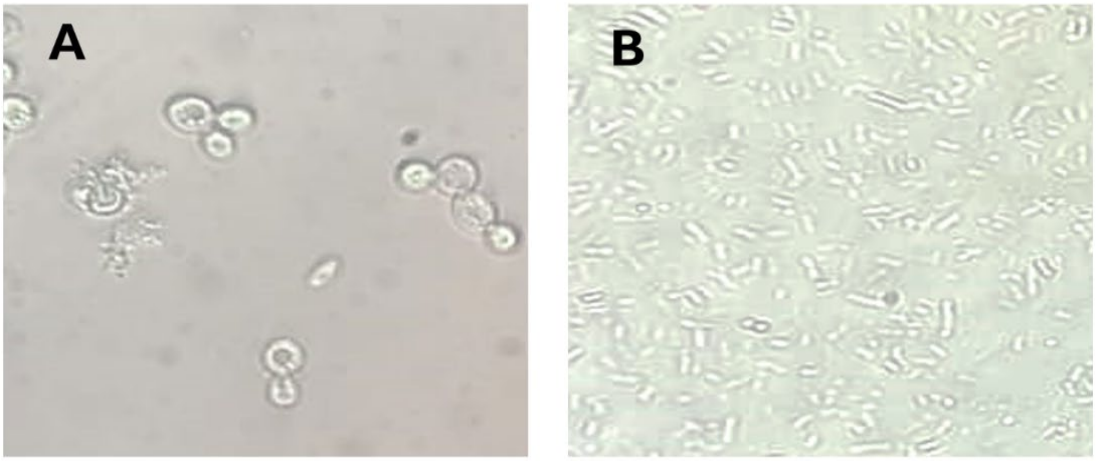

Figure 1. Microscopic characterization of some isolates. (a) Yeast isolate shape and (b) bacterial isolate shape.

This morphological identification revealed that the number of bacterial isolates was 47, while the number yeast isolates was only 3 (ZU-2, YE-10, and CJ-12). Moreover, *S. cerevisiae* primers (SC1/SC2), designed by (Josepa *et al.,* 2000*),* were used to identify the *S. cerevisiae* isolates among the 50 collected isolates. Agarose gel electrophoresis (Figure 2) confirmed that only 3 isolates (ZU-2, YE-10 and CJ-12) were able to amplify the PCR specific band (1170 bp), so they were molecularly identified as *S. cerevisiae*. The isolates that did not exhibit any amplification were considered as non-*Saccharomyces*.

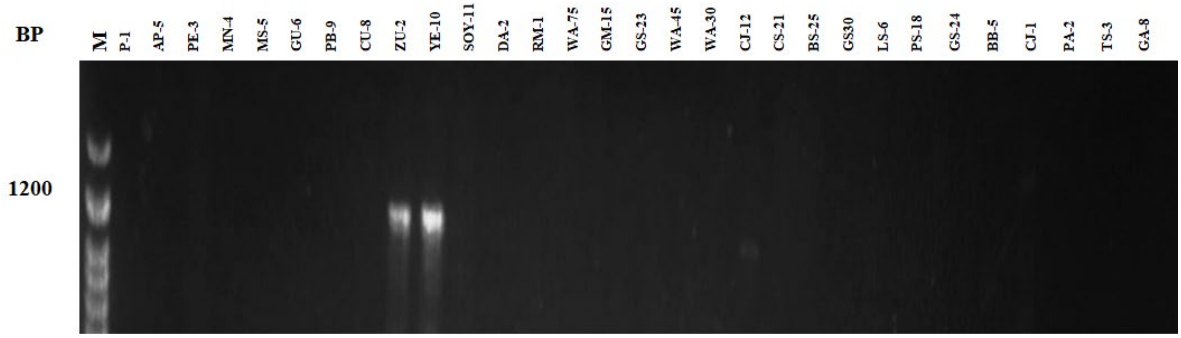

Figure 2. Agarose gel for the PCR which used *Saccharomyces*-species-specific primers for yeast isolates identification. Positive bands were identified as *S. cerevisiae* and negative bands as non-*Saccharomyces*.

Table 2. Details of collected samples for isolation of bacteria.

