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Isolation and Characterization of Yeast Inhabiting Alcohol Processing Environment in Bayelsa State, Nigeria

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Abstract: Distillation environments represents a rich resource of microbial diversity, some of which are of high value to industry. This study was undertaken to isolate, screen and identify naturally evolved indigenous yeast with ethanol tolerant capabilities. Data obtained revealed that using 0.2% Chloramphenicol supplemented potato dextrose media, yeast isolates were attainable from soil samples obtained from a local distillery in Bayelsa state, Nigeria. The obtained colonies were capable of fermenting glucose, sucrose, fructose and galactose giving off distinct yellow colour using phenol red broth method. Morphological examination revealed that the isolates obtained were white, round shaped, smooth textured and flat elevation with transparent opacity. A dip in its growth curve was observed in broth cultures consisting of yeast extract, peptone, malt extract, glucose containing 10-20% (v/v) absolute ethanol between 24-48 hours of incubation at 30°C. In this broth cultures, a progressive growth curve was observed between 48-120 hours at the same parameters for incubation. The isolate also demonstrated good growth in ethanol supplemented medium with pH ranging from 5.2-6.6 at 30°C. Growth measurements were determined by measuring optical density of the cells in broth using spectrophotometer at 570nm. The basic local alignment search tool (BLAST) of the genetic sequence obtained revealed a 98% similarity to *Meyerozyma guilliermondii*. The results obtained suggested that non-Saccharomyces species possess ethanol tolerant ability, particularly those obtained from alcohol rich environments. Such yeast species could be applied towards the fermentation of ethanol for industrial uses.

Keywords: Ethanol, Ethanol Tolerance, *Saccharomyces cerevisiae*, Non-saccharomyces, *Meyerozyma guilliermondii*

1. Introduction

Industrial ethanol production via the direct fermentation of glucose is easily achieved using *Saccharomyces cerevisiae*. One of the major factors of importance for this product towards its use as a fuel is the reduction of excess greenhouse gases, thereby placing ethanol poised as a key renewable biofuel to service industries where it serves as component of anti-freezing agents, germicides or as industrial solvents [1]. However, microbial cultures employed in ethanol fermentation are subjected to a range of stress factors including high concentrations of ethanol [2]. Studies confirm that during fermentation, ethanol is toxic to the cell whereby it causes a cascade of events particularly the disruption of amino acid and glucose transport systems, which leads to structural alterations to both the cell membrane and functions proteins; this in effect reduces the cells viability thereby causing cell death [3], [4].

Therefore, to meet the increasing demand for bioethanol, an ideal candidate for fermentation would be one that can ensure increasing concentrations of ethanol, thereby increasing productivity [5]. Biotechnology proffers an array of solutions ranging from the identification and introduction of genes that confers tolerance to ethanol stress, thereby

creating mutant strains or the screening and isolation of naturally evolved ethanol tolerant strains [6]. The later which possess peculiar resistance genes that synthesize certain categories of proteins or effector molecules which together coordinate stress resistance, can be obtained from unexplored habitats [7], [8].

Studies show that non-Saccharomyces yeasts like *Candida sp., Hansenula sp., Kloeckera sp., Torulaspora sp., Kluyueromyces sp*., *Pachysolen tannophilus, Pichia stipitis* and many more possess the ability to grow and participate in alcoholic fermentation [9], [10], [11]. Just like *Saccharomyces cerevisiae* which produce ethanol by metabolize fermentable sugars which include glucose, xylose, etc, (figure 1) these natural occurring non-saccharomyces yeast are capable of metabolizing the same simple sugars to produce ethanol via the Embden-Meyerhof-Parnas pathway [12].

