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Isolation, identification and characterization of ethanol tolerant yeast species from fruits for production of bio-ethanol

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Abstracts: Ethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels. This study focused on the isolation, identification and characterization of indigenous yeast species involved in ethanol production using morphological and Biolog identification techniques. Varieties of fruit (mango, papaya and orange) samples were collected from Benshangul Gumize and Metehara at 9479-1570m altitudinal ranges. Streak plate techniques were done on yeast extract peptone dextrose agar (YPDA) by taking 0.1ml of serially diluted fruit juice and incubated at 30°C. Pure cultures were transferred on to BUY agar plate and incubated at 30°C. Single Colony of morphologically identified yeast species were transferred in to micro plate containing different types of nutrients and biochemicals. Incubated for 24, 48 and 72 hours at 30°C temperature and micro plate reading were carried out using MicroLog 3 Software. Seven ethanol tolerant yeast species were accurately identified i.e. *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, *Zygosaccharomyces fermentati*, *Candida sorboxylosa*, *Candida apicola*, *Kluyveromyces delphensis*, and *Issatchenkia orientalis* and two of them not tolerate ethanol. Most of the identified yeast species are tolerate temperature and different concentration of alcohol up to 16% and assimilating pentose and hexose sugars so possible candidates for the production of high yield ethanol which can be exploited in future by industries for alternative fuel production from the renewable sources such as fruits.

Key words: - Assimilation, Biolog, Fruits, Micro station, Oxidation

BACKGROUND

Global crude oil production is predicted to decline five times below its current level by 2050. Based on World Energy Council (WEC) calculations, the world-wide primary energy consumption is approximately 12 billion tons coal equivalent per year. United Nations calculations have shown that the world's population will increase to about 10 billion people by 2050 which will in turn increase energy demands to at least 24 billion tons coal equivalent per year (twice of what we consume today) depending on economic, social and political developments^[1,2].

Recently they have been used in the production of bio fuels, a potentially important alternative energy source. Renewable energy is one of the most efficient ways to achieve sustainable development. Increasing its share in the world matrix will help prolong the existence of fossil fuel reserves, address the threats posed by climate change, and enable better security of the energy supply on a global scale^[3]. Successful fermentations to produce ethanol using yeast require tolerance to high concentrations of both glucose and ethanol. These cellular characteristics are important because of high gravity (VHG) fermentations, which are common in the ethanol industry, give rise to high sugar concentrations, at the beginning of the process, and high ethanol concentration at the end of the fermentation *Saccharomyces cerevisiae* is an important micro organism in bio- industry and its tolerance to ethanol is one of main characteristics to decide whether it can be used as bio-fermentation resources^[3]. Yeast has been isolated from variety of natural sources like leaves, flowers, fruits etc^[4, 5, 6, 7]. Being a sugar-loving microorganism, it is usually isolated from sugar rich materials. Fruits contain high

sugar concentration and hence yeast species are naturally present on these and can be easily isolated from fruits. Distinct wild yeast species are supposed to be present and associated with different fruits in natural environments^[6]. Because of yeast fermentative characteristic, there is always a need for yeast strains with better features of fermentation especially high ethanol tolerance for production of ethanol as bio fuel on commercial scale^[8]. The population of micro flora on the substrate always depends on the pH of the substrate. Since fruits are acidic in nature they are predominantly inhabited by yeasts^[9]. Yeast strains associated with fruit surfaces are capable of converting wide range of sugars into alcohol. Ethanol tolerance, sugar tolerance and invertase activities are some of the important properties for use in industrial ethanol production. Yeast has also been isolated from many fermenting sources including fermenting cassava tubers. Many research workers found yeast in large numbers in a wide variety of natural habitats as different as leaves, flowers, sweet fruits, tree exudates, grains, roots fleshy fungi, insects, dung, soil^[3]. During the last three decades both Ascomycetous^[10, 11, 12, 13, 14] and Basidiomycetous yeasts^[11, 12, 15, 16] belonging to the genera *Candida*, *Pichia*, *Clavispora*, *Issatchenkia*, *Kluyveromyces*, *Kloeckera*, *Torulaspora*, *Geotrichum*, *Cryptococcus* etc., have been identified for their ability to produce ethanol from D-xylose. Therefore, it is likely that yeasts capable of assimilating hexoses and (or) pentoses could be possible candidates for the production of ethanol. With this in view, it was logical to assume that yeasts of the genera *Rhodotorula*, *Cryptococcus*, *Sporobolomyces*, *Saccharomyces*, *Candida*, *Pichia* etc., isolated from fresh and rotten fruits^[17, 18, 19, 20] could produce ethanol.