No	Source sample	Sample Type	Code	Type
1	Fruit	Plum	P-1	Bacteria
2	Fruit	Apple	AP-2	Bacteria
3	Fruit	Pear	Pe-3	Bacteria
4	Fruit	Mango	Mn-4	Bacteria
5	Soil	Mango	Ms-5	Bacteria
6	fruit	Guava	Gu-6	Bacteria
7	Fruit	Pomegranate	PB-9	Bacteria
8	Vegetable	Cucumber	CU-8	Bacteria
9	Legume product	Soybean	SOY-11	Bacteria
10	Fermented products	Yoghurt (Danone)	Da-2	Bacteria
11	Fermented products	(Rayeb milk)	Rm-1	Bacteria
12	Water	Water	Wa75	Bacteria
13	Milk	Goat milk	Gm-15	Bacteria
14	Soil	Guava	GS-23	Bacteria
15	Water	Water	Wa-45	Bacteria
16	Water	Water	Wa-30	Bacteria
17	Water	Water	Wa.p1	Bacteria
18	Soil	Sugar cane	CS-21	Bacteria
19	Soil	Banana	BS-25	Bacteria
20	Soil	Guava	GS-30	Bacteria
21	Soil	Lemon	LS-6	Bacteria
22	Soil	Pomegranate	PS-18	Bacteria
23	Soil	Grapes	GS-24	Bacteria
24	Fruit	Banana	BB-5	Bacteria
25	Juice	Cane juice	CJ-1	Bacteria
26	Fruit	Pomegranate	PA-2	Bacteria
27	Soil	Tangerine	TS-3	Bacteria
28	Fruit	Guava	GA-8	Bacteria
29	Juice	Cane juice	CJ-10	Bacteria
30	Juice	Cane juice	CJ-13	Bacteria
31	Soil	Banana	BS-14	Bacteria
32	Soil	Pear	PS-15	Bacteria
33	Soil	Fig	FS-16	Bacteria
34	Soil	Grape	Gs-17	Bacteria
35	Soil	Orange	Os-20	Bacteria
36	Soil	Olive	Ols-21	Bacteria
37	Soil	Apple	AS-22	Bacteria
38	Soil	Apple	AS-27	Bacteria
39	Soil	Grape	Gs-28	Bacteria
40	Soil	Tangerine	TS-29	Bacteria
41	Fruit	Banana	Ba-30	Bacteria
42	Vegetable	Tomato	To-31	Bacteria
43	Fruit	Annona	An-32	Bacteria
44	Fruit	Dates	Da-33	Bacteria
45	Fruit	Kiwi	Ki-34	Bacteria
46	Juice	Juice sugar cane	Sc-35	Bacteria
47	Water	Water	Wa-00	Bacteria
48	Vegetable	Zucchini	ZU-2	Yeast
49	Instant dry yeast	Yeast	YE-10	Yeast
50	Juice	Cane juice	Cj-12	Yeast

These primers permit the amplification of a 1170 bp DNA fragment located between the ITS-1 region and the LSU gene of *S. cerevisiae* strains. This method offers a quick and accurate way to distinguish between non-*Saccharomyces* and *S. cerevisiae*. These primers were also used by other researchers to identify the *S. cerevisiae* isolates from several isolates (Guimarães *et al.,* 2006).

2-Screening and estimation of ethanol productivity.

Fifty isolates of bacteria and yeast were evaluated for bioethanol productivity at 37°C and 24 hours later. LS-6 Isolate of bacterial showed the highest ethanol productivity (50%) (Figure 3), while OS-20 isolate showed the lowest bioethanol production (10%).

According to Mostafa *et al.* (2024), bacterial samples were isolated from molasses and vinasse, and after a 24-hour incubation period at 37°C with a pH of 7, the ethanol productivity was examined. The findings demonstrated that the bacterial isolates produced high ethanol, ranging from 74% to 27%.