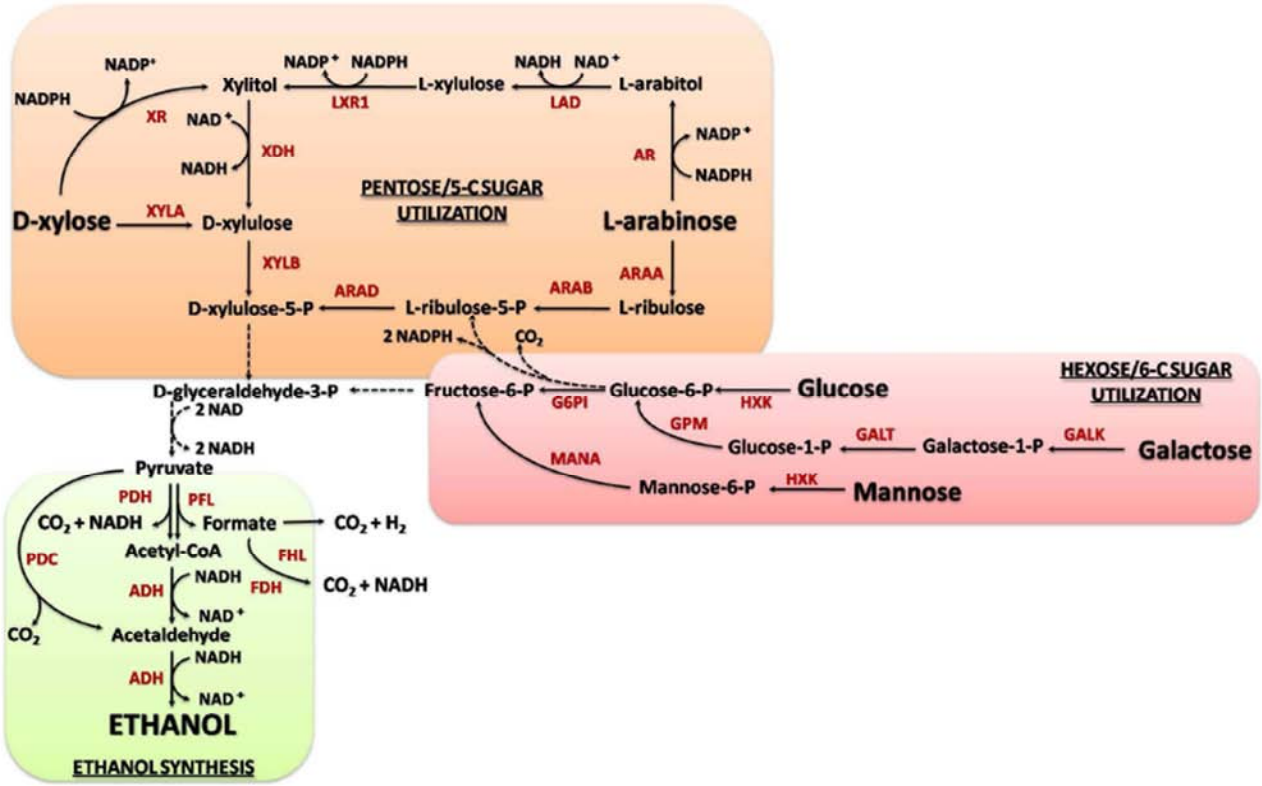

Figure 1. *Ethanol production via Xylose and Glucose utilization pathway.*

Non-*Saccharomyces* yeast strains like *Pichia stipitis* was reported to produce ethanol from different sugars, yielding between 0.42-0.47 grams of ethanol per substrate utilized [13], [14]. *Kluyveromyces marxianus* was found to produce ethanol yields of about 37.1g/L compared to 40.9 g/L obtained from *Saccharomyces cerevisiae*, both with some growth optima of 30^{o}C, pH range 4.5-5.5 [15]. In another report, a specie of *Pichia*; *Pichia anomala* isolated from a local distillery in China was reported to endure ethanol concentrations of up to 14% [16], [17]. It then stands that screening for high ethanol tolerant wild-type yeasts of *Saccharomyces* or non-*Saccharomyces* origin could be sourced from sugar-rich environments or from local distilleries or wineries. The difference in strains and species obtained as a result of the degree of stress involved therefore infers a genetic diversity which supports adaptation of peculiar environments. The objective of this study is then to acquire an ideal wild-type yeast candidate for ethanol production that possesses good ethanol tolerant ability from sources.

2. Materials and Methods

2.1. Isolation of Yeast

Sources selected for isolation of ethanol tolerant yeasts were soil samples from a local distillery in Imiringi village of Yenogoa Local Government Areas, Bayelsa state, Nigeria. Isolation and morphological identification was done using potato dextrose agar supplemented with 0.2mg/ml chloramphenicol [18].