Yeasts of the genera *Kloeckera*, *Hanseniaspora* and *Candida* predominate in the early stages of the fermentation, followed by several spp. of *Matschnikowia* and *Pichia* in the middle stages, when the ethanol rises to 3-4 %^[21]. The latter stages of spontaneous wine fermentation invariably are dominated by the alcohol-tolerant strains of the *Saccharomyces cerevisiae*. The study therefore, was aimed at isolating, identifying and characterizing ethanol producing yeast species from different fruits types with its potentials for industrial ethanol production.

METHODS AND MATERIALS

Study area

Study was conducted at Benshangul Gumiz (Asosa) and Oromia region (Metehara). Asosa is located in the Asosa Zone of the Benshangul Gumiz Region; it has a latitude and longitude of of 10°04'N 34°31'E, with an elevation of 1570 meters. Metahara is Located in the Misraq Shewa Zone of the Oromia Region, it has a latitude and longitude of 08°54'N 39°55'E, with an elevation of 947 meters above sea level (Figure.1).

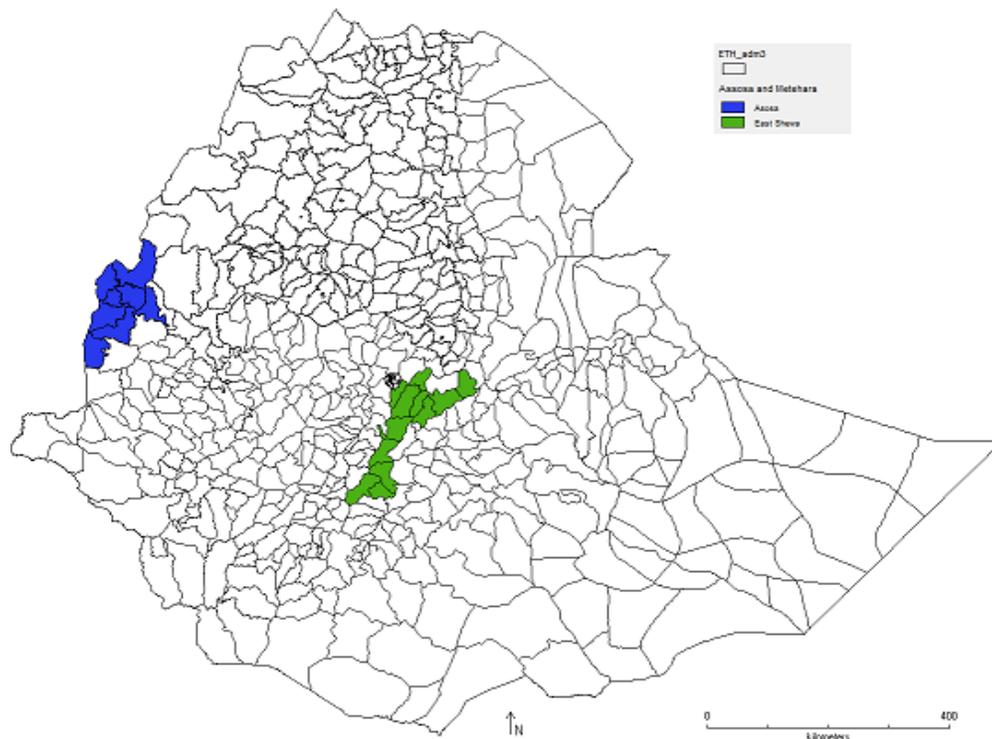


Figure. 1. Map of study area

Collection of samples

Varieties of fruit (mango, papaya and orange) samples were collected from Benshangul Gumiz and Oromia region in sterile poly ethylene plastic bags from 2006-2007 G.C and transport to mycology laboratory, Ethiopian Biodiversity Institute.

Laboratory work

Isolation of yeasts

The fruits were washed and rinsed many times in distilled water. They were then cut, squeezed and collected in the sterile test tubes. Mixed samples were dilute serially and 0.1 ml of diluted fruit juice was plated on yeast extract peptone-dextrose agar medium (YEPD) supplemented with antibiotics (chloramphenicol) and incubated at 30°C for 48hours. Yeast colony was sub-cultured until the Purified cultures were maintained and kept at 4°C.

