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Isolation and Characterization of Local Yeast Strains from Fermented African Breadfruits for Use in Pentose Sugars Fermentation

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ABSTRACT

The strong pressure to improve the economic viability of second generation bioethanol production and the identified limits of what conventional *S. cerevisiae* can offer in terms of fermentation performance in lignocellulose hydrolysates, necessitated the motivation to explore alternatives beyond the conventional *Saccharomyces* species. The non-conventional yeast strain, Pichia kudriavzevii, with an Accession number LC 375240.1, was isolated from African Breadfruit and screened for its potential to ferment D-xylose and L-arabinose directly to ethanol. 15.7153 ppm, 3.5285 ppm, and 0.1504 ppm of ethanol, Carbon(iv)oxide and acetic acid respectively were produced by this yeast strain after 72 hours fermentation from D-xylose.
Keywords: Breadfruit, Pichia kudriavzevii, Xylose, Fermentation, Bioethanol.

INTRODUCTION

Intense research has been carried out for obtaining efficient fermentative organisms, low-cost fermentation substrates and optimum environmental conditions for fermentation to occur. Traditionally, ethanol production is usually accomplished by microbial conversion of carbohydrates present in agricultural products [1].

Currently, bioethanol is produced either from starch or from the sucrose fraction of some edible agricultural crops, such as corn, sugar cane, and sugar beet. For economic and environmental reasons agricultural residues and other low-value sources of carbohydrates are highly considered for bioethanol production [2]. These include corn stover, sugar cane bagasse, wheat straw, non-recyclable paper, and Lignocellulosic materials. Lignocellulosic biomass is essentially composed of cellulose, hemicellulose, pectin, and lignin [3], with glucose being the main sugar constituent, but pentose sugars, such as D-xylose and L-arabinose, may represent up to 20% [4]. The hexose sugars (glucose, mannose and galactose) are relatively easily fermented to ethanol by the traditional yeast, *Saccharomyces cerevisiae*, whereas the pentose sugars (xylose and arabinose) are not fermented. The amount of arabinose is so small that it does not present a real problem. The xylose fraction, on the other hand constitutes 10 - 40% of the total carbohydrate [5]. Hence, the total ethanol yield could theoretically be increased by 25%, through the use of efficient xylose-fermenting yeast that could convert both hexoses and pentoses to ethanol [6].

Pentose fermentation is accomplished by non-Saccharomyces yeasts, such as *Kluyveromyces marxianus*, *Scheffersomyces (Pichia) stipitis*, *Pachysolen tannophilus*, and *Candida shehatae*, [7]; [8]. Hence, most work involving pentose fermentation in yeast is focused on generating genetically-modified *S. cerevisiae* strains with heterologous xylose metabolic pathways. As few yeast strains have been found to possess appreciable characteristics for ethanol production, there is a dire need to explore the potential of indigenous strains of yeasts to meet the national

requirements and to save the foreign exchange.

There are different sources for the isolation of yeast species. However their presence, were reported mostly from the citrus juice, and sugarcane juice, molasses, sugar mill effluents, fermented foods and fermented pineapple juice. African breadfruit (*Treculia africana*), is the highest yielding food plants with a single tree producing up to 200 or more fruits per season. Due to the high carbohydrate content of African Breadfruit, numerous microorganisms including *Aspergillus sp., Rhizopus sp., Staphylococcus sp.*, and *Mucor* sp. inhabit the fruit at different stages of ripeness. In the present study, several local samples of African Breadfruit were analyzed for isolation and subsequent characterization of yeast strains, with the hope of identifying species with high potential for alcohol production.

MATERIALS AND METHOD

Sample Preparation

Freshly fallen Samples of African breadfruit were collected from Nneogidi in Agulu, Anaocha Local Government Area of Anambra state, packaged in a sterile polythene bag and transported to NAFDAC Zonal Laboratory Agulu, (Anambra State) for analysis. The samples were allowed to ferment for 4 days in a sterile room and were cut into equal halves, from which different parts of the African breadfruit were aseptically cut and put into sterile petri dishes and brought to the laboratory within an hour of collection.