2.2. Identification by Fermentation Assessment

Colonies with distinct morphological characteristics were randomly selected and purified by repeat streaking on the antibiotic supplemented potato dextrose agar (PDA). The pure strains were transferred, maintained on PDA slants using 20% glycerol and stored at 4^{o}C until needed for further studies. Distinct isolates were subjected to morphological identification aided by gram staining with the following parameters: Colour, Shape, Texture, Elevation, Margin and

Opacity [18]. The carbohydrate utilization capability was determined using phenol red broth technique in which were fructose, galactose, lactose and sucrose were the test sugars [18]. Sugar utilization formed a basis of biochemical identification [19].

2.3. Screening for Ethanol Tolerance

A modified ethanol tolerance test method was adopted [18]. The isolate was grown in yeast extract, peptone, malt extract, glucose broth (10mls) in the presence of 0, 5, 10, 15, 20% (v/v) ethanol in 250ml flasks. Periodic (24 hour) measurements of growth was achieved using a UV-VIS spectrophotometer, measurements were taken at 570nm. Viability determination as well as pH measurements were analysed [20].

2.4. Molecular Characterization

Species diagnostics was achieved through the amplification of the PCR (polymerase chain reaction) products using the ITS1 (5^1-TCCGTAGGTGAACCTGCGG-3^1) and ITS4 (5^1-TCCTCCGCTTATTGATATGC-3^1) primers [21]. The PCR cycle ran at 94°C for 2 min, 52°C for 1 min and 72°C for 2 min. Cloning was achieved using a pGEM vector prior to sequencing. Sequence analysis was done using corresponding neighbour sequences from GenBank-NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). The results obtained were further imported into MEGA software for the construction of a phylogenetic tree using Bootstrap analysis and the statistical method used was Neighbor-joining.

3. Results

3.1. Isolation and Identification of Isolates

The obtained isolate was considered to be a yeast isolate based on the isolating media, and colony characteristics. Morphological examination of the isolate revealed a creamy white, round shaped, smooth textured and flat elevation with transparent opacity (figure 2, table 1). The budding stage of the yeast isolates was observed under (40X) microscope (Table1).

In the sugar fermentation test, the obtained isolate demonstrated a dexterous ability to consume all tested sugars; fructose, glucose, galactose and sucrose using phenol red broth assay (Table 1). The morphological and sugar fermentation data steered towards the preliminary assumption that the isolate was a strain of *Saccharomyces cerevisiae*.

Figures 4 & 5 were obtained from the analysis of amplified DNA fragments and sequence analysis between 500-750bp, from the yeast isolate. The partial nucleotide sequences obtained were subjected to BLAST analysis and the identity was established on the basis of sequence similarity and closest neighbour. The blast sequence query showed that predominantly *Meyerozyma guilliermondii* strains with accession numbers KF91353.1, KT897919.1, LN626313.1, KU883323.1, KT282394.1, KT385726.1, KT923173.1 and KR063216.1 from the Genebank Library database, has 98% sequence homology with the genomic DNA sequence of the isolate at both ITS regions (Table 2, figure 6). The isolate also shared the same percentage sequence similarity with other yeast strains that are anamorphs of *Meyerozyma guilliermondii.*

3.2. Ethanol Tolerance of Yeast Isolates

The ethanol tolerant yeast would prompt extended batch production processes, thus promoting high yields of ethanol over a sustained duration. The effect of ethanol on the growth rate of the isolate is depicted in figure 3. Good growth performance was observed in the samples containing 5% ethanol. The growth curve persisted in sample broths containing higher concentrations (10-20% (v/v)) of exogenous ethanol although a degree of reticence was observed.

3.3. pH Analysis

Variations in the pH traversing the period of incubation duration in the broth samples containing different concentrations of ethanol was examined (table 3). Analysis of the data obtained indicates fluctuations in pH levels within the range of pH 5.2-6.8 in broth samples without any initial ethanol. This addresses the veracity of the cell membrane coupled with the cells acidification activity.