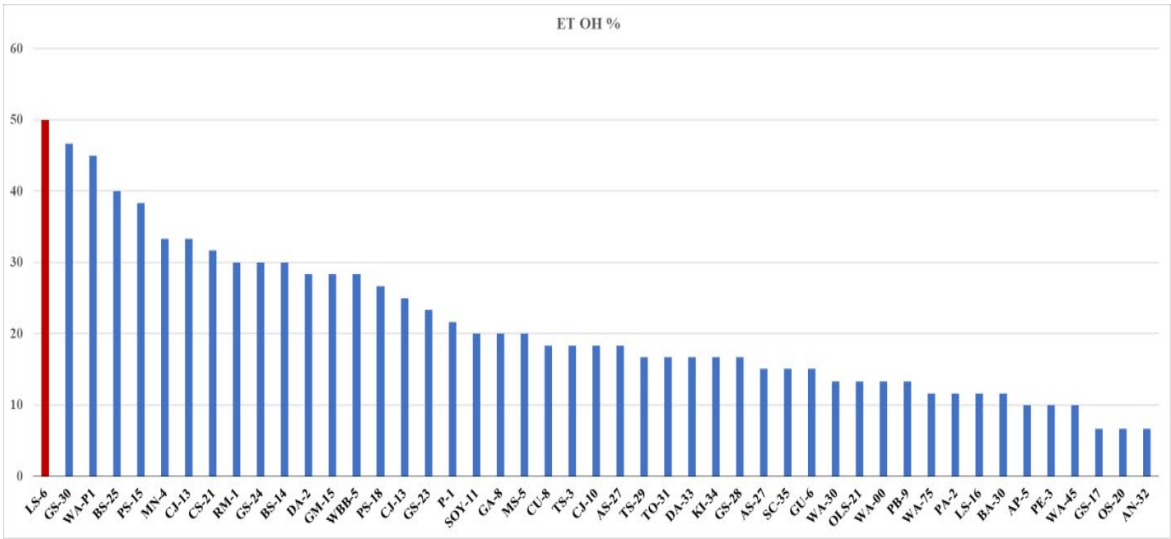

Figure 3. Screening bacterial isolates for ethanol productivity.

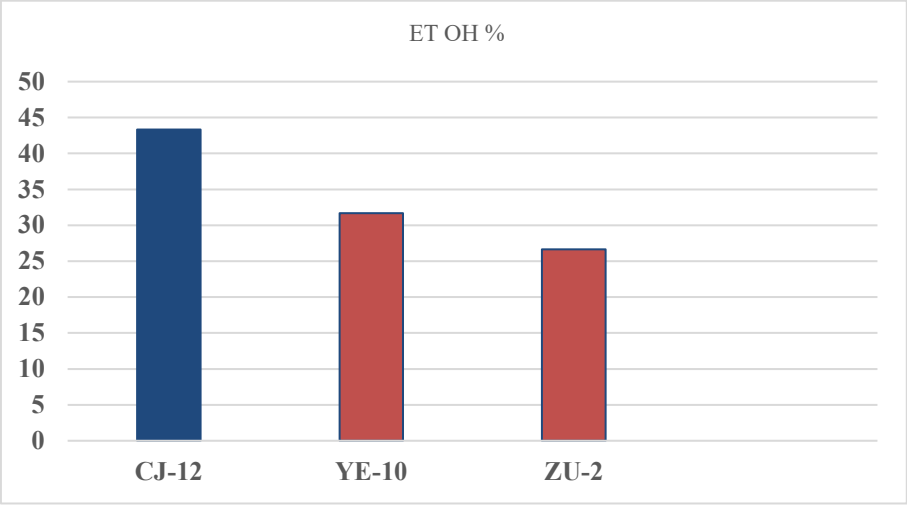

Figure 4. Screening yeast isolates for ethanol productivity.

Bacillus subtilis strain DSM 10 16S ribosomal RNA, partial sequence

Sequence ID: NR_027552.1 Length: 1517 Number of Matches :1

Score	Except	Identities	Gaps	Strand
2621 bits (1419)	0.0	1419/1419 (100%)	0/1419 (0%)	Plus/Plus

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Figure 5. Sequence alignment of the LS-6 isolate (Query) against the partial 16S rRNA gene sequence data of *B. Subtilis* in GenBank.

