Chapter

Isolation, Characterization and Preliminary Analysis of Sugar Supplementation on Alcohol Fermentation by Non Saccharomyces Yeast 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 for which non Saccharomyces yeast strains have been considered due to their ability to utilize a diverse range of sugars. The ability of such wild type indigenous strains to do so and compete with industrial strains of Saccharomyces cerevisae is not common in Nigeria. This study was undertaken to isolate, screen and identify naturally evolved indigenous yeast with ethanol tolerant capabilities. It also aimed at comparing the ability of the indigenous non Saccharomyces strain to that of Saccharomyces cerevisiae, to consume sugars (fructose, galactose, glucose, lactose, sucrose and molasses) and to convert them into ethanol during fermentation. 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. Yeast extract (6g/L), peptone (10g/L), malt extract (6g/L) broth was supplemented with different concentrations (5g/L, 10g/L, 20g/L, 30g/L) of fructose, galactose, glucose, lactose and sucrose respectively. Sugar utilization post incubation for 96 hours at 120 rpm, 30°C was measured using a refractometer. The basic local alignment search tool (BLAST) of the genetic sequence obtained revealed a 98% similarity to Meyerozyma guilliermondii. The alcoholic yield using molasses for Meyerozyma guilliermondii 9.2±0.45 (mg/ml) was significantly higher than that of Saccharomyces cerevisiae strain T (4.8±1.15 mg/ml) at 96 hours. Ethanol production from the consumption of fructose as the sole carbon source was more favourable for M. guilliermondii 2.1, 3.0, 8.11 and 9.06 (mg/ml) compared to 1.08, 3.12, 8.06 and 6.0 (mg/ml) for S. cerevisiae. Both strains displayed similar adaptation to galactose metabolism at all tested concentrations. With glucose, M. guilliermondii yielded more than its S. cerevisiae counterpart at 1.0% (4.15, 3.18 mg/ml) and 2.0% glucose (4.25, 3.3 mg/ml). At 3.0% glucose broth content, 8.15 and 9.08 mg/ml ethanol was obtained for M. guilliermondii and S. cerevisiae respectively. Sucrose utilization resulted in a 10.18 mg/ml yield of ethanol compared to a 7.06 mg/ml yield for M. guilliermondi and S. cerevisiae respectively at 3.0% sugar supplement. Meyerozyma guilliermondii displayed its ability as a highly adaptable, ethanol tolerant, non-Saccharomyces yeast specie capable of producing ethanol from a variety of sugars indicative of local feedstock as a suitable alternative.

Keywords

Ethanol; Ethanol Tolerance; Saccharomyces Cerevisiae; Non-Saccharomyces; Meyerozyma Guilliermondii

Introduction

Amongst the rich diversity of microorganisms in the world, yeasts species represent one of the most studied and documented

species [1]. Several studies geared towards their industrial applications, response to different stress conditions and genetic modification attempts via knowledge of its complete genome sequence, makes Saccharomyces cerevisiae the most prominent specie known to man [2-4]. 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 [5].

Furthermore, the preference for Saccharomyces cerevisiae as the preferred starter culture, particularly in the brewing is due to its ease of converting sugar-rich substrates into ethanol coupled with its unique tolerance to the end-product and other inhibitory metabolic intermediates [6,7].

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 [8,9].

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 [7]. To this end, several governments are sourcing for sustainable energy solutions whilst attempting to balance the long-term, and in some cases, the short-term needs of the environment [10]. In Nigeria and many developing countries, there is a lot of untapped feedstock material that are rich in a range of sugar types (arabinose, galactose, glucose, mannose, galactose, xylose, xylitol). Preliminary studies on the production of alcohol from these alternative carbon sources would aid in the supply chain, emulating countries like Brazil and America who generate bioethanol from their excess sugar cane and corn resources [10]. For that reason, research into the consumption and fermenta-

tion of pentose sugars would be a logical step in the production of ethanol from alternative organic stock [11].

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 [12]. The later which possess peculiar resistance genes which synthesis certain categories of proteins or effector molecules that together coordinates stress resistance, can be obtained from unexplored habitats [13,14].

