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Original article

Isolation and Characterisation of pH and Temperature Tolerant Yeast from Local Brews for Bio-ethanol Production

[*1]Omoniyi, A. M., [1]Egwim, E. C., [1]Akanya, N. O., [1,3]Shittu, O. K. and [2,3]Hamidu, A.

[1]Biochemistry Department, Federal University of Technology, Niger State, Nigeria
[2]Department of Microbiology, Federal University of Technology, Niger State, Nigeria
[3]Centre for Genetic Engineering and Biotechnology, Federal University of Technology, Niger State, Nigeria

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ABSTRACT

The use of yeast for ethanol production from biomass has been well reported, however the performance of various yeast strains is limited due to high temperature involved of the fermentation process as well as the ethanol concentration. Efficient production of bioethanol, therefore, requires strains that are both thermo-tolerant and ethanol tolerant. This paper aimed at producing bioethanol using ethanol, pH and temperature tolerant yeast species from isolated from local brews in Nigeria. The study involves the isolation, identification and characterisation of yeast strains using morphological techniques from local brews samples in Minna, Niger state, Nigeria. All isolates assimilated xylose sugar; however they showed significant variations in sugar fermentation pattern. The growth of all the yeast isolates at 45 ℃ was observed in temperature tolerant activity and between pH range of 2 to 6; also, all isolates were tolerant to 14% ethanol, however, some showed the ability to grow at 17% ethanol. All yeast isolates produced ethanol in quantities between 0.73 – 18.43 g/L, however the highest production was observed by isolate B21 on day 2. The study demonstrated that most of the identified yeast species are temperature and alcohol tolerate up to 45 ^{o}C and 14% ethanol. The assimilation of pentose sugars further suggests that these isolates could be applied for the production of high yield bioethanol and the use of indigenous local brews could be exploited by various industries for alternative fuel production.

Keywords: Bio-ethanol production, Local Brews, Thermo-tolerant yeast, pH-tolerant Yeast, Xylose utilisation.

Corresponding author's email: crownofwealth2707@gmail.com, +234 7033300738

INTRODUCTION

The global energy consumption has been estimated as 12 billion tons' coal equivalents annually, however crude oil production is predicted to decline five times below its current level by 2050 [1, 2]. The world's population is also predicted to increase to about 10 billion people by 2050 according to the United Nations which indicates a significant increase energy demands with the current rate of economic, social and political developments [3, 4]. The use of renewable energy has been suggested as one of the most efficient ways of achieving sustainable development especially with current challenges depletion of fossil fuel reserves [4], the negative environmental effects of using them, climate change [5] and global energy security [6,7].

Ethanol, being a sustainable biofuel [8], presents a hopeful resolution to this predicament. Nevertheless, the effective synthesis of ethanol, from lignocellulosic biomass sources such as xylose, encounters significant difficulties [9]. The primary obstacle in using xylose in the biofuel industry, which is the second most prevalent sugar in biomass [10], is the restricted capacity of traditional ethanol-producing microbes, such as *Saccharomyces cerevisiae*, to convert xylose into ethanol via fermentation [11]. This constraint greatly impedes the overall effectiveness and economic feasibility of bioethanol production [12]. An essential aspect of tackling this difficulty is to identify and cultivate microorganisms that can efficiently convert xylose into ethanol [13] while simultaneously exhibiting strong resistance to the restrictive conditions often encountered during fermentation [14].

Isolates from local drinks and brews have been suggested to be potential sources of ethanol production [15]. Various studies have identified yeast strains such as *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, and other genera of yeasts from indigenous sources like hibiscus sabdariffa drink, millet meal, burukutu, grape juice, fermented fruits and vegetables. *Citrus aurantifolia*, bee honey, toddy, and fermented fish samples [16-18]. These yeast isolates have shown peculiar characteristics such as high temperature tolerance, ethanol tolerance, flocculation ability, fermentative capacity, and stress resistance, making them suitable for industrial applications in the bakery and brewing industries [19]. This highlights the potential of utilizing locally isolated yeast strains from drinks and brews for bioethanol production.