3-Bacterial isolates identification by 16S rRNA sequencing

The highest ethanol producing bacterial isolate (LS-6) was chosen for DNA molecular identification by 16S rRNA sequencing, which was carried out by the Gene Analysis Unit (Macrogene Inc., Seuol, Korea) using universe primers, PS-1 (5'-AGT CGA ACG GCA GCG GGG G-3') and Ps-2 (5'-GGG GAT TTC ACA TCG GTC TTG CA-3'). The obtained partial sequences of the 16S rRNA were first analyzed using the advanced BLAST search program at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) to molecularly identify the isolate. The sequencing results showed that LS-6 isolate was 100% similar to *B. subtilis* (Figure 5).

The sequencing results indicated that LS-6 isolate can be identified as *B. subtilis* (Maleki *et al.,* 2021) also found that *B. subtilis* demonstrated a high potential for ethanol production.

Several sequences were selected from GenBank database to construct the phylogenetic tree to compare the LS-6 isolate with other closely related species (Figure 6). Phylogenetic trees were constructed with MEGA ver. 3.1, using a neighbor joining algorithm. The results showed that LS-6 isolate was clustered with *B. subtilis* with a similarity of 100%.

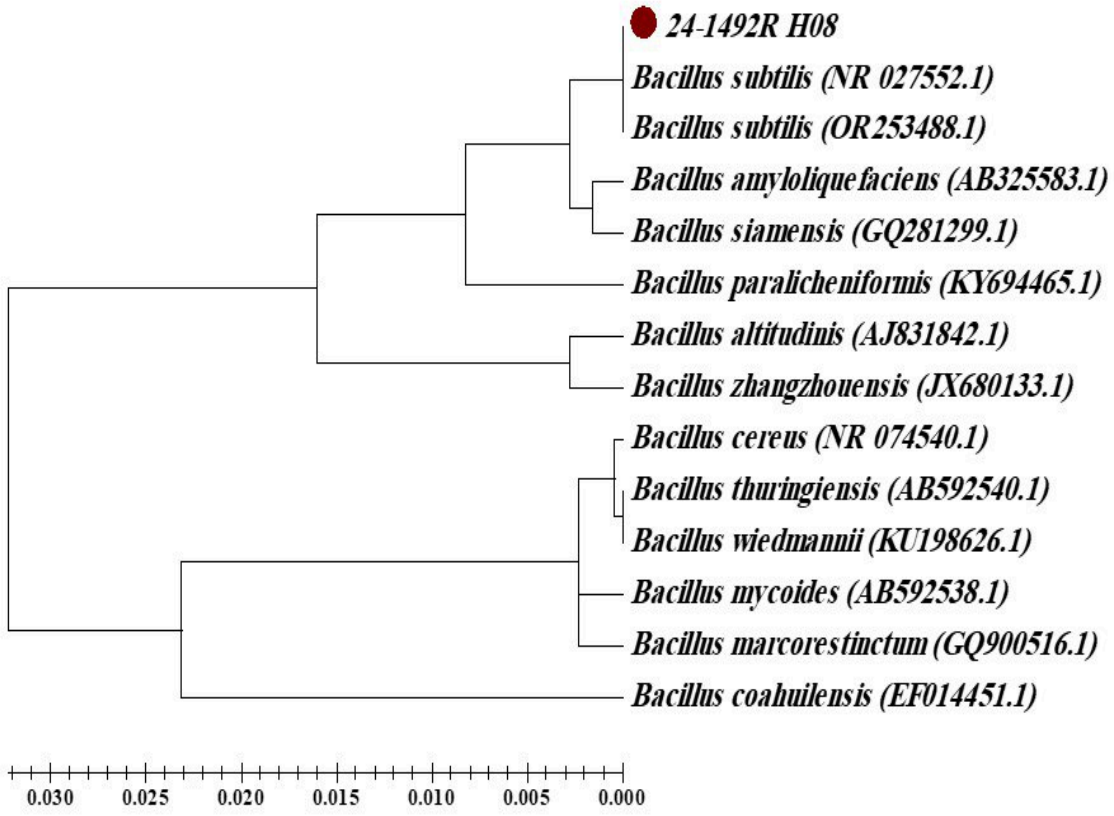

Figure 6. Phylogenetic tree for *B. subtilis* and related species.

4-Mutagenesis of the best ethanol producing isolate by UV irradiation.