As research is turning to the exploration of unconventional organic substrates for ethanol production, known limits of S. cerevisiae in alcoholic fermentation are being stretched [15,16]. Different organic raw materials rich in lignocellulose is the foremost choice as these are selected based on the fact that its use does not hamper for production nor deplete agricultural resources materials [17]. The ability for non-Saccharomyces yeast strains to consume intricate nutrient types whilst showcasing industrially robust traits to fermentation process inhibitors (such as weak acids, furaldehydes, phenolics, etc) makes them ideal starter cultures that can compete effectively with S. cerevisiae [18,19].

Studies show that non-Saccharomyces yeasts like Candida sp., Hansenula sp., Kloeckera sp., Torulaspora sp., Kluyueromyces sp., Pachysolen tannophilus, Pichia stipites, Zygosaccharomyces rouxii [20-23]. Kluyveromyces marxianus [24,25], Pichia kudriavzevii [26-28] Dekkera bruxellensis [29], Zygosaccharomyces bailii [30] and many more possess the ability to grow and participate in alcoholic fermentation [31-33]. 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 [34]. 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 [35,36]. Kluyveromyces marxianus was found to pro-

duce ethanol yields of about 37.1g/L compared to 40.9 g/L obtained from Saccharomyces cerevisiae, both with a growth optima of 30°C, pH range 4.5-5.5 [37].

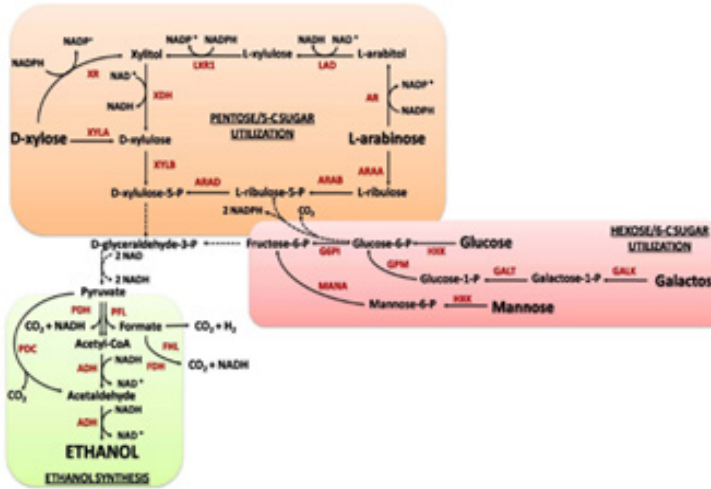


Figure 1: Ethanol production via Xylose and Glucose utilization pathway.

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% [38,39]. 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. Furthermore, this study would also examine the ability of any non-saccharomyces yeast obtained to consume selected

simple sugars coupled with its ethanol production potential in comparison to a known strain of Saccharomyces cerevisiae.

Materials and Methods

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. The source samples were serially diluted in sterile distilled water and inoculated on potato dextrose agar (PDA) medium plates by spread plate method. These plates were incubated at 30°C for 72 hours. After incubation developed colonies were observed for their morphology and microscopic characteristic. Colonies obtained from the isolation step were sub-cultured by streaking on PDA supplemented with 0.2mg/ml chloramphenicol to inhibit bacterial growth. Colonies with morphologically distinct characters were observed and purified by repeat streaking on PDA medium. Pure culture of the strain was kept on potato dextrose agar slants and stored at 4°C until needed for further studies. The obtained isolate were subjected to morphological identification with the following parameters: Colour, Shape, Texture, Elevation, Margin and Opacity and crosschecked using the methods of [40]. Wet mounts of isolated cell samples were prepared in distilled water and examined using 40X objective magnification.

Identification by Fermentation Assessment

The isolate obtained was analysed for their ability to utilize carbon sugars like fructose, glucose, galactose and sucrose, which also served as a method of biochemical identification using Phenol red broth (yeast fermentation base). 1g of each sugar type was placed in a test tube containing 5ml of Phenol Red broth medium. After inoculating each tube with the test isolates, the tubes were incubated for 48 hours at 30°C. A colour change from red to yellow due to acid production gave a positive indication of their fermentative capability [41].

