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RESEARCH ARTICLE

Enhancement of sustainable bioethanol production from microorganisms isolated from molasses and venasses

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Abstract

Ethanol is one of the most important biofuels that can be produced from different renewable sources. Molasses and Venasses are used as cheap raw materials in the isolation of microorganisms and used molasses as renewable materials for ethanol production. Molasses and Venasses are considered important by-products in the sugar industry. This study aims to isolate and identify yeasts and bacteria present in both molasses and venasses to use them in the production of bioethanol. Molasses and venasses samples were collected from ten different sugar factories (Guirga, Savola, Deshna, Komombo, Abokorkaus, Delta, Dakahlia, Qus, Nag-hamdy, Armant) and were used to isolate different microorganisms that were screened for their bioethanol productivity. The results showed that the molasses samples contained more microbes than venasses. Twelve isolates were molecularly identified as S. cerevisiae by PCR-specific primers, while 64 isolates were bacterial isolates. All the yeast and bacterial isolates were screened for bioethanol productivity. Isolate M3 showed the highest bioethanol productivity (74%) and was identified by 16S rRNA gene sequencing as Klebsiella pneumoniae. Several factors affected the production of bioethanol, including sugar concentration, urea, and ammonium sulfate. When molasses was used as the carbon source, Klebsiella pneumoniae produced 1% (v/v) bioethanol by utilizing 20% molasses (sugar concentration), 0.4% urea, and 0.4% ammonium sulfate. When UV- mutagenesis was used to improve the bioethanol productivity, all the obtained mutants showed lower productivity compared to the wild-type (M3 isolate).

Keywords: Molasses; Venasses; Bioethanol; *Klebsiella pneumoniae*; Mutagenesis**.**

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Introduction

Bioethanol is a promising type of biofuel produced by sugar fermentation and used as a partial gasoline replacement in several parts of the world (Bhatia et al. 2012; Lee and Shah 2012; Sadik and Halema 2014). Bioethanol has many advantages over gasoline such as broader flammability limits, higher flame speeds, higher octane number(108) and increased heat of evaporation (Balat and Balat 2009). In contrast to petroleum fuel, bioethanol is less toxic, readily biodegradable and produces fewer airborne pollutants (John et al. 2011).

It is also used for cosmetics, medicines, industrial materials and its production is increasing every year (Cardona and Sánchez 2007). The sugarcane and beet molasses are by-products of sugar industries in Egypt and are cheap raw materials, readily available, and ready for conversion with fewer pretreatments as compared with starchy or cellulosic materials for bioethanol fermentation on an industrial scale. The majority of the sugars in molasses are available for fermentation (Razmovski and Vučurović 2011). Sugarcane molasses is a dark viscous fluid, and rich in nutrients necessary for the growth of microorganisms such as nitrogen, carbon, sodium, potassium, phosphorus and non-nitrogenous compounds. Beet molasses also is used as feedstock to produce bioethanol (Dodić et al. 2009).

Molasses composition is typically influenced by cane and beet variety and maturity, climate, soil, and factory processing conditions (El-Gendy et al. 2013). The primary components of molasses generally consist of sucrose (30–35%), fructose and glucose (10–25%), non-sugar compounds (2–3%), mineral and moisture content and about 45–55% of total fermentable sugars (Solomon 2011). Sugarcane and beet molasses are the final byproduct of sugar factories that is easily available and economically low in price for using it as a raw material for ethanol production (Khoja et al. 2015).

Molasses have a significant quantity of sugar, around 40% to 50%(w/v), and ash content of around 5-15%, making it a suitable substrate for rum and bioethanol production for many years (Doelle and Doelle 1990).