The (CJ-12) and (LS-6) isolates were mutagenized by the exposure to UV to improve their ethanol productivity.

The result showed that the CJ-12 isolate had an ethanol production of 36.6 % but when it was subjected to UV. Nine mutants out of 15 produced more ethanol than the wildtype isolate (Figure 7).

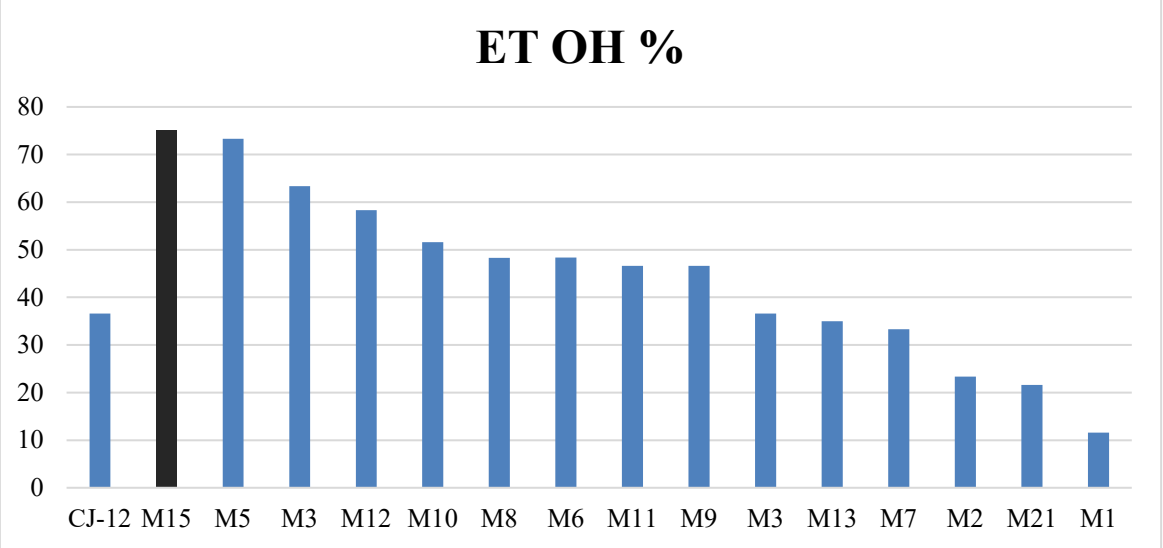

Figure 7. Screening the mutants obtained from the CJ-12 yeast isolate for their ethanol productivity.

On the other hand, the LS-6 bacterial isolate had an ethanol productivity of 58.33% but when it was subjected to UV, 15 mutants out of 20 produced more ethanol than the wildtype isolate (Figure 8).