During fermentation, some factors are critical to achieving high ethanol yield; for instance, high temperatures, which are common in tropical regions and can raise the risk of contamination and increase cooling expenses; different pH levels, which can influence the microorganisms' growth and fermentative activity; and high concentrations of ethanol, which can be harmful to the microbial cells and lower their viability and capacity for fermentation [20]. It is essential therefore, to search for organisms that can tolerate ethanol, pH, and temperature to improve the efficiency of fermenting lignocellulosic biomass. These organisms would allow the bioethanol industry to surpass the current constraints of conventional fermentation methods [21], enhance the economic viability of bioethanol production, and make a substantial and sustainable contribution to the world's energy supply [22]. This study therefore describes the isolation of ethanol producing yeast strains which can tolerate to high temperature and high ethanol concentrations to get high yield of ethanol.

MATERIALS AND METHODS

Study area

Study was conducted in Minna Niger State, Nigeria. Minna is in the northern hemisphere and has a 9° 35' 0.798" N and 6° 32' 46.7376"

E with an elevation of 250.6 meters as seen in Figure 1.

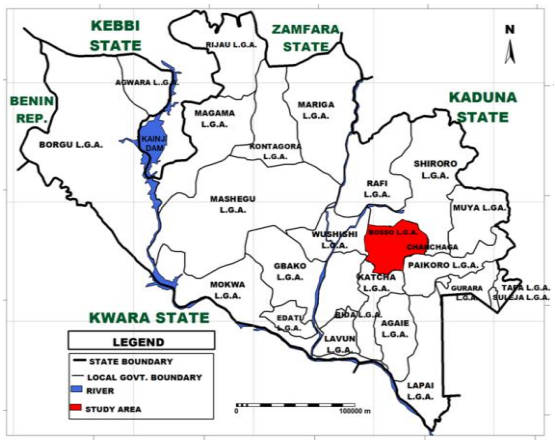

Figure 1: Map of Study Area

Sample collection and Isolation of yeasts

Samples of local brews, specifically pito and burukutu, were collected from brewing spots in Minna. The samples were placed in sterile bottles, properly labeled, and transported to the Centre for Genetic Engineering and Biotechnology (CGEB) at the Federal University of Technology, Minna, Niger State. The samples were then serially diluted, and 0.1 ml of each diluted sample was plated on Sabouraud dextrose agar (SDA) medium supplemented with chloramphenicol. The plates were incubated at 30ºC for 48 hours. Yeast colonies were subsequently sub-cultured until pure cultures were obtained, which were then maintained and stored at 4ºC.

Identification and characterization of yeast species

The morphology and appearance of ethanol-tolerant yeasts on solid medium (SDA) were assessed based on their cultural characteristics, such as colony shape, size, pigment, elevation, edge, and surface texture. Additionally, the cellular morphology of yeast isolates was analyzed using the Gram staining method under a microscope with a 100x objective lens. The ability of these yeasts to anaerobically assimilate (ferment) various carbohydrates was tested using peptone water broth (composed of 20 g Peptone and 5 g NaCl per liter) with Durham tubes or by incorporating phenol red (2% solution) as an indicator, according to the method outlined by [21]. Observations were made daily for up to seven days of incubation.

Temperature, pH and ethanol tolerance of yeast isolates

The tolerance of the yeast strains was assessed following the protocols outlined by [23]. To evaluate thermo-tolerance, Sabouraud dextrose (SD) broth medium was prepared initially. Yeast isolates were introduced into each tube of broth with an initial optical density (OD) of 0.1 and incubated at varying temperatures: 25°C, 30°C, 37°C, 40°C, and 45°C. After 48 hours of incubation, cell concentrations were measured using a UV spectrophotometer (Shimazu, 1800 series) at 600 nm.

For the pH tolerance test, SD broth was prepared at different pH levels (2, 4, 6, 9, and 11). The initial inoculum of each yeast isolate was adjusted to an OD of 0.1 and then incubated at 37°C. SD broth served as the control. Following 48 hours of incubation, cell concentrations were measured at 600 nm using a spectrophotometer.

For ethanol tolerance SD medium was prepared and supplemented with different ethanol concentration (3%, 5%, 8%, 11%, 14%, 17%, 20% and 23%). The medium was used as a control for the appropriate concentration after yeast isolates were inoculated with an initial optical density of 0.1 and cultured for 48 hours at 37 °C. The spectrophotometer measured each cell density at 600 nm.