A large variety of microorganisms can produce ethanol from polysaccharides. But an ideal microorganism used for ethanol production must have rapid fermentative potential, improved flocculating ability, appreciable osmo-tolerance, enhanced ethanol tolerance and good thermotolerance (Brooks 2008). The yeast (Saccharomyces cerevisiae) has been used widely on a commercial scale, but bacteria have not been commercially practiced yet due to some constraints. such as industrial robustness, substrate utilization, productivity and yield. Bacteria have some advantages over yeasts (Hahn-Hägerdal et al. 2006; Khoja et al. 2014; Sadik and Halema 2014). Industrially, bacteria are preferred over fungal strains as bacterial strains show higher yield, higher tolerance, shorter generation time, lower biomass generation, more effective substrate utilization and simpler downstream processing steps than fungal strains (Yang et al. 2016). There have been numerous reports of bacterial strains with the potential to generate ethanol, which Zymomonas mobilis is the oldest known. Apart from these, Lactobacillus plantarum M24, L. sakei, Weissella viridescens and Pediococcus acidilactici are also reported for efficient bioethanol production (Sharma et al. 2007). With the progress of molecular engineering, various recombinant strains with higher ethanol production capabilities have been created, including Escherichia coli and Klebsiella oxytoca (Soleimani et al. 2017). Certain yeast strains such as Pichia stipitis (NRRL-Y-7124), S. cerevisiae (RL-11) and Kluyveromyces fagilis (Kf1) were reported as good ethanol producers from different types of sugars (Mussatto et al. 2012). Most yeasts can convert a range of hexose sugars to ethanol via glycolysis. However, Saccharomyces cerevisiae is the most used yeast organism for alcoholic fermentation due to its robustness and tolerances. S. cerevisiae has several advantages over other yeasts as it is a facultative anaerobe capable of growing under both aerobic and anaerobic conditions in the presence of glucose (Krantz et al. 2004). Yeast is the most widely utilized microorganism for ethanol fermentation. S. cerevisiae is one of the well-known ethanol producer (Izmirlioglu and Demirci 2012). Yeast plays an essential role in bioethanol production by fermenting a wide range of sugars to ethanol. S. cerevisiae is used in industrial plants due to valuable properties in ethanol yield (> 90.0% theoretical yield), ethanol tolerance (> 40.0 g/L), ethanol productivity (> 1.0 g/L/h), growth in simple, inexpensive media and undiluted fermentation broth with resistance to inhibitors and retard contaminants from growth condition (Dien et al. 2003). There are many factors which affect the production of bioethanol including temperature, sugar concentration, pH, fermentation time, agitation rate, and inoculum size (Zabed et al. 2014). The growth rate of the microorganisms is directly affected by the temperature (Charoenchai et al. 1998). Inoculum concentration affects the consumption rate of sugar and ethanol productivity but does not give significant effects on the final ethanol concentration (Laopaiboon et al. 2007). One important element in the production of ethanol has also been identified as the initial sugar content. Using a greater initial sugar concentration during batch fermentation will result in increased ethanol production and yield. But it requires more time for fermentation and higher recovery cost (Zabed et al. 2014). The aim of this study was to isolate yeasts and bacterial isolates and characterize their ethanol production and enhance the production by mutagenesis and investigating the factors that affect the production from molasses.

Materials and Methods

Samples collection

Several types (20 samples) of molasses and vinasse samples were collected from different Sugar factories (Guirga, Savol, Deshna, Komombo, Abokorkaus, Delta, Dakahlia, Qus, Nag hamdy, Armant) in Egypt which were used to isolate several bacterial and yeast isolates.

Isolation of microorganisms from molasses and vinasses

One ml of the molasses sample was suspended in 9 ml sterile distilled water and mixed well. A 0.1 ml of the suspension was plated on Luria broth medium which contains the following composition (g/L dist. H2O): yeast extract, 5 g; peptone,10 g and NaCl, 10 g (Nasiri et al. 2017). The plates were incubated at 37°C overnight, single colonies were re-streaked onto fresh plates. Colonies were picked up individually repeatedly sub- cultured for purification and maintained on sterile LB slants, coded and stored at 4 °C with regular transfer at monthly intervals and glycerol stock was used for culture preservation. These cultures were then screened for their ability to produce bioethanol.

