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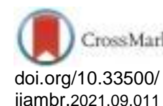
Comparative ethanol tolerance of different yeast isolates obtained from spontaneously fermenting cocoa (*Theobroma cacao* L.) pod husk



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ABSTRACT

Yeast strains are commonly associated with sugar rich environments. Spontaneously fermenting Cocoa pod husk samples were selected as source for isolating yeast cells, with the intent to genetically modify the selected yeasts for biofuel production from cocoa pod husk. The isolated yeast cultures were identified at Genus level by colony morphology, biochemical characteristics and cell morphological characters. The viability of yeast cells were monitored under different concentrations of ethanol. Ethanol tolerance of each strain was studied by allowing the yeast to grow in liquid yeast extract peptone dextrose (YEPD) medium having different concentrations of ethanol. A total of fifty yeast strains isolated from different samples and five were carefully selected based on frequency of occurrence for the study. Five strains of yeast obtained were screened for ethanol tolerance at different concentrations of 4 to 10%. The results obtained in this study show ranges of selected yeasts tolerance levels. *Saccharomyces uvarum* (MX_3) was less tolerance at 6% concentration compared to MX_4 and MX_5. However, two strains of *Saccharomyces cerevisiae* (MX_1 and MX_2) had the highest level of tolerance (1.65 ± 0.00600^b and 1.79 ± 0.05550^a) respectively. Based on these qualities, genomic DNA of these two strains was carried out for further genetic modification studies and ethanol production.

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INTRODUCTION

Yeasts are the safest and most effective microorganisms for fermenting sugars to ethanol and have traditionally been used in industry to ferment glucose based agricultural products to ethanol and carbon dioxide (Hossain et al., 2017; Sergi, 2020). The yeast, *Saccharomyces cerevisiae* is a unicellular eukaryotic, microorganisms classified as fungus and they are also known as baker's yeast or beer yeast (Hough et al., 1994). The yeast, *S. cerevisiae* was the first microorganism known to possess the ability to ferment sugars for the production of ethanol and carbon dioxide both aerobically and anaerobically (De Haas and Kreuger, 2010). Yeast is ubiquitous in the environment, but is most frequently isolated from sugar rich substrates like fruits berries and exudates from plants. Some yeast strains are also found in association with soil and insects. Yeast has also been isolated from many fermenting sources including fermenting cassava tubers (Osho et al., 2010). Many research workers had found yeast in large numbers in a wide variety of natural habitats such as leaves, flowers, sweet fruits, tree exudates, grains, roots fleshy fungi, insects, dung, soil (Chiranjevi et al., 2013).

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In assessing yeast strains for industrial use, specific physiological properties are required (Chiranjevi et al., 2013). Ethanol tolerance and sugar tolerance are some of the important properties for use in industrial ethanol production (Jimenez and Benetez, 1986). The production and tolerance to ethanol, organic acids and SO_2 are also important tools to differentiate among species (Sergi, 2020). Also, some yeast cells are able to withstand adverse environmental conditions, such as lack of nutrients or high temperatures in the medium through sporulation until the conditions are favourable for reproduction and then they start to sprout all over again (Neiman, 2011). Recently, yeasts have been used in the production of bio fuels, a potentially important alternative energy source. Renewable energy is one of the most efficient ways to achieve energy sustainable development. Increasing its share in the world matrix will help prolong the existence of fossil fuel reserves, address the threats posed by climate change, and enable better security of the energy supply on a global scale (Chiranjevi et al., 2013). For successful fermentations to produce ethanol using yeast requires tolerance to high concentrations of both glucose and ethanol. These cellular characteristics are important because of high gravity (VHG) fermentations, which are common in the ethanol industry, give rise to high sugar concentrations, at the beginning of the process, and high ethanol concentration at the end of the fermentation. *S. cerevisiae* is an important microorganism in bio-industry and its tolerance to ethanol is one of main characteristics to decide whether it can be used as bio-fermentation resources. Ethanol-tolerant and thermotolerant strains which can resist stresses can be isolated from natural resources such as soil, water, plants and animals. This is because cells adapt to their environment over time by natural selection (Siti et al., 2017).

No work has been done in assessing the yeasts isolated from cocoa pod husk for any characteristics of industrial importance. The aim of this work was the isolation and identification of tolerant yeast strains from fermenting cocoa pod husk, which serves as a substrate for isolating adapted starter cultures of yeasts, screening of the yeast strains for ethanol tolerance with the intent to genetically modify the yeasts for industrial biofuel production from cocoa pod husk.