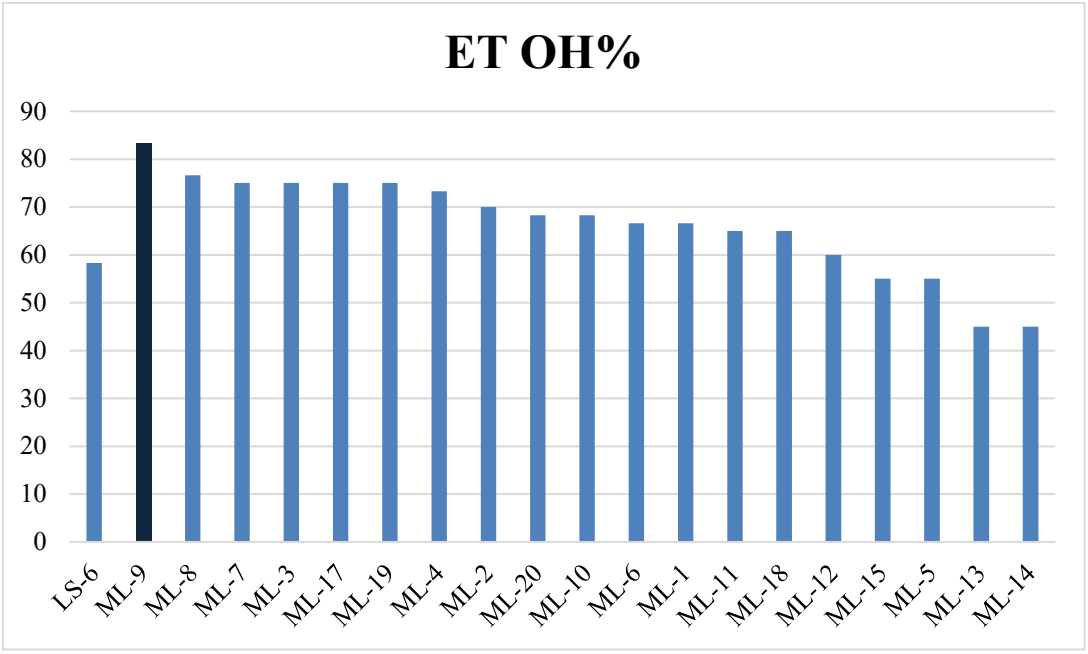

Figure 8. Screening the mutants obtained from the LS-6 bacterial isolate for their ethanol productivity.

The results indicated that UV mutagenesis has an effective role in improving ethanol productivity in both (CJ-12) and (LS-6) isolates since some mutants were higher than the wild-type in the productivity (Figure 7 and Figure 8).

All Results obtained from the selected mutants showed higher amounts of bioethanol compared to the wild-type (CJ-12 isolate). These results agreed with (Sridhar *et al*., 2002) who found that mutagenesis always improves productivity. Moreover, Shivsharan and Kadam (2019) reported that a mutant strain of *S. cerevisae* caused an increment in production of ethanol.

Conclusions

Fifty different isolates were collected from contaminated fruit and vegetables, Yeast isolates were identified by specific primer, while one of the bacterial by 16S rDNA sequencing. Screened yeast isolates showed less production of ethanol than bacterial isolates, sequenced the highest ethanol producing strain and the result was *B. subtilis*, UV- mutagenizes could improve the bioethanol productivity in all the selected mutants.

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التعريف والتوصيف الجزيئي للكائنات الحية الدقيقة عالية الإنتاج للإيثانول المعزولة من مصادر طبيعية مختلفة في محافظة أسيوط

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المخلص

ويعتبر الإيثانول أحد أهم المنتجات الحيوية المستخدمة في مجال الطاقة النظيفة. يتم إنتاجه من خلال تخمير السكريات بواسطة الكائنات الحية الدقيقة المختلفة. توجد الخميرة والبكتيريا في البيئات الأرضية والمائية ويمكن عزلها عن المواد الطبيعية مثل التربة والفواكه والخضروات.

حيث أن الخمائر، وخاصة *Saccharomyces cerevisiae*، هي اهم الكائنات المستخدمة في إنتاج الإيثانول بسبب كفاءتها وقدرتها. كما تلعب البكتيريا مثل *Bacillus subtilis* أيضًا دورًا في هذه العملية. تهدف هذه الدراسة إلى عزل والتوصيف الجزيئي لعدد من الخمائر والعزلات البكتيرية ثم تقدير إنتاج الإيثانول بواسطة هذه العزلات وتحسين بعض هذه العزلات لإنتاج المزيد من الإيثانول. تم جمع خمسين عزلة مختلفة وتم توصيف ثلاث عزلات على أنها *S. cerevisiae* جزيئياً بواسطة تفاعل الـــ PCR. تم تقدير إنتاجية الإيثانول الحيوي لجميع عزلات الخميرة والبكتيريا. أظهرت العزلة البكتيرية LS-6 أعلى إنتاجية للإيثانول الحيوي بنسبه (50%) وتم تعريفها جزيئيا من خلال تحديد تتابعات جين الـــ S16 على أنها *B. subtilis*، تم استخدام الأشعة فوق البنفسجية لاستحداث بعض الطفرات بغرض تحسين إنتاجية الإيثانول الحيوي في كلا من عزلة الخميرة (عزلة CJ-12) والعزلة البكتيرية (عزلة LS-6). أظهرت جميع الطافرات تحسن في إنتاجية الإيثانول الحيوي وذلك بالمقارنة بالطراز البري.

الكلمات المفتاحية: *الإيثانول الحيوي، التوصيف الجزيئي، الطفرات، S. cerevisiae.*