Screening for bioethanol production

Screening for bioethanol productivity was conducted on the following media which contains (g/L dist. H2O): glucose 10 g; yeast extract,5 g; peptone,10 g and NaCl,10 g. Isolates (76 isolates) were cultured and incubated at 37°C for 24 h. After 24 h, the culture samples were centrifuged for 3 minutes at 10000 rpm and 750 µl of the culture supernatant were mixed with 750 µl of Tri-n-butyl phosphate (TBP) and then vortexed vigorously for 10 min.

After phase separation,750 µl of solvent phase (upper) was transferred to a new tube and 750 µl of dichromate reagent was added, and vortexed vigorously for 10 min. After phase separation, the lower phase was transferred and the optical density were measured using spectrophotometer at OD595 (Seo et al. 2009). All the obtained OD were compared to the standard curve (Figure 1) to determine the ethanol productivity. All the isolates were tested in 3 replicates.

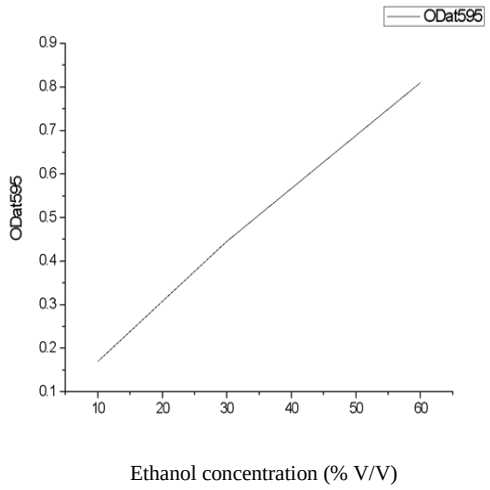

Figure 1. Standard curve used for bioethanol concentration determination.

Molecular identification of isolates

The isolates were identified molecularly by 2 different methods. Firstly, the yeast isolates were identified by species-specific PCR primers (the 5′ specific primer (SC1) was designed from the ITS-1 region (between position 161 and 161 from the 3′-SSU end forward), the 3′specific primer (SC2) was located in the LSU gene (between positions 562 and 582 from the 5′ end of this gene, backward) (Josepa et al. 2000), while the bacterial isolates were identified by 16S rRNA gene Sequencing.

DNA Isolation

Isolation of DNA was performed according to (Saghai-Maroof et al. 1984) with some modifications. A 1 ml of overnight liquid culture (Bacteria and Yeasts) was placed in a 1.5 ml disposable centrifuge tube. The cells were collected through centrifugation at 7,500 rpm for 10 minutes. The supernatant was discarded, and the pellets were resuspended in 0.2 ml of phosphate buffer. A 10 µl of lysozyme was added to bacterial isolates and incubated at 37°C for 60 minutes. A 0.4 ml of Cetyltrimethyl Ammonium Bromide (CTAB)extraction buffer was added followed by 40 µl of β-mercabtoethanol and mixed gently. The tube was placed in 60 ºC water bath for 60 min. After cooling an equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed. This mixture was centrifuged for 5-10 min at full speed, and the aqueous supernatant was transferred to a new tube. An equal volume of cold ethanol 100% was added then cooled at -4ºC for 30 min. Then centrifuge for 5 min. at 1300 rpm to pellet the DNA. 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 warm dist. The quality and the quantity of the genomic DNA were checked spectrophotometry using nanodrop at wavelength of 260/280 nm.

Molecular identification of yeasts using PCR

PCR was used to identify yeast of S. cerevisiae. The SC1/SC2 (table1) primers were designed by (Josepa et al. 2000) to distinguish between yeast and bacteria.

Table 1. Primer used for Molecular identification of yeast isolates.

Primer	Primer sequence	Size	Reference
SC1-F	5′-AACGGTGAGAGATTTCTGTGC-3′	1170bp	(Josepa et al. 2000)
SC2-R	5′-AGCTGGCAGTATTCCCACAG-3′		

PCR Master Mix reactions (GeneDirex) were conducted in a 20µL total volume, containing 1x PCR master mix, 1 µL of primer (100 ng / µL) and 2 µL of DNA template, and 7 µL dH2O. The PCR program was as follows: Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, 30 s of annealing at 50°C, 30 s of extension at 72°C, and a final extension for 5 min at 72°C. PCR products were electrophoresed onto a submerged 1% agarose gel and the results were compared with a 100 bp ladder marker. The gel documentation system was used to visualize the banding patterns.