Media Preparation

Peptone powder, yeast extract, Sabouraud dextrose broth and Sabouraud dextrose agar (All product of TM media, India) were prepared according to manufacturer's instructions for the isolation of yeast organisms.

Liquid media

According to the manufacturers instruction the following media were prepared as follows;

Sabourad dextrose broth (SDB) preparation

Approximately 3g of Sabouraud 4% dextrose broth was dissolved in 100ml of distilled water and stirred thoroughly to dissolve. Then 10ml of the solution was dispensed into McCartney bottles and autoclaved at 121^{o}C for 15minutes.

Yeast extract and peptone water (YEP) preparation

Approximately 2g of yeast extract and 1g of peptone powder were dissolved in 100 ml of deionized water in a conical flask and stirred until it completely dissolved. 9ml of the YEP solution was dispersed in McCartney bottles, while 1ml of the glucose solution was dispersed into the same McCartney bottles and autoclaved at 121^{o}C for 15minutes. This medium is regarded as YEPG medium.

Determination of pH and Temperature of Fermenting African Breadfruit

50g of fermenting African breadfruits were obtained and crushed with pre-sterile mortar and pestle, after which it was transferred to a conical flask and 100ml of deionized water was added to form suspension, it was then stirred vigorously, sealed and left under room temperature for 6 hrs to form a homogeneous mixture, After homogenizing, the pH and temperature of the mixture were determined using a digital pH meter and thermometer (VORZ, Russia).

Isolation of Yeast

Using a sterilized forcep and spatula, 2-3g of the African breadfruit sample was inoculated into the McCartney bottle containing Sabouraud dextrose broth (SDB) and YEPG medium respectively. The culture was incubated in a fungi incubator at 25-28^{o}C for 24-48hrs. After 48hrs the microorganisms observed at the different broth medium were sub cultured on Sabouraud dextrose agar (SDA) and then incubated at 28^{o}C for growth of the Yeast.

Pentose sugars (D-Xylose and Arabinose) preparation and Fermentation using the isolated Yeast strain

To assess the yeast for fermentation of pentose sugars (xylose and arabinose), the

strain was pre-cultivated in a YEP medium for 4 days and at the end of the growth period, the biomass was centrifuged, watched twice with sterile distilled water and a suspension of the biomass was used as inoculum at 1g of dry biomass per litre of culture medium composed of KH_2PO_4 (2g/L), $(NH_4)_2SO_4$ (2g/L), $MgSO_4.7H_2O$ (1g/L), Urea (0.3g/L), $CaCl_2$ (0.3g/L) and D-Xylose or Arabinose (30g/L). The alcoholic fermentation was assayed in 125mL Erlenmeyer flasks adapted for alcoholic fermentation, containing 60m/L of medium closed with a valve containing sodium metabisulfite solution at 1g/L to ensure that no oxygen entered. The incubation was done at 28°C for 72 hours in a rotary shaker [9].

Molecular Characterization of Isolated Yeast

The DNA of the Yeast isolated was extracted using Quick-DNA™ Miniprep Plus Kit (Zymo Research), amplified by PCR and separated by Agarose Gel Electrophoresis. The amplification products were then visualized in 1% agarose gel, stained with DNA gel stain (GR green) and 1 kb DNA ladder (Invitrogen, USA) was used as gene ruler [10].

The PCR products were purified with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA), packaged and sent to INQABA BIOTECH (South Africa) for sequencing. The nucleotide sequence result was analyzed by BLAST, ClustalW and BioEdit software packages.

Quantification of Genomic DNA using a Nanodrop

1 µl of DNA sample is loaded onto the pedestal of the Nanodrop to quantify the genomic DNA [11].