Screening for Ethanol Tolerance

The yeast isolate were inoculated unto 10mls of YPMG broth (yeast extract, peptone, malt extract, glucose) supplemented with different concentrations of ethanol (0, 5, 10, 15, 20% v/v) differing by 5% (v/v) from one flask to another. The tubes were incubated for 120 hours at 30°C. Samples were drawn every 24 hours and growth was measured using a UV-VIS spectrophotometer at 570nm. Also, the viability of yeast cells were checked inoculating the isolates unto 10ml of YPMG broth supplemented with different concentrations (0, 5, 10, 15 and 20% v/v) of ethanol. Viability was measured following 48 hours of incubation at 30°C by serially diluting with sterile distilled water and plated on YPMG agar medium. The number of CFU/ml was calculated [42]. Variations in pH was also monitored in broth cultures.

Ethanol Production Using Molasses

A loop of each isolate obtained from previous experiments was used to inoculate 100ml of autoclaved yeast extract, peptone, dextrose (YPD) broth in 250ml Erlenmeyer flasks. The flasks were incubated at 30°C, 120rpm for 24 hours. 10ml of each yeast cell suspension was transferred into 125ml broth composed of 6g/L yeast extract; 10g/L peptone; 6g/L malt extract; 2g/L glucose media. The pH of each medium was adjusted to 5.5. 25mls of autoclaved molasses was then introduced into each flask under the laminar flow hood. The flasks were then incubated for 96 hours at 30°C, 120rpm. At 24 hour intervals, samples were collected to measure sugar utilization and ethanol production. Ultra Violet visible (UV-Vis) spectrophotometry was used to measure yeast growth. All experiments were performed in triplicate and the data reported is the average of the three replications.

Sugar Uptake

24 hour cultures (10ml in YPD broth) were used to inoculate flasks containing yeast extract (6g/L), peptone (10g/L) and malt ex-

tract (6g/L) supplemented with different concentrations of fructose, galactose, glucose, lactose and sucrose (5g/L, 10g/L, 20g/L, 30g/L) respectively. The growth kinetics was characterized via absorbance measurements (OD_{580}) after 4 days to ensure a reasonable degree of fermentation. Sugar utilization was measured using a refractometer.

Molecular Characterization

The isolate was genetically identified via amplification and sequence analysis of the ribosomal DNA internal transcribed spacer region (ITS) [21]. The primers; ITS1 (5^1-TCCGTAGGTGAACCTGCGG-3^1) and ITS4 (5^1-TCCTCCGCTTATTGATATGC-3^1), were used for rDNA amplification. The amplification reaction was performed in a final volume of 50 micro litre containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, each dNTPs at a concentration of 0.2 mM, 1.25 IU of Taq polymerase, each primer at a concentration of 0.2 mM and 10 micro litre of DNA (50ng) template.

The reactions were run for 36 cycles with denaturation at 94°C for 2 min, annealing at 52°C for 1 min and elongation at 72°C for 2 min. The PCR products were purified using the Nucleic Acid and Protein purification Kit and cloned into the pGEM vector then sequenced. Sequences of the PCR product were compared with known ITS region sequences deposited in the GenBank (http://www.ncbi.nlm.nih.gov) and the percentage of similarity among the fragments was calculated using the BLAST program and compared to the 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.

Statistical Analysis

All the experiments were conducted in triplicate and analysed using one way ANOVA.

Results

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.*

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 (figure 2). The isolate grew well in the presence of 5% ethanol. From 10-20% (v/v) exogenous ethanol presence, growth still continued although a certain degree of inhibition was detected.