Ethanol-producing activity of yeast isolates

The potassium dichromate oxidation method was used to evaluate the yeast isolates' ethanol-producing capacity [24–25]. The range of ethanol-water solutions used to make ethanol standards was 0–10% ethanol (v/v). That is, adding 1 ml of potassium dichromate

solution to a test tube containing 1 ml of each concentration of the standard solution [0–10% (v/v)]. The samples were heated for five minutes, then cooled and diluted with 50 ml of distilled water. Spectrophotometer absorbance was measured at 660 nm wavelength. Each sample's ethanol content was ascertained by use of the ethanol standard curve.

RESULTS AND DISCUSSION

Isolation and characterization of yeast strain

The yeast cultures used in this work were taken from samples of local breweries. Five distinct isolates in all were separated from the collected samples, and their colony morphology (pigmentation, texture, size, elevation, shape, and margin) allowed them to be classified as yeast [21].

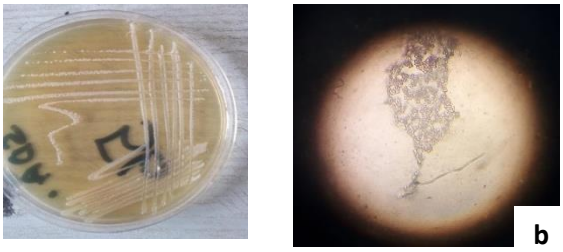

Figure 1: Sample of (a) Colony Morphology of yeast isolates (b) Microscopic morphology of yeast isolate

Table 1: Microbial Population of Isolates from the Local Brews Samples

Source	Sample code	Total fungi count (CFU/ml)	Physical Observable Features on SDA	Microscopic Features	Suspected organism
Pito	P2	5.1×10^4	Small, white colonies with smooth texture and uniform distribution	Presence of single-celled structures with no observable septations	Yeast
	P12	4.8×10^4	Medium-sized, white colonies with smooth texture and well-defined edges	Presence of single-celled yeast structures with no observable septations	Yeast
Burukutu	B15	1.7×10^4	Colonies of a moderate size that seem silky or fluffy	Chains of elongated cells that resemble hyphae but are not true hyphae	Yeast
	B18	2.0×10^4	Irregular shapes of colonies which develops a characteristic hue colouration after 4 days	Presence of single-celled structures with no observable septations	Yeast
	B21	1.8×10^4	Cream-colored, circular colonies with smooth texture	Uniform shape and size within a colony.	Yeast

We investigated the morphological features of yeast isolates grown on SD broth. There were noticeable differences in their microscopic morphology even though their colony morphological traits were largely comparable (Table 1). Table 2 lists the patterns of yeast isolate fermentation and sugar absorption. Similar outcomes were observed in specific carbon sources for yeast isolates' sugar assimilation patterns. All yeast isolates were able to ferment glucose and trehalose but not lactose. Most of the yeast isolates tested for xylose fermentation were able to ferment the sugar.

Table 2: Sugar fermentation profile of Local Brews Isolates

Isolates	Simple Sugars											
	Sucrose		Glucose		Lactose		Xylose		Maltose		Trehalose	
	Gas	Colour change	Gas	Colour change	Gas	Colour change	Gas	Colour change	Gas	Colour change	Gas	Colour change
P2	+	+	+	+	+	-	+	-	+	-	+	+
P12	+	+	+	+	+	+	+	+	+	-	+	+
B15	-	-	+	+	-	-	-	-	+	+	+	+
B18	-	-	+	+	-	-	+	+	+	+	+	+
B21	-	-	+	+	+	+	+	-	+	-	+	+

+ represent positive response; - represent negative response

Factors influencing bioethanol production

When sugar ferments with microorganisms, a number of variables, including pH, temperature, and ethanol level, affect the ethanol production and fermentation pace [21]. Therefore, the ethanol, temperature, and pH tolerance—three crucial elements needed in the synthesis of ethanol—were examined in all yeast isolates. In this study, all the five yeast isolates could tolerate 45 °C (Figure 2). [26] reported that their selected yeast strains were thermo-tolerant and could grow up to 46°C. The result of this study is in line with other researchers who have isolated xylose fermenting organisms. For example, [27] identified *Saccharomycodes ludwigii* APRE2, *Pichia kudriavzevii*, and *P. manshurica* as thermotolerant yeasts with significant fermentative capabilities at elevated temperatures. Additionally, *Meyerozyma guilliermondii* H1, isolated from lychees, demonstrated both thermotolerance and the ability to ferment xylose and arabinose, positioning it as a promising candidate for ethanol production from agricultural wastes [28]. Out of all the isolates collected for their study, [29-30] reported that three yeasts could thrive at a high temperature of 45 °C.