Preparation of Sample for GC/MS Analaysis

About 10ml of sample was extracted with 200ml of dichloromethane. The mixture was separated using separating funnel and the dichloromethane layer was concentrated in rotary evaporator. 1ml of acetonitrile was added into the concentration and transferred into a vial ready for analysis [12].

RESULTS

Temperature and pH of The African Bread Fruit after Seven Days of Fermentation

Table 1: Changes in Temperature and pH of African Breadfriut during Fermentaion

Days of sampling	Ambient Temperature (°C)	Temperature (°C)	pH
1st	28.8	31	7.2
3rd	30	34	6.4
5th	29.6	39	5.5
7th	29	37	4.3

The result of changes in temperature and pH during the 7 days fermentation period are presented in table 1. The ambient temperature readings were the temperature conditions of the laboratory as at the time temperature of the fermentation medium was taken for each day. The temperature reading recorded highest value on the 5th day of fermentation followed by the 3rd day. These values also correspond to the degradative activities of the fermenting microorganisms. It was observed that on the 4th and 5th days of the fermentation, the colour of the African Breadfruit changed from green to brown indicating elaborate activities of the organisms inhabiting the fruit. It is our opinion that activities of the fermenting organisms are exothermic in nature as the temperature rose from 31°C to 39 °C on the 5th day. The pH value of the fermentation medium decreased progressively from 7.2 which was near neutrality to acidic value of 4.3 on the 7th day.

Table 2 Fermentation ability of the isolated Yeast strain

Parameters	Composition ppm	% composition
Carbon (IV) oxide	3.5285	16.622
Methane	1.2288	5.788
Methanol	0.6019	2.835
Ethanol	15.7153	74.029
Carbon (II) oxide	0.0034	0.160
Acetic acid	0.1504	0.708
Total	21.2285	

Table 2 shows the percentage compositions of the fermentation products of D-xylose and arabinose by the isolated yeast strain. It was found that the identified strain has the ability to produce alcohol to significant quantity. The ability of the isolated yeast strain to grow axenically in African Breadfruit is subject to further scientific investigation.

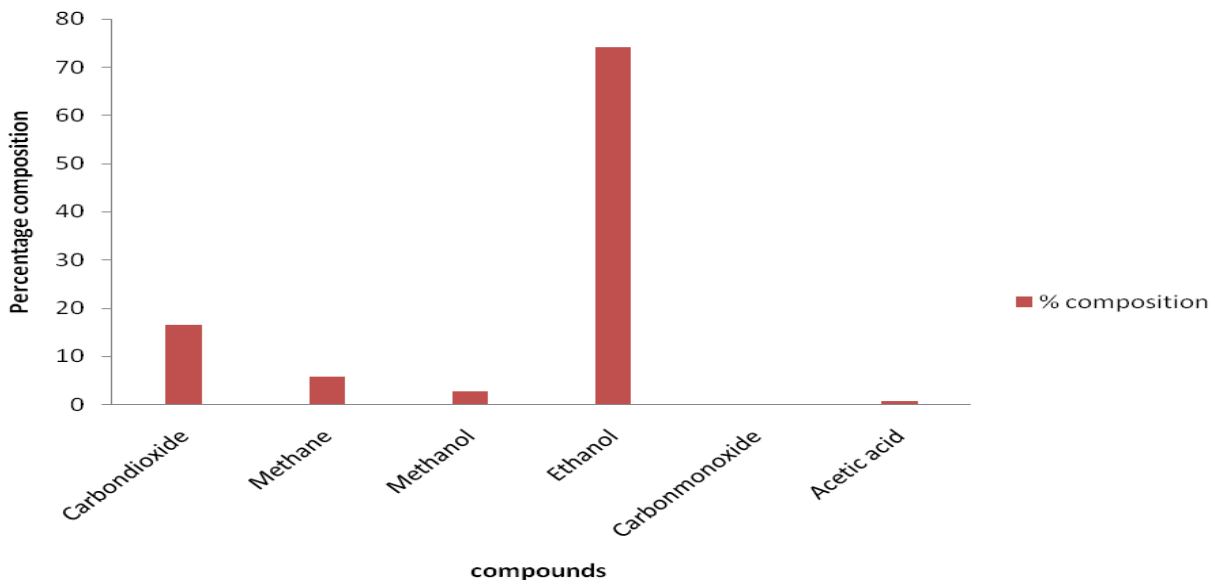


Figure 1: Composition of Ethanol Content and Other Compounds Produced by Isolated Yeast