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

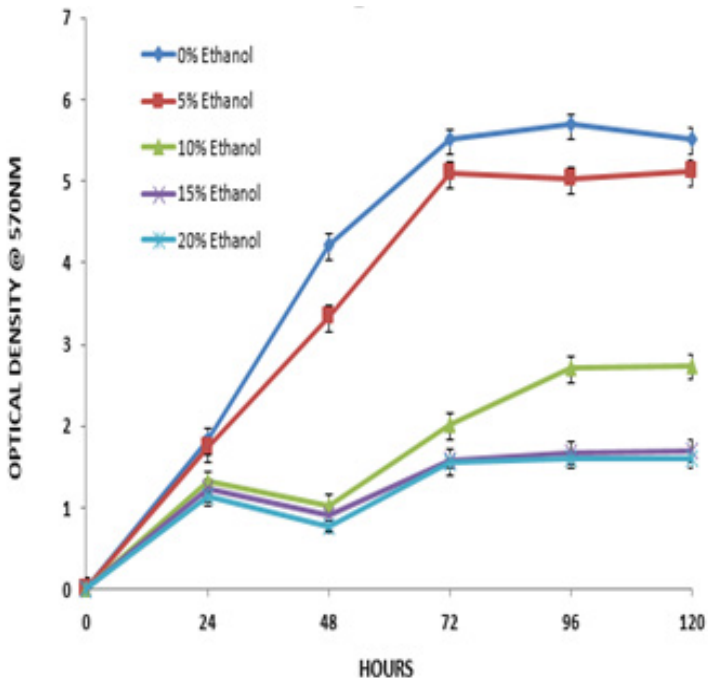
Table 1: Morphological identification and sugar fermentation of yeast isolate.

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

Key: += Present, - = Absent

pH Analysis

Measurements of the pH from the broth cultures spanning the incubation duration with increasing concentrations of ethanol was examined (table 2). 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.



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

Sugar Utilization and Ethanol Production

The isolate was grown and used in the fermentation of molasses against the control (*Saccharomyces cerevisiae*) for an incubation period of 96 hours at 28°C (Figure 3). The results showcase a steady increase in ethanol production of 0.9 ± 0.15ml, 2.0 ± 0.35ml, 3.4 ± 0.85ml and 4.8 ± 1.15ml for the control strain and 1.6 ± 0.10ml, 3.9 ± 1.20ml, 6.5 ± 0.95ml and 9.2 ± 0.45ml for the studied isolate respectively (figure 3).

Table 2: 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±

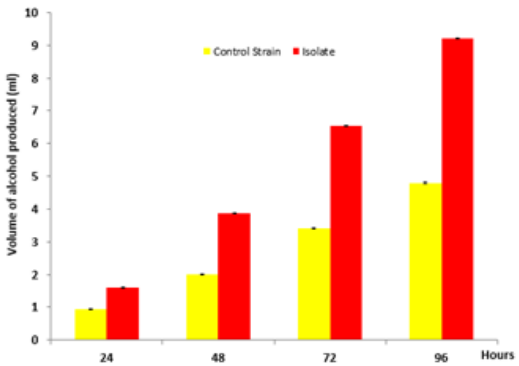


Figure 3: Graphical comparison of the ethanol production rate from molasses by Saccharomyces cerevisiae and Meyerozyma guilliermondi.

Consumption of simple pentose sugar tests revealed that at the different concentrations (0.5%, 1%, 2% and 3%) of fructose, the isolate did considerably better than its *Saccharomyces cerevisiae* counterpart (figure 4A, 4B). The residual fructose concentrations in the broth was 1.7 ± 0.55, 1.9 ± 0.35, 2.1 ± 0.5 and 2.5 ± 0.25 g/L respectively for the isolate.

Concentrations of 1.8 ± 0.15, 2.0 ± 0.45, 2.1 ± 0.75 and 3.1 ± 0.35 (g/L) for the control sample was obtained. The corresponding values for ethanol obtained in the fructose supplemented YMP broth were 2.1 ± 0.25, 3.0 ± 0.55, 8.11 ± 0.95 and 9.06 ± 1.05 (mg/ml) in the broth medium contain the studied isolate while that of the control resulted in 1.08 ± 0.10, 3.12 ± 0.30, 8.06 ± 0.35 and 6.0 ± 0.75 (mg/ml) as depicted (figure 4B). In the glucose consumption test, the best ethanol producing concentration was 3.0% glucose which produced 8.15 ± 0.20 and 9.08 ± 0.45 mg/ml of ethanol (figure 5A, 53B). Galactose Adaptation to galactose test (figure 6A) revealed that at either concentration of the simple sugar, both strains of yeast consumed fractional quantities for 0.5%, 2.0% and 3.0%.