Identification and characterization of yeast species

Morphological and Biolog identification system of the yeast species was studied using the conventional methods described by Kurtzman and Fell^[22], and also conventional methods of the Biolog identification system.

Morphological identification and characterization

According to the method of Kurtzman and Fell^[22], morphology of the yeast cells was observed.

Cultural (colonial) characterization

Morphology of ethanol producing yeasts and their appearance on solid Medium (YPD) was examined based on their cultural characteristics (Colony shapes, size, pigment, elevation, edge and surface appearance).

Biolog identification and characterization

Biolog system for yeast identification consisted of the Micro Station and YT micro plate. Micro station is semi automated machines used for reading YT micro plate. YT Micro Plate are prefilled and dried with all necessary nutrients and biochemicals in to the 96 wells of the plate. YT Micro Plate is configured with both metabolism test and turbidity tests. The first 3 rows of the panel (rows A-C) contain carbon source metabolism tests using tetrazolium violet as a colorimetric indicator. The next five rows of the panel (rows D-H) contain carbon source turbidity tests. Results from this test are scored turbid metrically. The last row of the panel (row H) has wells that contain 2 carbon sources. These wells test for the co-utilization of various carbon sources with D-Xylose. Yeast suspension was prepared in 15ml sterile distilled water and

adjusted to 47-49% T using Biolog YT turbidity standard. One hundred microliters of inoculum was added to each well of the YT Micro Plate (BiologInc) and incubated at 28-30°C for 24, 48 or 72 hours until a sufficient metabolic pattern is formed. During incubation, yeast respiration in wells containing compounds that can be utilized will either reduce the tetrazolium dye forming a formazon purple color or initiate growth leading to an increase in turbidity. Each metabolic pattern was read by a Micro Station (BiologInc) at a single wavelength of 590 nm and interpreted by micro log software ver. 4.20.05 (Biolog, Hayward, CA).

RESULT

Isolation of yeasts

In the present study yeast cultures were isolated from different fruit samples as mentioned in the methods and materials. A total of 20 different isolates were isolated from all collected fruit samples (*Citrus sinensis*, *Carica papaya* and *Mangifera indica*). The isolates were identified as yeast based on their colony morphology (pigmentation, shape, size, texture, elevation and margin) (Figure.1).



Figure.2. Pictures of some isolated yeast strains

Morphological identification and characterization of ethanol producing yeast species

The primary identification of the yeast isolates from different fruit samples was done on the basis of morphological characteristics of colonies on solid media. Their morphology on culture media (BUY) agar was obtained. The highest percentage occurrence on culture media recorded as 19%

Candida sorboxylosa, 18% *Pichia holistii*, 15% *Kluyveromyces delphensis*, 12% *Zygosaccaromyces fermentati*, 10% *Issatchenkia orientalis*, 9 % *Saccharomyces cerevisiae B*, 6% *Saccharomyces boulardii*, and 5% *Candida zeylanoides*. The lowest percentage occurrence on culture media was 4% *Zygosaccharomyces bisporus* and 3% *Candida apicola* (Table. 1).

Table .1 Morphological characteristics of the isolated yeasts

Isolated code	Name of organisms	Pigmentation	Colony morphology	Cell size
Y1	<i>Saccharomyces boulardii</i>	white	Raised, Ovoid, smooth	medium
Y2	<i>Zygosaccharomyces bisporus</i>	Yellowish	Raised, circular, smooth	Medium
Y3	<i>Kluyveromyces delphensis</i>	Creamy	Flat, Fury	medium
Y4	<i>Candida apicola</i>	Brown	Mucoid, circular	medium
Y5	<i>Issatchenkia orientalis</i>	white	Dry, flat, rough	large
Y6	<i>Candida sorboxylosa</i>	white	Raised, circular	Medium
Y7	<i>Pichia holistii</i>	white	Raised , circular, smooth	Medium
Y8	<i>Saccaromyces cerevisiae B</i>	white	Raised , circular, smooth	large
Y9	<i>Zygosaccharomyces fermentati</i>	white	Raised , circular, smooth	Large

Biolog identification and characterization of ethanol tolerant yeast species

The isolates were identified at species level by using omnilog micro station and YT micro plates. During the present investigation a selective medium that is BUY agar was used for isolating yeast species from different fruit types for Biolog identification. 9 yeast species were accurately identified which is involved in ethanol production. Among these yeast species Omni log \geq 90% probability and 0.60 similarity results read

seen in 9 yeast species, i.e. 100% *saccharomyces boulardii*, 99% *Saccharomyces cerevisiae B*, 99% *Candida apicola*, 98% *Pichia holistii*, 97% *Issatchenkia orientalis*, 97% *Zygosaccharomyces bisporus* and they are followed by 86% *Kluyveromyces delphensis* has 86% probability and 0.55 similarity, *Zygosaccharomyces bisporus* has 83% probability and 0.66 similarity and *Candida sorboxylosa* has 76% probability and 0.52 similarity (Table. 2) respectively.