Molecular identification of bacterial isolates by 16S rRNA Sequencing

The16S rRNA gene sequencing was performed to identify the highest ethanol producing bacterial isolates. The sequencing was carried out as follows: the extracted DNA was used as a template in a PCR reaction to partially amplify the 16S rRNA gene with two universal bacterial primer sets, PS-1 (AGT CGA ACG GCA GCG GGG G) and Ps-2 (GGG GAT TTC ACA TCG GTC TTG CA) (Pastrik and Maiss 2000). Sequencing was done at SolGent Company, Daejeon, South Korea. The obtained sequence was compared with sequences available in the GenBank database (NCBI) using a BLAST search, and a phylogenetic tree was constructed using mega software (version 11).

Enhancing bioethanol productivity by mutagenesis

The Bacterial isolate producing the highest amount of bioethanol was selected to increase its bioethanol production by UV mutagenesis following the method used by (Al Makishah and Elfarash 2022). A 1 ml an overnight bacterial culture was spread on the Petri dish and irradiated with a UV lamp for different time periods (10 s, 20 s, 40 s, 1 min, 2 min, and 3 min). Plates were covered and incubated overnight to generate mutants. Different mutants were screened for ethanol production as described before.

Optimization for bioethanol production using molasses as a carbon source.

Several factors affect ethanol production, including sugar molasses (150, 180, 200 and 240 g/L), urea and ammonium sulphate concentration (1, 2, 3 and 4 g/L). Therefore, these factors were studied to increase ethanol production. Twenty-four hours old bacteria inoculum was used to inoculate the molasses at the rate of 15%,18%,20% and 24%. Production of ethanol was conducted in 100ml glass bottles that included 47ml of pretreated molasses and 3ml of 24h old culture. The bottles were incubated on a rotary shaker (150rpm) at 37oC and pH (7). An empty media without bacterial inoculum was used as Negative control.

These different concentrations were used to prepare several media inoculated that were incubated with the highest ethanol producing isolate and was incubated overnight statically at 37°C. After fermentation, the ethanol concentration in the samples were estimated based on volatility by an Ebulliometer used in distillation factories (Iland et al. 2000).

Results and Discussion

Isolation of different microbial isolates from molasses and vinasse

In the present investigation, seventy-six microbial isolates were isolated from the collected molasses and venasses (Figure 2). The microscopic investigation showed that the number of bacterial isolates was 64 while the yeast isolates were 12 (Figure 2).

As shown in Figure 2, the highest number of bacterial isolates obtained from Armant Sugar Factory, while the highest number of yeast isolates recovered from Nag Hamdy Sugar Factory.

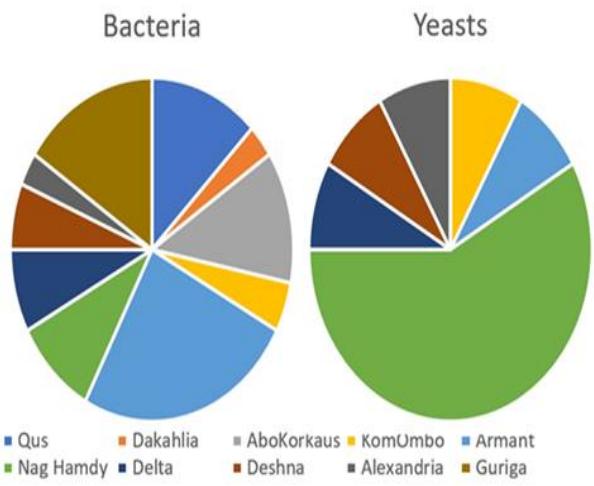

Figure 2. Number of bacterial and yeast isolates collected from different sugar factories.