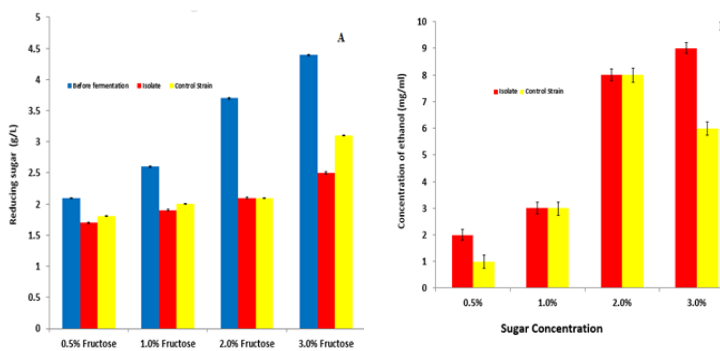


Figure 4: Graphical representation of sugar consumption rates (A) pre and post fermentation for variable concentrations of fructose in YPM broth. B represents rate of ethanol production using fructose following 96 hours of incubation.

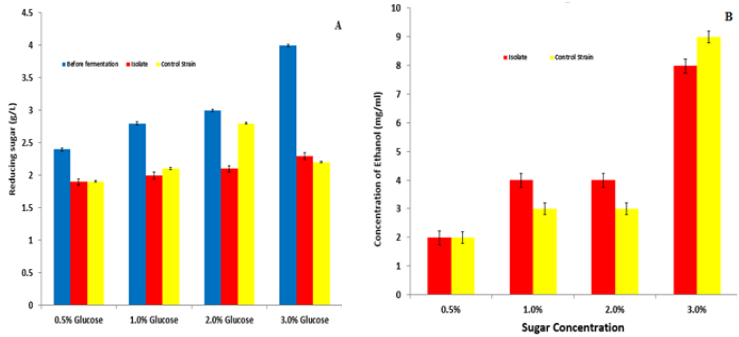


Figure 5: Graphical representation of sugar consumption rates (A) pre and post fermentation for variable concentrations of glucose in YPM broth. B represents rate of ethanol production using glucose following 96 hours of incubation.

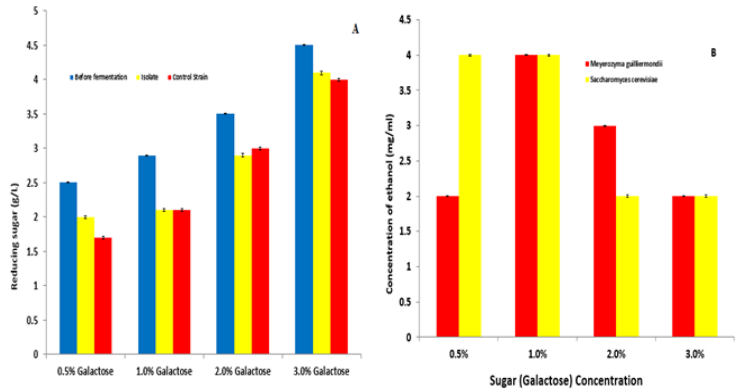


Figure 6: Graphical representation of sugar consumption rates (A) pre and post fermentation for variable concentrations of galactose in YPM broth. B represents rate of ethanol production using galactose following 96 hours of incubation.