Table.2. Biolog identification and characterization result

Species	Probability	Similarity	Incubation hour	Host fruits	Status
<i>Saccharomyces boulardii</i>	100%	0.847	48	<i>Mangifera indica</i>	>>
<i>Saccaromyces cerevisiae B</i>	99%	0.722	72	<i>Mangifera indica</i>	>>
<i>Candida apicola</i>	99%	0.504	72	<i>Mangifera indica</i>	>>
<i>Pichia holstii</i>	98%	0.683	48	<i>Carica papaya</i>	>>
<i>Issatchenkia orientalis</i>	97%	0.83	48	<i>Citrus sinesis</i>	>>
<i>Zygosaccharomyces bisporus</i>	97%	0.66	48	<i>Carica papaya</i>	>>
<i>Kluyveromyces delphensis</i>	86%	0.553	72	<i>Citrus sinesis</i>	>>
<i>Zygosaccharomyces fermentati</i>	83%	0.58	48	<i>Mangifera indica</i>	>>
<i>Candida sorboxylosa</i>	76%	0.52	72	<i>Citrus sinesis</i>	>>

Temperature tolerance test

The ability of the yeast to grow at higher temperatures was verified by plating the yeast species onto YPG medium and incubated at 2 different temperatures i.e.26, 30, 37 and 45°C for 72 hours. All identified yeast species (*Saccharomyces cerevisiae*, *Saccharomyces blourdeous*, *Candida apicola*,

Candida sorboxylosa, *Issatchenkia orientalis*, *Zygosaccharomyces fermentatii*, *Kluyveromyces delphensis*, *Zygosaccharomyce bisporus* and *Pichia holistii*) were tolerate to the temperature up to 37°C but not at 45°C (Table.3.).

Table.3. temperature tolerance test

Yeast species isolated from local fruits	Temperature			
	26	30	37	45
<i>Saccharomyces cerevisiae</i>	+	+	+	-
<i>Saccharomyces blourdeous</i>	+	+	+	-
<i>Zygosaccharomyces fermentatii</i>	+	+	+	-
<i>Zygosaccharomyce bisporus</i>	+	+	+	-
<i>Candida apicola</i>	+	+	+	-
<i>Candida sorboxylosa</i>	+	+	+	-
<i>Pichia holistii</i>	+	+	+	-
<i>Issatchenkia orientalis</i>	+	+	+	-
<i>Kluyveromyces delphensis</i>	+	+	+	-

Ethanol tolerance test

The ability of the identified yeast species to grow in ethanol concentrations medium were tested by growing them in YPG broth containing 4 different concentration of ethanol, 2%, 6%, 10% and 14% (v/v), respectively and incubated at 30°C for 72 hours. Most of identified yeast species (*Saccharomyces cerevisiae*, *Saccharomyces blourdeous*, *Candida apicola*,

Candida sorboxylosa, *Issatchenkia orientalis*) were grow well at 2%, 6%,10% ,14% and 16% of concentration of alcohol, (*Zygosaccharomyces fermentatii* and *Kluyveromyces delphensis*) are grown well at 2% and 6% alcohol concentration of alcohol, (*Zygosaccharomyce bisporus* and *Pichia holistii*) are not grown at different concentration of alcohol (Table.4.).

Table.4. ethanol tolerance test

Yeast species isolated from local fruits	Different concentration of alcohol				
	2%	6%	10%	14%	16%
<i>Saccharomyces cerevisiae</i>	+	+	+	+	+
<i>Saccharomyces blourdeous</i>	+	+	+	+	+
<i>Zygosaccharomyces fermentatii</i>	+	+	-	-	
<i>Zygosaccharomyce bisporus</i>	-	-	-	-	
<i>Candida apicola</i>	+	+	+	+	+
<i>Candida sorboxylosa</i>	+	+	+	+	+
<i>Pichia holistii</i>	-	-	-	-	-
<i>Issatchenkia orientalis</i>	+	+	+	+	+
<i>Kluyveromyces delphensis</i>	+	+	-	-	-

DISCUSSION

Yeast Micro Plate has two different tests: (metabolism test and turbidity tests) used for yeast species identification and characterization. All necessary nutrients and biochemical's are prefilled and dried into the 96 wells of the plate. Tetrazolium violet is used in some of the wells as a redox dye to calorimetrically indicate the oxidation of the carbon sources. The utilization of carbon sources in the other wells is indicated by an increase in turbidity.