Figure 2. *Pictures of pure yeast isolate on PDA medium.*

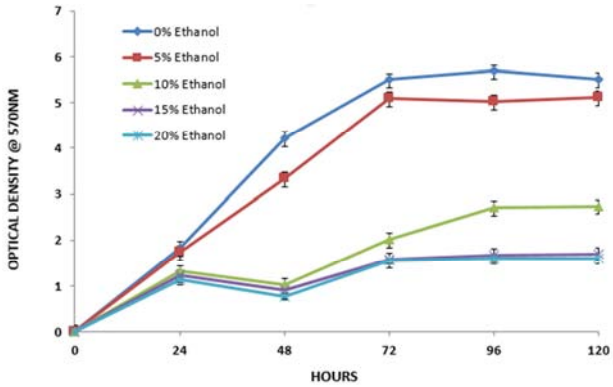

Figure 3. *Growth determination of yeast isolate over time with increasing concentrations (%, v/v) of ethanol. Data was obtained from the mean triplicates.*

Table 1. *Morphological identification and sugar fermentation of yeast isolate.*

Parameter	Observation
Colour	Creamy white
Shape	Round
Texture	Smooth
Elevation	Flat
Margin	Entire
Opacity	Transparent
Bud Presence	+
Sugars	
Fructose	+
Galactose	+
Glucose	+
Sucrose	+
Suspected microorganism	*Saccharomyces cerevisiae*

Key: += Present, - = Absent

Table 2. *Identification of yeast isolate based on sequence alignment (BLAST).*

Nearest phylogenetic relative	Strain	Accession number	Sequence similarity (%)
M. guilliermondii	HBLA20	KF913531.1	98
M. guilliermondii	M1	KT897919.1	98
M. guilliermondii	KW2680	LN626313.1	98
M. guilliermondii	ZAG8	KU883323.1	98
M. guilliermondii	AP.MSU5	KT282394.1	98
M. guilliermondii	2D0101	KT385726.1	98
M. guilliermondii	18S rRNA gene	KR063216.1	98
C. parapsilosis	HNC11	KT959262.1	98

Table 3. *pH read-out for yeast isolate.*

% Ethanol (v/v)	24 (hr)	48 (hr)	72 (hr)	96 (hr)
0	6.2±	5.7±	5.2±	6.8±
5	5.8±	5.6±	5.1±	6.5±
10	5.8±	5.6±	5.3±	6.5±
15	5.7±	5.5±	5.3±	6.6±
20	5.7±	5.5±	5.3±	6.2±

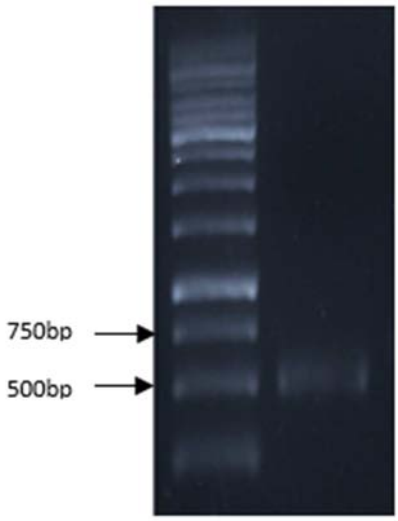

Figure 4. *Gel electrophoresis micrograph of amplified product.*

TCCGTAGGTGAACCTGCGGTGATTTGAGGCGAGCTTTTTGTTGTCTCGCAACACTCGCTCT
ITS1
CGGCCGCCAAGCGTCCCTGAAAAAAAGTCTAGTTCGCTCGGCCAGCTTCGCTCCCTTTCA
GGCGAGTCGCAGCTCCGACGCTCTTTACACGTCGTCCGCTCCGCTCCCCCAACTCTGCGC
ACGCGCAAGATGGAAACGACGCTCAAACAGGCATGCCCCCCCGGAATGCCGAGGGGCGCA
ATGTGCGTTCAAGAACTCGATGATTCACGATGGCTGCAATTCACACTAGGTATCGCATTTC
GCTGCGCTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTTGTTTGTTTTTT
CGTAGATTTCTCTTGTCGACTATATGCTATATTCCACATTTTAGGTGTTGTTGTTTTCGTTCC
GCTCACGCAGTGTAGTAGTAAATCACAGTAATGATCCTTCCGCAGGTCACCCTACGAAAGC
TCATTACTGTGATTTACTACTACACTG TCCTCCGCTTATTGATATGC
ITS4