Several investigation used molasses and venasses to isolate yeast and bacterial isolates. (Kechkar et al. 2019) used molasses, dates and figs to isolate three yeasts isolates. (Faiz Rasul et al. 2015) isolated 26 bacteria isolates from soil and molasses. (Farjana Islam and Narayan Roy 2018) isolated from molasses some bacterial isolates that were identified as Paenibacillus sp., Bacillus sp and Aeromonas sp.

Molecular identification of S. cerevisiae isolates

Agarose –gel electrophoresis showed that only 12 isolates (E2, G11, D2, D3, D4, D8, D10, D11, D12, C6, Z2 and f1) were able to produce the PCR specific band (1170 bp), so they were identified as S. cerevisiae (Figure.3). While this band disappeared by other isolates (64 isolate).

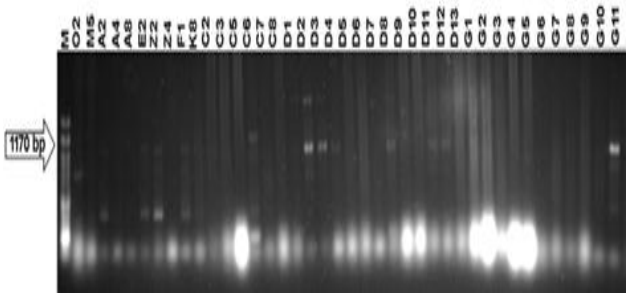

Figure 3. The agarose gel electrophoresis of the PCR product shows the presence of the 1170 bp band in some tested isolates.

These primers were also used by other researchers to identify the S. cerevisiae isolates from several isolates (Guimarães et al. 2006; Josepa et al. 2000).

Screening for bioethanol production microorganisms

Both bacterial and yeast isolates (Seventy-six isolates) were screened for their bioethanol productivity after 24 hours, at pH 7 and 37°C. When the bacterial isolates were screened for bioethanol productivity (Figure 4), isolates M3 showed the highest ethanol productivity (74%). While the lowest bioethanol productivity was 1% in C2 isolate. Figure (6) showed that the productivity divided the bacterial isolates into 3 different groups (High, Moderate, and Low). The high isolates (8 isolates) produced ethanol with a percentage range from 40% to 74%, while the moderate isolates (15 isolates) ranged from 27%-36%, and 41 low productivity isolates which were only able to produce less than 27% of bioethanol.

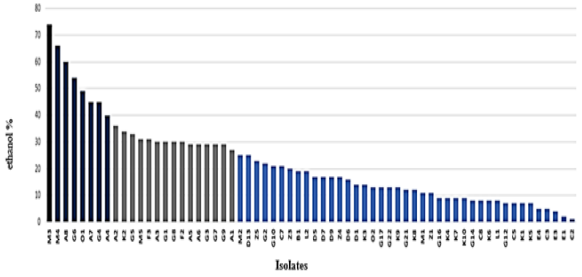

Figure 4. Bioethanol production by bacterial isolates

Yeast isolates showed lower ethanol productivity range (Figure 5) compared to the bacterial isolates (74% to 1%), since the range of yeast productivity was only from 29% to 2%. The highest yeast isolate (F1 isolate) in the productivity was 29%, while the lowest bioethanol productivity was 2% (G11 isolate). Since most yeast isolates were able to produce less than 21% of bioethanol, they were all considered as low ethanol producers.

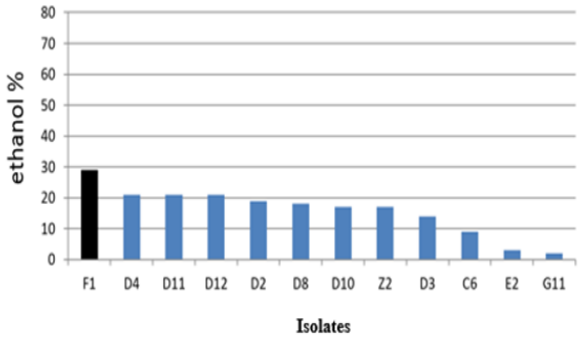

Figure 5. Ethanol production by yeast isolates.