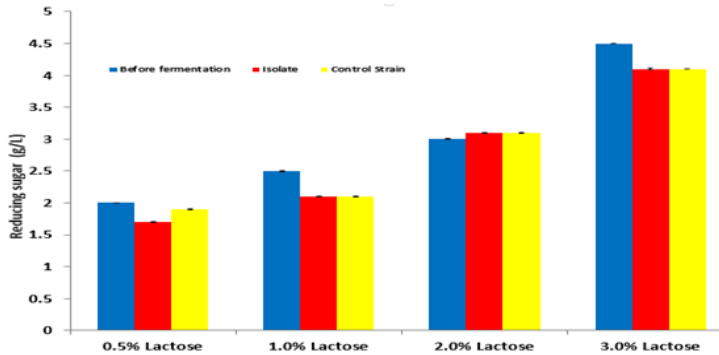


Figure 7: Graphical representation of sugar consumption rates (lactose) in YPM broth following 96 hours of incubation.

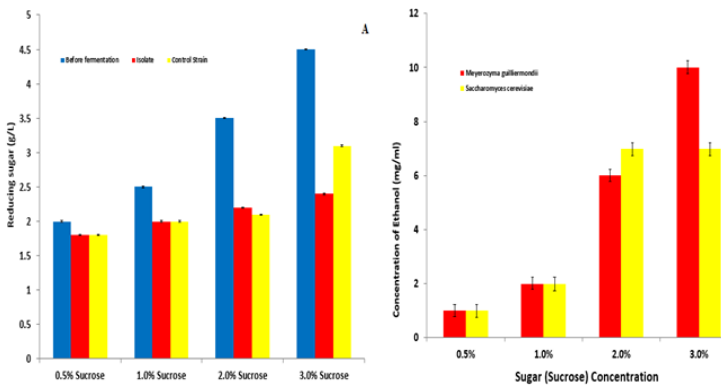


Figure 8: Graphical representation of sugar consumption rates (A) pre and post fermentation for variable concentrations of sucrose in YPM broth. B represents rate of ethanol production using sucrose following 96 hours of incubation.

Broth samples containing 1.0% galactose was the most utilized of this sugar, which resulted in the highest measured concentration of ethanol produced (figure 6B). The negative control; lactose, employed in this study was not converted into ethanol (figure 7). The consumption of this sugar did not give any substantial value. Sucrose utilization (figure 8A, 8B) revealed that the studied isolate produced more ethanol (10.18 ± 1.25mg/ml) than that of the control (7.06 ± 0.90mg/ml) at 3.0% sugar supplement.

Molecular Identification

Figures 9 & 10 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 3, figure 11). The isolate also shared the same percentage sequence similarity with other yeast strains that are anamorphs of *Meyerozyma guilliermondii.*

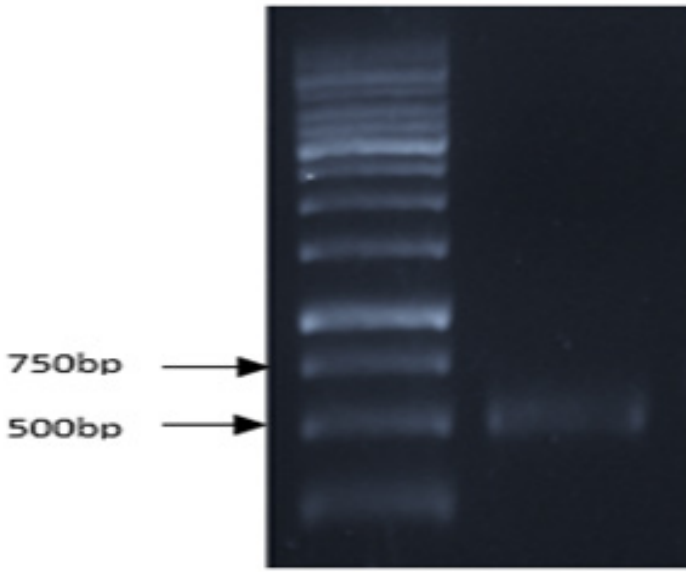


Figure 9: Gel electrophoresis micrograph of amplified product.

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

Figure 10: Sequencing results of the ITS region of yeast isolate. Primer sequences were underlined.