MATERIALS AND METHODS

Collection of cocoa pod husk samples

Fresh cocoa pod husks from which cocoa beans have been removed were obtained from the fermentary section of Cocoa Research Institute of Nigeria (CRIN), Ibadan. Suitability was determined to ensure that diseased pods were not selected. The selected cocoa pods were then sundried.

Isolation of starter cultures from a spontaneously-degrading cocoa pod-derived ecosystem

Dried cocoa pod husk was obtained and fractionated to different particle size forms- coarse, fine particle size and powdered using electric blender. Spontaneous fermentation of the different samples of cocoa pod husk (coarse, fine and powder) was carried out by steeping 10 g substrate in 100 ml of sterile distilled water in seven different flasks each and allowed to ferment naturally at room temperature for 7 days. On each day of spontaneous fermentation, 1 ml of the samples was put in 9 ml of sterile distilled water in a test tube and serially-diluted in 9 ml of sterile distilled water in different test tubes (Meynell and Meynell, 1970). One milliliter (1 ml) each of appropriate dilutions was inoculated by pour plate method in yeast extract agar (YEA) medium in duplicate containing Streptomycin antibiotics at a concentration of 40.0 μg/ml. The plates were incubated at 30°C for 48 h. Representative colonies were picked randomly and transferred by streaking unto sterile fresh YEA plates until pure cultures were obtained (Harrigan and MacCance, 1966).

Identification of yeast isolates

Yeast isolates with the highest frequency of ecological occurrence during isolation were selected. Preliminary identification of yeast isolates was done based on their cultural, colony characteristics, microscopic appearance of the cell and other biochemical characteristics. Smear of yeast isolates were air dried, flooded with Lactophenol in cotton blue and then viewed under the microscope using oil immersion lens (Barnet et al., 1990). The selected isolates were given specific names for easy recognition.

Determination of yeast viability

This was carried out according to the modified methylene blue staining method adopted by Rocken and Staruss (1976) using Thoma counting Chamber. Exactly 0.1 g of each type of yeast under test was weighed in to 10 ml warm sterile distilled water. Thereafter 1 g of glucose was added and the content was properly shaken to dissolve yeast and sugar completely, this was left in an incubator at 30°C for 3 h. The stock was diluted 10 fold by taking 1 mL of sample (stock), plus 1 mL of methylene blue and 1 mL of 5 N acetic acid and finally made up to 10 mL by the addition of 7 mL of sterile distilled water. This process was repeated further to make the dilution to 10-2 such that the cell concentration was between 15-300 cells present per

microscope field. The drop of the mixture was applied to the ruled grids of the Thoma haemocytometer chamber. By counting the total number of cells in the number of squares and counting the number of blue cells in the same group of squares, the percentage of dead cells were calculated from the total number of cells present.

Thus:

$$\%\ \text{viability} = \frac{\text{Number of live yeast (unstained cells)}}{\text{Total number of yeast cells (dead and living)}} \times 100$$

Screening of the selected yeasts for ethanol tolerance

This was necessary for further selection of two organisms amongst the five selected yeast starters for intended genetic modification. Isolates were screened for ethanol tolerance on the criterion of Rose et al. (1990) using the medium of Novak et al. (1981). The medium containing (per litre), 5 g of $MgS0_4.7H_2O$, NH_4SO_4 2 g, yeast extract 1.0 g and 2.0 g of glucose. The medium was dispensed into flasks and autoclaved at 121°C for 15 min. It was allowed to cool to room temperature before ethanol was added to each flask of the same medium to constitute varying percentages of ethanol differing by 1% (v/v) from one flask to the other. The samples were then dispensed into pre-sterilized flasks and inoculated with the selected yeast strains in duplicates. The flasks were then incubated with shaking at 150 rpm in a rotatory shaker incubator at 30°C for 48 h. Initial Optical Density of the flask was taken at 600 nm in a spectrophotometer using un-inoculated medium as blank. Any increase in the optical density (OD) reading was taken as evidence of growth. The OD is directly proportional to the cell mass or growth. The increase in optical density in a flask was recorded as evidence of growth. Ethanol tolerance is defined as the amount of ethanol (% v/v) which completely inhibited the growth under the conditions stated (Rose et al., 1990).