Moreover, the results in Figure 6 showed a significant difference between the highest yeast isolate (G11 isolate) and the highest bacterial (isolates M3) in ethanol productivity when tested under the same condition (incubation time 24 hours and pH 7 and at temperature 37 °C.)

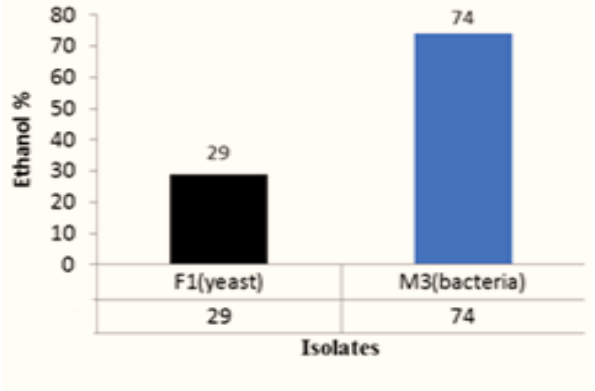

Figure 6. Comparison between the highest yeast isolate and the highest bacterial isolate in ethanol production.

Dien et al. (2003) reported that ethanol-producing bacteria have attracted much attention in recent years because their growth rate is substantially higher than that of the Saccharomyces. Among these ethanol-producing bacteria, Z. mobilis is a well-known species that has historically been employed in tropical regions to produce alcohol from plant sap (Skotnicki et al. 1983).

Bacterial isolates Identification by 16S rRNA sequencing

The best bacterial isolate that produces bioethanol (M3 isolate) was selected for molecular identification by 16S rRNA gene sequencing. The sequencing was done by the Gene Analysis Unit (Macrogene Inc., Seuol, Korea) using universe primers; PS-1 (AGT CGA ACG GCA GCG GGG G) and Ps-2 (GGG GAT TTC ACA TCG GTC TTG CA).

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 indicated that M3 isolate can be identified as K. pneumonia (Figure 7). (da Silva et al. 2020; Oh et al. 2011) also found that K. pneumonia demonstrated a high potential for ethanol production.

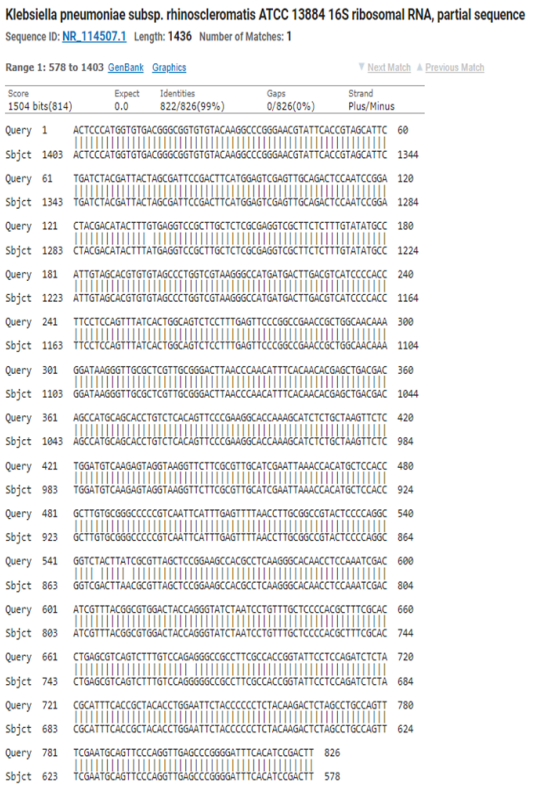

Figure 7. Sequence alignment of the M3 isolate (Query) against the partial 16S rRNA gene sequence data of K. pneumonia in GenBank showing 4 base substitutions.

Several sequences were selected from GenBank database for the construction of a phylogenetic tree to compare the M3 isolate with other closely related species. These strains were: K. pneumonia (NR 114507.1), K. quasipneumonia (NR 134063.1), Kluyvera ascorbate (NR 114589.1), K. intermedia (NR 028802.1), K. aerogenes (NR 113614.1).