Figure 5. *Sequencing results of the ITS region of yeast isolate. Primer sequences were underlined.*

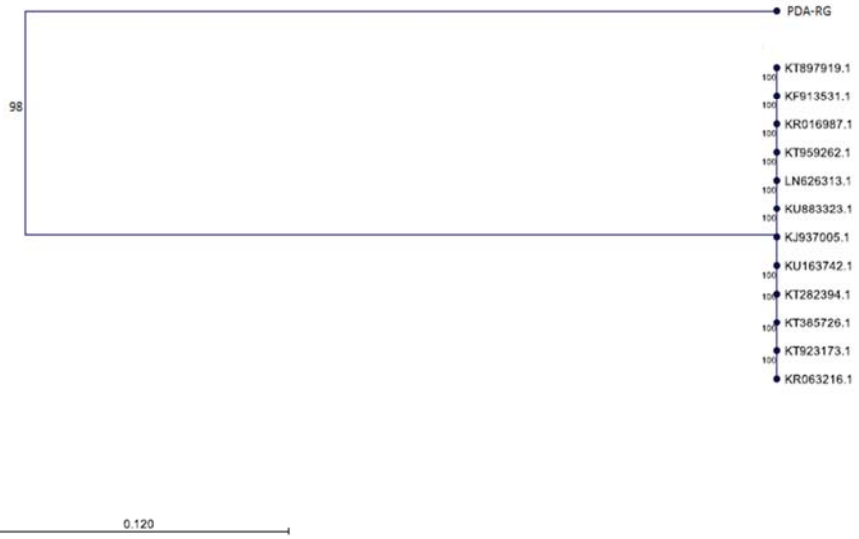

Figure 6. *Neighbour-joining tree showing the isolated strain (PDA-RG and related Meyerozyma guillermondi strains. Numbers below tree nodes represent the percentage bootstrap support for 1000 replicates, respectively.*

4. Discussion

Species-level identification of yeasts commonly involved in alcoholic fermentation placed *Saccharomyces cerevisiae* at the top of the fermentative yeast chart [21], [22], [23].

Although this still serves as the model organism for ethanol production by yeast, non-Saccharomyces strains of yeast have been identified that achieve the same feat like the model organism [24], [25]. These non-saccharomyces yeasts represent a unique diversity of unexplored natural resources which possess exceptional genetic makeup and mechanisms that permits their ability to grow well in under diverse environmental conditions in addition to participate in a number of industrial processes [26]. Wide speculation on the mode of adaptation of such species suggests mechanisms which may not be present in *Saccharomyces cerevisiae* thus making them preferable to the latter in different industrial sectors like the beverage and food industries [27], [28]. The unique yeast strains include *Candida famata, Candida palmioleophila, Debaryomyces hansenii, Dekkera bruxellensis, Kluyveromyces marxianus, Ogataea polymorpha, Pichia kudriavzevii,* etc [29], [30], [31].

Through the advances in molecular engineering, it is now possible to distinguish between the diverse arrays of non-Saccharomyces yeast, thereby elucidating a clear relationship between target yeast strains and the model Saccharomyces yeast strain.

In this study, features of the colonies obtained via the spread plate technique revealed a smooth surfaced, creamy white coloured organism with a transparent opacity, flat elevation, possessing no pseudo-hypha (figure 2, table 1). The data obtained also revealed that with regards to its fermentative ability, the obtained isolate possessed the inherent ability to consume carbon sugars like fructose, glucose, galactose and sucrose using Phenol red broth (yeast fermentation base). The unique adaptation towards the consumption of other sugars than glucose infers that regulatory mechanisms for metabolism exists in such cells thereby allowing for the production of either several products or a single product from a wide range of starting materials. In other reports were researchers searched for non-Saccharomyces yeast species, it was speculated that the environment from which such yeast strains were obtained could play a role in their ability to utilize non-conventional sugar sources for their growth and survival. This was apparent in *Pichia kudriavzevii* which over the last decade has been isolated from different reported niches including sour dough [32], cocoa bean fermentation and mango pulp peel compost [33], sugar cane juice [34], fermented pineapple juice [35], soil [36]. The results given in table 1 is similar to that obtained by Schnierda and colleagues who observed that non Saccharomyces yeast like *P. kudriavzevii* can ferment a number of sugar types [37].