Interpretation of Results: The color density or turbidity increase in each well is referenced against the negative control wells, A-1 and D-1. All wells optically resembling the negative control wells are scored as "negative" (-) and all wells with a noticeable increase in absorbance at 590 nm are scored as "positive" (+). Wells with an extremely slight increase in absorbance at 590 nm are scored as "borderline" {}. Reports that indicated a probability and similarity were chosen only for species identity. For Micro Plates read at 24 hours of incubation, the similarity index must be at least 0.75 to be considered as acceptable species identification. At 48 or 72 hours of incubation, the similarity index must be at least 0.50 to be considered acceptable (table.2). After completion of micro station read a single colony of identified yeast cell was picked and transferred to cryo preservative media under aseptic conditions and preserved at -80°C.

Fruits are locally available and thus served as readily available raw materials for the isolation of ethanol tolerant yeasts. Wild yeast species are supposed to be present and associated with different fruits in natural environments^[6]. Because of yeast fermentative characteristic, there is always a need for yeast strains with better features of fermentation especially high ethanol tolerance for production of ethanol as bio fuel on commercial scale^[8]. Eghafona *et al.*,^[23] isolated various strains of indigenous yeasts capable of producing ethanol from local fermented pineapple and orange juice. In this study from the different fruit samples (*Citrus sinensis*, *Carica papaya* and *Mangifera indica*), isolated 9 different isolates of yeasts belonging to 7 genera: *Candida*, *Issatchenkia*, *Kluyvermyces*, *Pichia*, *Saccharomyces*, *Zygosaccharomyces*. Results are greatly supported by Hashem^[25], who isolated many of yeast species from vegetables, fruits, honey. *Zygosaccharomyces rouxii* was isolated previously from food products such as sugar syrups and fruit juices^[26]. The association of wide spectrum of yeast species with fruits is because fruits contain adequate sugars and nutrients that are important for several yeasts^[27].

Among the identified yeast species (*Saccharomyces cerevisiae*, *Saccharomyces blourdeous*, *Candida apicola*, *Candida sorboxylosa*, *Issatchenkia orientalis*) were relatively show rapid growth in the growth media having 10%-16% of ethanol concentration and (*Zygosaccharomyces fermentatii* and *Kluyveromyces delphensis*) are growth in the media containing 2%-6% alcohol concentrations were as (*Zygosaccharomyces bispurus* and *Pichia holstii*) are not growth in media containing different concentration of alcohol. All the nine identified yeast species were growth up to 37°C temperature (table). The result is supported by Nwachukwu *et al.*^[33]. *Saccharomyces cevisiae* isolated from raffia palm wine are

tolerated up to 16 % (v/v) ethanol concentration. Ethanol tolerance, temperature tolerance, sugar tolerance and invertase activities are some of the important properties for use in industrial ethanol production^[34].

Out of all isolated yeast cultures, most of the identified yeast species were assimilated different types of carbohydrates as sole source of carbon which few were oxidized. *Issatchenkia orientalis*, *Kluyveromyces delphensis*, *Candida sorboxylosa* and *Candida apicola* are commonly assimilated carbohydrates:- D-glucose, sucrose, D-raffinose, D-trehalose, D-xylose, cellobiose, maltitol, methyl succinate + D-xylose and thus yeast species are tolerate temperature up to 37°C and alcohol concentration up to 16% so those are candidates for high yield ethanol production. The results are supported by Nigam *et al.*^[11] Alexandra *et al.*^[13] and Limtong *et al.*^[26] The yeast genera *Candida*, *Issatchenkia*, *Kluyveromyces*, *Pichia* and *Cryptococcus* produced more than 1 g of ethanol per litre from (20 g l/L) D-xylose. Therefore, it is likely that yeasts capable of assimilating hexose and pentose sugars could be possible candidates for the production of ethanol. *Zygosaccharomyces fermentatii* and *Pichia holstii* are commonly assimilated carbohydrates:- D-glucose, D-galactose, D-galactose + D-xylos, D-trehalose, D-raffinose, dextrin, inullin, salicin maltito,l maltose, sucrose, turanose, cellobiose, and arabinose. The results also supported by other investigator Bhadra *et al.*^[19] which state that species isolated from fresh and rotten fruits that assimilating xylose and arabinose as ability to produce ethanol. *Zygosaccharomyce bispurus* do not assimilate any types of carbohydrates; an occasional species ferment glucose. Thus result supported by other investigators Paraggio^[28]. The more frequently encountered yeasts in the early fermentation phase are non-Saccharomyces species.