The phylogenetic tree (Figure 8) of 16S rDNA gene sequences indicated that the M3 isolate and the K. pneumonia shared one clear clade with 99 % similarity.

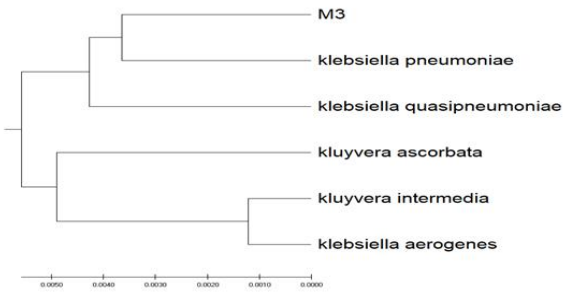

Figure 8. Phylogenetic tree relationships between the M3 isolate and other 16S rDNA gene sequences selected from GenBank database. In the phylogenetic tree, M3 and K. pneumonia were clustered together in one group.

Mutagenesis of the best ethanol producing isolate by UV irradiation.

Mutagenesis is a technique commonly used to improve the performance of enzymes as well as for high productivity in several organisms such as bacteria (Joshi et al. 2013) and Fungi (Hasan et al. 2019). Mutagenesis has been used to enhance bioethanol production levels in the best ethanol producing wild type isolate (M3). Mutagenesis was performed for different periods of exposure to UV irradiation. Mutants were selected from surviving bacteria after exposure. A total of 20 different mutants obtained from the mutagenesis of M3 isolate were screened for bioethanol productivity. Results in Figure 9 showed that all the selected mutants produced lower amounts of bioethanol compared to the wild-type (M3 isolate),

while (Oh et al. 2011) reported that a mutant strain of K. pneumoniae showed increment in production of ethanol. This results agreed with Strub et al. (2004) who found that mutagenesis did not always improve productivity.

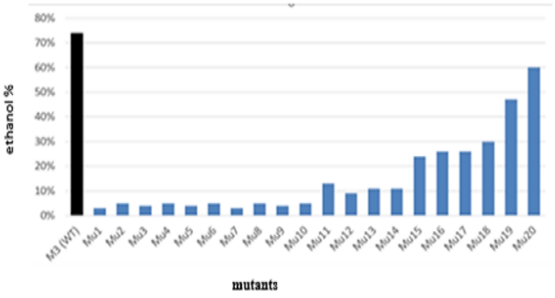

Figure 9. Bioethanol production of the 20 selected mutants produced from M3 isolate mutagenesis.

Table 2. Bioethanol production at different conditions.

Molasses sugar concentration (%)	Urea	Ammonium sulphate	3 days
15%	1	1	0
15%	2	2	0
15%	3	3	0.2
15%	4	4	0.8
18%	1	1	0
18%	2	2	0.5
18%	3	3	0
18%	4	4	0
20%	1	1	0
20%	2	2	0.2
20%	3	3	0
20%	4	4	1
24%	1	1	0
24%	2	2	0
24%	3	3	0.8
24%	4	4	0.1

The obtained results are nearly similar to those recorded by (Al-Talibi et al. 1975; Rasmey et al. 2018), who observed that the alcohol produced by S. cerevisiae grown increased with increasing sugar concentration in the juice from 10-25% and then decreased.

Conclusions

Seventy -six different isolates were collected from contaminated Molasses and Vinasse. Yeast isolates were identified by specific primer, while one of the bacterial isolates were identified by 16S rDNA sequencing. When bioethanol productivity was screened, yeast isolates showed less production of bioethanol than bacterial isolates. The effect of different parameters in bioethanol production were studied for k. pneumoniae. The highest achieved production was 1%(v\v) of bioethanol production when the media were supplied with 20% of molasses, 0.4% of urea and 0.4% of ammonium sulphate after 3 days at 37 °C and pH=7. UV- mutagenesis could not improve the bioethanol productivity in all the selected mutants.

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