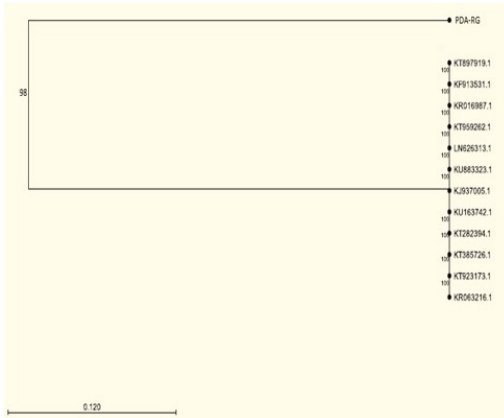


Figure 11: Neighbour-joining tree showing the isolated strain (PDA-RG and related *Meyerozyma guillermondi strains*. Numbers below tree nodes represent the percentage bootstrap support for 1000 replicates, respectively.

Table 3: 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

Discussion

Species-level identification of yeasts commonly involved in alcoholic fermentation placed *Saccharomyces cerevisiae* at the top of the fermentative yeast chart [43-45]. 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 [46,47]. 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 [22]. 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 [20,48]. The unique yeast strains include *Candida famata, Candida palmioleophila, Debaryomyces hansenii, Dekkera bruxellensis, Kluyveromyces marxianus, Ogataea polymorpha, Pichia kudriavzevii*, etc [49-51].

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 there-

by 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 [52], cocoa bean fermentation and mango pulp peel compost [53], sugar cane juice [54], fermented pineapple juice [55], soil [56]. 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 [57].

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 [58,59].

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 [60].

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* [61-63]. Data from figure 3 indicates that the indigenous yeast isolate was capable of withstanding increased concentrations of ethanol up to 20% (v/v).

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 of the studied yeast isolate containing different concentrations of ethanol maintained at 30°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 [27,53]. Reports indicated that the generation of these by-products diffuse across plasma membranes of the cells and acidifies the cytosol [64,65]. 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-organization of its lipid composition to thwart the effect of such by-products [66]. 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 [67].

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 [68]. The DNA fragment generated following genomic DNA extraction and amplification was approximately 600bp in length (figure 9).

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 3). Interestingly, *Meyerozyma guilliermondii* possesses close homology with *Candida parapsilosis* (figure 11) as these other strains are anamorphs to each other [69]. 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 2). This makes it conceivable that the indigenous strain obtained should be a strong candidate for ethanol production.

Total yield of ethanol from *M. guilliermondii* and *S. cerevisiae* using molasses medium (initial reducing sugar concentration of 18.3 w/v %) revealed that M. guilliermondii produced higher volumes (10 ± 0.2 mg/ml) than that of S. cerevisiae (9 ± 0.2 mg/ml) at 96 hours (figure 3). The demand curve for assortment of low, medium to high alcohol content wines has informed the decision to study various tactics towards the production of low-medium [70]. Sluggish fermentation reported in the wine-making industry is associated with the incomplete conversion of pentose sugars into ethanol [71]. Studies suggest that most yeast species are more inclined towards high ethanol production from the conversion of pentose sugars like glucose than most other simple organic carbohydrate materials [72-74]. Research into the use of sugars like fructose for ethanol production revealed that only a small quantity of the carbon fluxes into ethanol while a large percentage lingers as unconverted fructose [75]. Yeast species with that exhibit good consumption of fructose is expected to be excellent in the conversion of glucose and sucrose, the former due to the ease or lateral conversion of its ring structure whereas the latter is via simple hydrolytic reactions. The correlation between sugar consumption and ethanol production was accessed (figures 4-8) with the hope of deducing alternative feedstock material with high concentrations of simple sugars like fructose, galactose, etc for indigenous yeast isolates. The data indicated that fructose consumption alongside ethanol production using *M. guilliermondii* increased concomitantly to increased sugar concentration (figure 4A, 4B). This was also observed in broth samples inoculated with *S. cerevisiae* (figure 4A, 4B). Assessment of strain performance revealed that the strain of *M. guilliermondii* used in this study was a better consumer of fructose at the different tested concentrations than that of *S. cerevisiae*, producing higher concentra-