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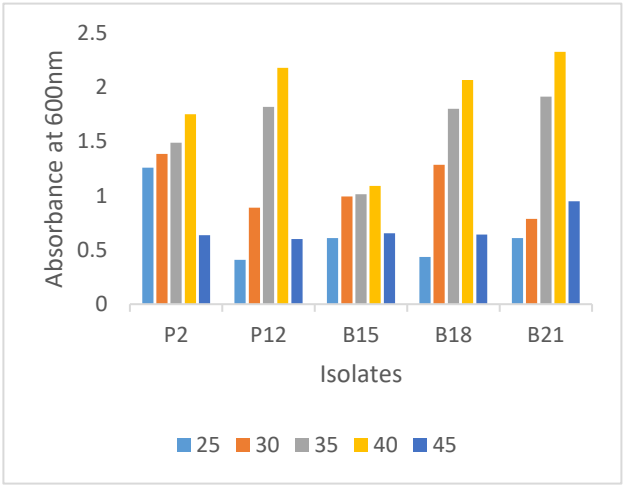

Figure 2: Temperature tolerance activity of local brews isolates

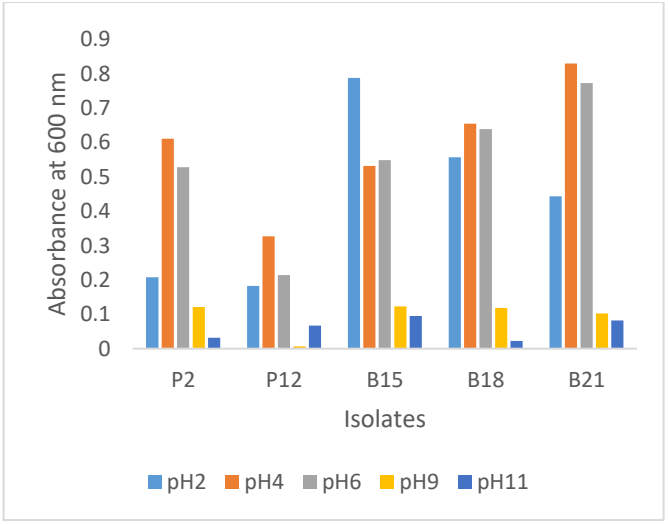

Figure 3: pH tolerance activity of local brews isolates

Utilizing thermotolerant xylose-fermenting microorganisms for bioethanol production offers several advantages, including high bioconversion rates [22], increased ethanol yield from biomass, reduced risk of contamination, and energy savings due to lower cooling costs [23,31].

The rate at which microorganisms produce ethanol could be greatly influenced by the pH of the fermentation media [11]. Though yeast function best in an acidic environment, with a pH range of 4.0 to 5.2, brewing and distilling strains could grow well at a pH range of about 3.5 to 6.22 [23]. The yeast isolated in this work grew best at pH 3 and 4, yet they could tolerate a pH range of 3–6 and were therefore appropriate for use in fermentation that was often carried out in acidic circumstances (Figure 3). The capacity of yeast isolates to proliferate and convert xylose into ethanol throughout a wide range of pH values guarantees the resilience of the fermentation process under different conditions [22], so improving its flexibility and stability. This adaptability allows for the fine-tuning of fermentation conditions to optimize the production of ethanol, hence enhancing the economic feasibility of the process. Similar studies such as [32-33] reported pH 6 as optimum pH for ethanol production from different pretreated agrowaste using microoganisms, while [22] reported a pH range 4-5 and [28] reported pH 4.