The percentage composition of the products of fermentation using D-xylose as the carbon source is displayed in fig 1. The concentration of ethanol was 74.029% followed by Carbon(iv)oxide which had a value of 16.622%.

Table 3: Quantification of genomic DNA of the Yeast

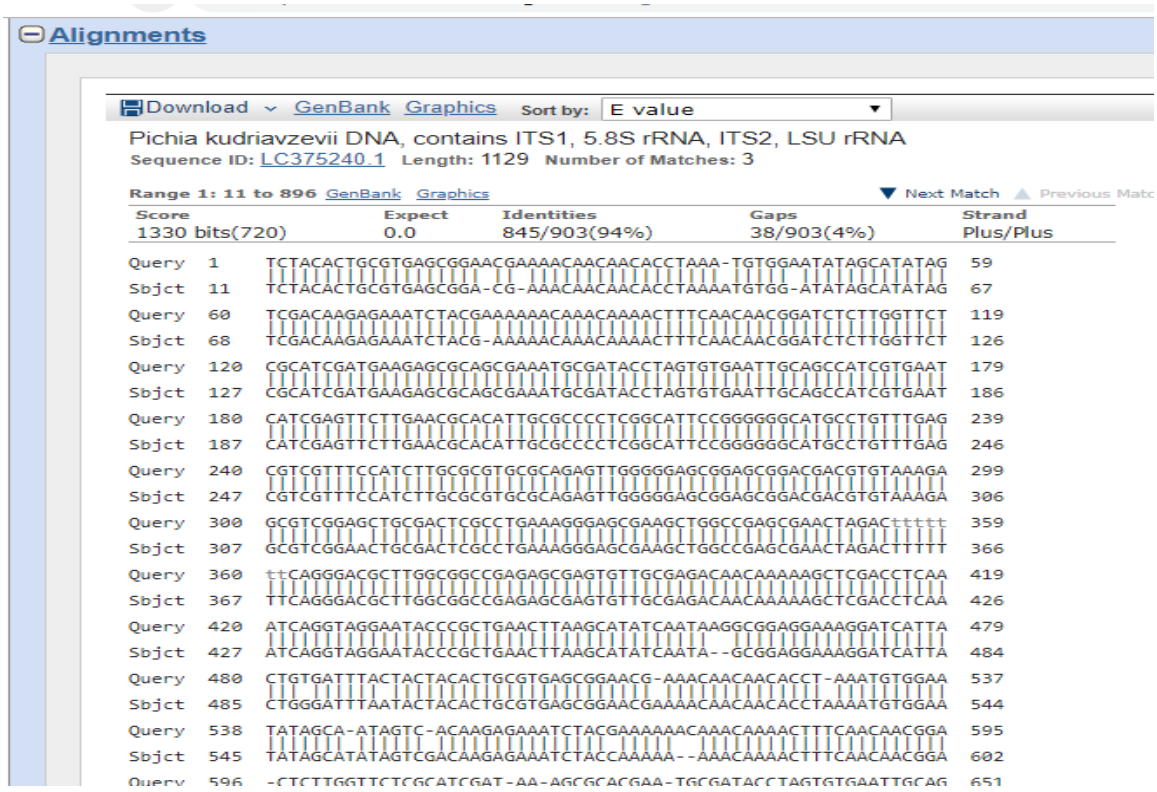
Organism	Concentration(µg/µl)	Purity	
		A260/A280	A260/230
Yeast	2.61	1.60	1.70

The values of the genomic DNA yield of 1.60 and 1.7 at A_{260}/A_{280} and A260/A230 ratios respectively shows that the DNA of the isolated yeast strain is pure and of good quality and can therefore be suitably applied for bio-ethanol production as starter culture.