tions of ethanol particularly at 0.5 and 3.0%. This is indicative that in feedstock materials with high concentrations of fructose, this non-Saccharomyces yeast strain would be a more suitable candidate for ethanol production than the conventional *Saccharomyces* yeast. Due to the unique metabolic relationship between fructose and glucose, it would be expected that the tested yeast strains would demonstrate good consumption as well as high production of ethanol using glucose as the fermenting material. This study showed that glucose consumption was significantly higher at the highest concentration in direct proportion to the rate of ethanol production by both the test and control yeast strains (figure 5A, 5B). Unlike in the broth samples containing fructose as the sole carbon source, the indigenous strain of *M. guilliermondii* produced ethanol at a slightly lower concentration than the foreign *S. cerevisiae*. This could be as a direct result of the up-regulation of genes involved in respiration, gluconeogenesis, in the uptake and absorption of fructose which inherently favours the forward reaction of fructose better than that of glucose [74-77]. It is conceivable that intermediate compounds and the availability of enzyme cascades via the activation of certain regulatory genes creates competition for the available carbon whereby some are diverted to the formation of intermediates rather than partake fully in the catalytic events by glucose transporters to produce ethanol. This concept would help in explaining the possibility of glucose repression whereby yeast strains demonstrate adaptation to alternative carbohydrates like galactose or lactose. Although acclimatization to galactose is uncommon, it is still worth investigating phenotypic traits as this to rule out the possibility to employing evolutionary distinct yeast species to fermentation of unique carbohydrate sources. In this study, both *M. guilliermondii* and *S. cerevisiae* exhibited a low degree of flexibility towards the conversion of galactose to ethanol (figure 6B). The weak assimilation of galactose tallies with reports that suggest most yeast species are not fully adaptable towards galactose metabolism [27,77]. This observation could be attributed to several factors including but not limited to its proton transport assembly and slow substrate af-

finities of its metabolic enzymes which could eventually cause feedback inhibition, thus decreasing the output. A lower concentration of galactose in the media of 0.5-1.0% mixed with glucose, fructose or sucrose may enhance ethanol production by these isolates.

Like most crab positive yeast species, *M. guilliermondi* and *S. cerevisiae* are not able to utilize lactose (figure 7). Using varying concentrations of lactose, this study revealed that both yeast strains were not capable of carrying out fermentation. This study also found that the rate of consumption increased parallel to increased sucrose concentrations, yielding increased amounts of ethanol. Also, *S. cerevisiae* appeared to have a sucrose optima of 2.0% as its level of ethanol production dipped at 3.0% (figure 8A, 8B). In contrast, consumption and production of ethanol with *M. guilliermondi* improved at higher concentrations of sucrose. Overall, the data obtained validates results from other studies that propose a partial bioconversion of sugar into ethanol, the outcome of which would be the low yield of ethanol in comparison with the quantity obtained using molasses [73,74]. Overall, the unique results obtained from *M. guilliermondi* with regards to its rate of consumption of the different sugar types tested, aligned with its high yield of ethanol and in comparison with the *Saccharomyces cerevisiae* control strain tested suggests a robust genetic and physiological make-up. The sugar consumption and ethanol production pattern spanning the entire duration of incubation suggested that for the purpose of commercial production of ethanol *Meyerozyma guilliermondi* is an effective alternative to *Saccharomyces cerevisiae* for use.

Conclusion

The data collected from this study show that non *Saccharomyces* yeast with close similarity to *Meyerozyma guilliermondii* strains, was able to tolerate ethanol concentrations of up to 20% (v/v). These indigenous isolate functioned efficiently at pH5-6, and temperature 30°C. The results obtained from this study support the use of other native non *Saccharomyces* yeast species in the production of ethanol,

utilizing locally available materials. The intracellular enzymes of this yeast seems to work on a range of substrates with a buffering capacity to mitigate against cytotoxic by-products. The present study has revealed the availability of indigenous non-saccharomyces yeast species that possess tolerance to ethanol therefore presenting alternative candidates for ethanol production, thus reaffirming data obtained in another study [63].

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