It is generally known that during industrial fermentation, the volume and concentration of ethanol increases as the fermentation process proceeds thereby hindering the activity and growth of the starter culture. Given that it was on the *Saccharomyces cerevisiae* native and modified strains that could tolerate increased concentrations of ethanol, non-Saccharomyces yeast species had not been considered for production of bioethanol in the past [38], [39].

Prior to this study, literature suggested that *Saccharomyces cerevisiae* was the most ethanol tolerant yeast specie, possessing an average level of tolerance in the region of 12% (v/v) ethanol, depending on the strain involved [40].

The present study corroborates the findings of other researchers who indicated that a number of non-*Saccharomyces* strains like *Dekkera bruxellensis, Pichia kudriavzevii, Schizo saccharomyces pombe, Torulaspora delbrueckii* and *Wickerhamomyces anomala*, show promise for ethanol production, coupled with a similar ethanol tolerance levels as those of *S. cerevisiae* [41], [42], [43]. Data from figure 3 indicates that the indigenous yeast isolate was capable of withstanding increased concentrations of ethanol up to 20% (v/v).

Species diagnosis via molecular typing is a much preferred technique for yeast and fungal identification owing to the fact that it is the most precise, rapid and easiest method [44]. The DNA fragment generated following genomic DNA extraction and amplification was approximately 600bp in length (figure 3).

The result from the sequence alignment and subsequent query (http://blast.ncbi.nlm.nih.gov) disclosed that the isolated yeast possessed a 98% sequence similarity pattern to that of *Meyerozyma guilliermondii* strain HBLA20 in the NCBI database which was isolated from a different source (table 2). Interestingly, *Meyerozyma guilliermondii* possesses close homology with *Candida parapsilosis* (figure 6) as these other strains are anamorphs to each other [45]. The fact that the isolate in this study was isolated from soil within close proximity to a distillery may account for its tolerance to increased levels of exogenous ethanol (figure 3). This makes it conceivable that the indigenous strain obtained should be a strong candidate for ethanol production.

The robust physiology of industrially significant yeast samples towards inhibitory compounds and environmental stress form part of the criteria for selection. In this investigation, changes in pH was monitored in broth cultures containing different concentrations of ethanol maintained at 30^{o}C over a 96 hour incubatory period (table 3). The data obtained revealed that variations in pH levels were with the ranges of pH 5.2-6.8 in the broths without any initial ethanol. The cultures containing 5-20% (v/v) ethanol has fluctuations between pH 5.1-6.6. In these exogenous ethanol containing broth samples, the generation of by-products such as acetic acid and formic acid could account for the conformational changes towards tolerance to aid in its survival in the presence of such cytotoxic agents [33], [46]. Reports indicated that the generation of these by-products diffuse across plasma membranes of the cells and acidifies the cytosol [47], [48]. To thrive under such conditions which if

left unchecked would be detrimental to cell metabolism, yeast cells would have to develop mechanism to maintain adequate intracellular pH which may include the re-organisation of its lipid composition to thwart the effect of such by-products [49]. The pH optima for alcohol fermentation with *Saccharomyces cerevisiae* is reported to be about pH 5.5 for both ethanol inhibited and uninhibited alcohol fermentation [50]. Thus, the data in this study is in harmony with established data. Furthermore, it also establishes that the yeast isolate obtained with close similarity to *Meyerozyma guilliermondii*, grows well under acidic conditions with an optimal pH range varied from pH 5-6. The intracellular enzymes of this yeast seems to work on a range of substrates with a buffering capacity to mitigate against cytotoxic by-products. This suggests that this strain is industrially significant and can be applied to a range of commercial processes.

The present study suggests that indigenous non-saccharomyces yeast species which are inherently tolerant to ethanol thrive within the distillation environment thus bestowing substitute contenders towards the production of ethanol, in addition to validating reports obtained in another study [43].

5. Conclusion

This study has shown that indigenous yeast which possess high sequence similarity to *Meyerozyma guilliermondii* are capable of withstanding ethanol concentrations of up to 20% (v/v). These yeast isolates maintained good metabolic functions between pH5-6, and at a temperature of 30°C. The data obtained ratifies reports that non *Saccharomyces* yeast species can be utilized for ethanol production.

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