Absorbance has a direct relationship with microbial tolerance activity such as ethanol, pH and temperature. This is explained from the point that temperature and pH can influence absorbance spectra as elevated temperature may enhance molecular motion [34] and pH shifts affect molecular ionization, altering absorbance properties. Similarly, elevated ethanol concentrations increase enzymatic reactions, leading to increasing absorbance [14]. The ethanol tolerance of the microorganism utilized in the fermentation process was one of the crucial aspects of the ethanol production. According to [22] ethanol tolerance of yeast cells is directly correlated with ethanol production, so the microorganism should develop and create ethanol in the presence of at least 4% (v/v) ethanol. Every isolated yeast strain could tolerate a 14% ethanol concentration. At 17% concentration of ethanol, some yeast isolates grew weakly (Figure 4). According to [38] and [14] ethanol tolerance is advantageous when yeast is being thought of for industrial usage, particularly when ethanol is being produced. Elevated levels of ethanol may have a harmful effect on microbial cells [31, 34], diminishing their ability to survive and impairing their capability for fermentation [35].

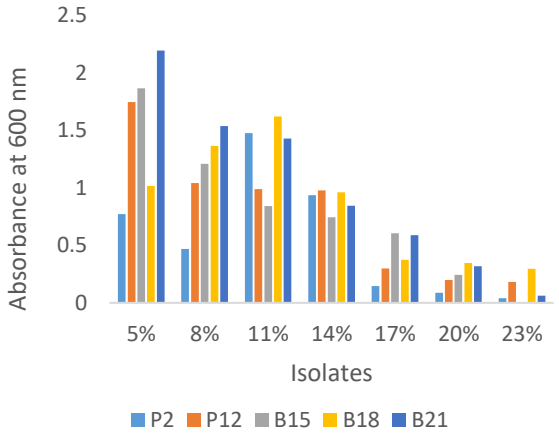

Figure 4: Ethanol tolerance activity of local brews isolates

Ethanol-producing activity of yeast isolates

While evaluating ethanol production ability of the local brew isolates, five yeast produce ethanol (0.73 – 18.43 g/L) (Figure 5). The variations in sugar fermentation patterns observed in the study suggest differences in the metabolic capabilities of the yeast isolates. The highest ethanol concentrations produced by certain isolates on specific days indicate varying rates of sugar utilization and ethanol production.

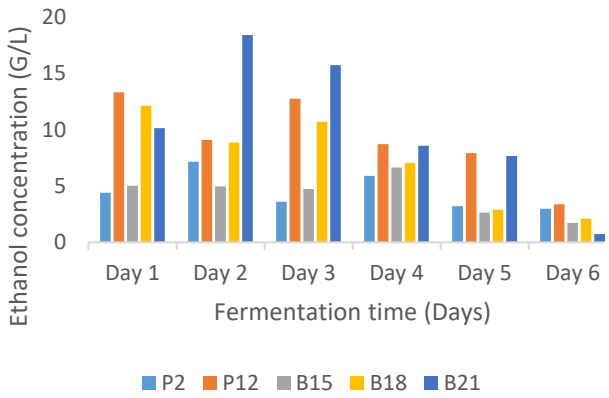

Figure 5: Ethanol production of local brews isolates

For instance, isolate B21 exhibited the highest ethanol concentration on day 2, while P12 peaked on day 1. These results underscore the potential of isolates P12, B18, and B21 for ethanol production from xylose fermentation (Figure 5). Previous research by [36] demonstrated similar findings, where specific yeast strains, such as *P. stipitis* CBS5773, displayed superior ethanol production from xylose fermentation (1.51% from xylose fermentation after 36 hours). A study by [37] revealed that the natural type of flocculant Saccharomyces cerevisiae could live in a concentration of 2.5% (v/v) ethanol, whereas two mutant strains from 0.8KGy dosage could survive in a concentration of 5% (v/v).

CONCLUSION

This study emphasises the substantial improvement achieved in the identification and characterization of yeast strains from local brews possessing the intended attributes of resistance to ethanol, pH, and temperature. These qualities are essential for improving the efficiency of converting lignocellulosic biomass into ethanol, thereby meeting the urgent need for environmentally and economically feasible biofuel production techniques.

Currently, the identification process relies on morphological and biochemical tests, which may not provide sufficient accuracy. While the isolated yeast strains show promise for ethanol production, precise identification and characterization are essential for optimising fermentation processes. It is therefore recommended that advanced molecular techniques, such as DNA sequencing and bioinformatics, which can enhance the accuracy of yeast identification, thereby improving fermentation efficiency should be undertaken.

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