Pichia holstii commonly assimilated and oxidized carbohydrates: - D-glucose, D-trehalose, cellobiose, salicin and gentiobiose. This is supported by other investigator Papalexandratou who studied the spontaneous fermentation of coca and mentioned that many yeast species were found during the early stage of fermentation. They reported that the genus *Pichia* was the dominant ethanol-producing yeast species followed by *Saccharomyces cerevisiae*.

Saccharomyces cervisiae and *saccharomyces boularidii* commonly assimilated carbohydrates: - D-glucose, D-galactose, turanose, maltose, sucrose, turanose, D-galactose +D-xylos and are the first candidates that produced high yield of ethanol. The results were supported by Al-Talibi *et al.*^[29] who found that the ethanol yield produced by *Saccharomyces cerevisiae* fermented 20% pure sugar solution reached 9.96%. Mehaia and Cheryan^[30] reported that the ethanol production by *S. cerevisiae* was 48.27% and 47.0% of the total sugar concentration when 9.8 and 13.83% sugars of date juice were used in batch fermentation, respectively. *Saccharomyce cerevisiae*, *Saccharomyces boulardii*, *Zygosaccharomyces bispurus*, *Pichia holstii* and *kluyveromyces delphensis* commonly oxidized:-D-glucose, D-galactose, maltose, L-glutamic acid, L- proline and xylitol. These results are greatly supported by Karczewska^[31] who was the first person to report direct conversion of xylose to ethanol by yeast. The first step in the metabolism of D-xylose is the transport of the sugar across

the cell membrane and this is mediated by glucose transporters in the absence of a specific transporter for xylose^[32]. Granstrom and Leisola^[33] and Rao *et al.*^[20] reported that the enzyme xylitol dehydrogenase oxidizes xylitol to xylulose which is then phosphorylated to xylulose-5-phosphate by xylulokinase. Xylulose-5-phosphate is then metabolized through the pentose phosphate pathway into ethanol.

CONCLUSION AND RECOMMENDATION

Yeast can be isolated from variety of natural resources such as leaves, flowers, fruits etc^[4, 5, 6, 7]. Being a sugar-loving microorganism, it is usually isolated from sugar rich materials. Fruits contain high sugar concentration and hence yeast species are naturally present on these and can be easily isolated from fruits. The population of micro flora on the substrate always depends on the pH of the substrate. Since fruits are acidic in nature they are predominantly inhibited by yeasts. Yeast strains associated with fruit surfaces are capable of converting wide range of sugars in to alcohol. Based on the results obtained from identification and characterization studies It can concluded that these nine identified yeast species belonging to *Saccharomyces cerevisiae*, *saccharomyces boulardii*, *Zygosaccharomyces fermentati*, *Candida sorboxylosa*, *Pichia holstii*, *Kluyveromyces delphensis* *Issatchenkia orientalis* and *Candida apicola* commonly assimilate hexose and pentose sugars which could be taken as possible candidates for the production of high yield of ethanol which can be exploited in the future by industries for bio-ethanol production. *Zygosaccharomyces bisporus* oxidized some carbohydrates; as any occasional organism ferment carbohydrate to produce alcohol. The result obtained from this study reveal a strong indication of yeast's great potential in the production of ethanol using locally available substrates especially high sugar containing fruits.

The following recommendation has been made to possibly isolate, identify and characterize ethanol tolerant yeast species for production of ethanol: The potential of ethanol producing yeasts from different fruits were not exploited. Therefore, efforts should have to be directed toward exploitation of ethanol producing yeast species especially from rotten fruit and other sugar containing fruit it with collected from rotten fruits damping sites of the country for novel strain discovery and application for industrial ethanol production. Further analysis is very important for studying molecular characteristics of the identified yeast species.

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