Table 4: Molecular Characterization of the Yeast strain

S/N	ORGANISM (YEAST) Genera	specie
1	*Pichia*	*kudriavzevii*

The 5.8S-ITS regions in the isolates and other strains identified from the GenBank database were compared by rRNA gene sequence comparison. High sequence similarities were observed among these regions, confirming the identification of these isolates to a species level (by ITS1 and ITS2 sequence analysis). In summary, all reference strains and isolates were correctly identified by ITS sequencing, yielding an identification rate of 94%



Pichia kudriavzevii has been isolated from a variety of niches, including sourdough [13], cocoa bean fermentation [14], mango pulp peel compost [15], fermented butter, fermented pineapple juice [16], soil [17], sugar cane juice [18]; [19], cornstalk, sweet sorghum stalk and rice straw [20]. This indicates the ability of *P. kudriavzevii* to grow on complex substrates. The ability of this isolated yeast strain to grow at 39°C and a pH of 4.3 indicates that it has potential to be active physiologically in high temperature and low pH conditions and several other environmental stress factors that are relevant to bioethanol production.

DISCUSSION

Utilization of xylose to improve ethanol yield from biomass hydrolysates is very important as well as viable economically. Bioethanol production was carried out using xylose as a sole carbon source by the yeast strain *P. kudriavzevii* with accession number LC 375240.1 which was isolated from African Breadfruits. The efficient use of natural resources for bioethanol production has been explored by several previous researchers [21]; [22]; [23]. With the exception of several fungal species, most alcohol fermenting fungi characterized to date are incapable of converting xylose into ethanol. Here, we demonstrated that yeast strain *P. kudriavzevii* with accession number LC 375240.1 is able to efficiently assimilate and convert xylose into ethanol. This in contrast to the work done by [3] which stated that *P. kudriavzevii* can grow on glucose, sucrose, fructose and mannose but it only weakly assimilates galactose and does not metabolize sugars like maltose, xylose, arabinose, cellobiose, raffinose or trehalose.

The presence of acetic acid in the fermentation medium up to 0.1504 ppm is an indication that the isolate in this study could be tolerant to high concentrations of inhibitory compounds such as formic acid, hydroxymethylfurfural and furfural [3]. Tolerance to weak acids is crucial for industrial yeast strains used in second-generation bioethanol production. [12] and several other studies reported that species of *Pichia kudriavzevii* are tolerant to several other fermentation inhibitory compounds relevant to second-generation bioethanol production. For example, it tolerates concentrations of acetic acid of up to 8–10 g L^{-1} [20].

The existence of the isolated yeast strain used in this study at a pH of 4.3 after the 7th day of fermentation of the African breadfruit shows that it has the potential to grow at lower pH conditions. This is in perfect agreement with the report of Daniel *et al.,* 2009. He reported that *Pichia kudriavzevii* can grow at extremely low pH (down to pH 2) and achieves 20% more ethanol yield compared to *S. cerevisiae* under low pH conditions (pH 4).

CONCLUSION

Yeasts that can ferment both hexose and pentose are important for the large-scale production of ethanol from hemicellulose. Agro-industrial wastes resulting from the processing of raw materials are rich in these carbohydrate polymers, which can be exploited for bioconversion by microorganisms. The result of this study indicated that the indigenous yeast, isolated from African breadfruit showed good fermentation attributes, which might enhance ethanol yield that would contribute to the cost effectiveness role in the production of bioethanol and enzymes of industrial importance. The production of other compounds such as acetic acid, methanol, and methane aside from ethanol is important because the application of this strain in industrial processes has not been well studied.

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