Extraction of genomic DNA from yeast (MX1 and MX2) and gel electrophoresis

Total genomic DNA of yeast was extracted from the two most ethanol tolerant yeast (MX1 and MX2) were carried out using the modified phenol chloroform method of (Kasiser et al., 1994). Three-milliliter suspension aliquots from each yeast broth were spun at 12000 rpm for 10 min, and the pellet was used to extract DNA. Extractions were conducted in duplicate. The extracted DNA were stored at -20°C in TE buffer. Small portion of the extracted DNA was run on 0.8% prepared agarose gel stained with ethidium bromide solution. DNA marker (1kb+) of known size was also loaded along- side the samples for verification. The gel was observed under the UV light.

Data management and statistical analysis

All data were analysed using one way analysis of variance (ANOVA) comparison.

RESULTS

Yeast isolation from cocoa pod husk (CPH) and selection

Thirty yeasts isolates were obtained from a sun-dried submerged cocoa pod husk subjected to spontaneous fermentation for seven days. The colonial morphologies, physiological and biochemical characteristics of some of the isolates were studied and used for preliminary identification. The colour varied from cream to white, some smells of palm-wine while some have fruity odours.

Five of the isolates were selected based on their frequency of occurrence as shown in Table 1. Majority of the isolates (80%) coded as MX1, MX2, MX4 and MX5 were identified as *S. cerevisiae* and (20%) coded MX3 was identified as *S. uvarum*.

The viability of the isolated yeasts was determined as shown in Table 2. Using this dye method allows the cells to be identified and distinguished into those alive and those that are dead. The use of the dye allows for analysis of individual yeast cells. Methylene blue penetrates into every cell. Living cells enzymatically reduce the dye to a colourless product and become unstained, whereas dead cells are stained blue.

Ethanol tolerance of the selected five yeast isolates is shown in Table 3. This provided a preliminary screening of all the isolates. It was observed that all the yeast isolates were able to grow at 4% ethanol concentration tested. Appreciable growth of the yeast were observed up to 8% of ethanol concentration, above this concentration, the tolerance decreases slightly for the *S. cerevisiae* isolate MX4 and MX5. However, *S. cerevisiae* isolates MX1 and MX2 were still able to tolerate ethanol to an appreciable extent above 9 to 10% concentration of ethanol. The tolerance exhibited by these two strains of yeast necessitated their selection for further genetic modification. Isolate MX3 was less tolerant to ethanol at higher concentration of 6% of ethanol, but it was selected based on the frequency of occurrence during preliminary isolation from the CPH medium. The genomic DNA of the two ethanol tolerant yeasts MX1 and MX2 was extracted using the chemical method. Gel electrophoresis was carried out using 0.8% concentration of gel on the extracted DNA as depicted in Figure 1 and 2 respectively. Also the DNA sizes of both yeasts are same.

DISCUSSION

The ability of the selected ethanol-tolerant yeast strains

Table 1. Frequency of occurrence of the different yeast isolates obtained from spontaneously fermenting CPH submerged medium.

Yeast Isolates	Frequency of occurrence (%)
MX1	28.5
MX2	27.3
MX3	15.2
MX4	14.6
MX5	14.4

Table 2. Viability test of selected yeasts.

Yeast Isolate	Cell counts cells/ml		No. of dead cells	Percentage viability
	A	B		
MX1	151	154	Nil	100
MX2	152	160	Nil	100
MX3	101	106	Nil	100
MX4	112	114	Nil	100
MX5	108	109	Nil	100

Table 3. Ethanol tolerance of the selected yeast isolates.

Yeast Isolates	Concentration of Ethanol (v/v)						
	4%	5%	6%	7%	8%	9%	10%
MX1	2.60± 0.0079[a]	2.45 ± 0.0020[a]	2.38 ± 0.00430[a]	2.34 ± 0.03700[a]	2.19± 0.00295[b]	2.15 ± 0.00110[a]	1.65 ± 0.00600[b]
MX2	2.54± 0.0041[a]	2.47 ± 0.00100[a]	2.41 ± 0.01190[a]	2.18± 0.13850[ab]	2.28 ± 0.01765[a]	2.10 ± 0.00505[a]	1.79 ± 0.05550[a]
MX3	1.95± 0.0233[b]	1.26 ± 0.00195[d]	0.95 ± 0.00480[d]	0.90 ± 0.00855[c]	0.81 ± 0.01140[d]	0.78 ± 0.00980[d]	0.58 ± 0.00045[d]
MX4	2.44± 0.09585[a]	2.34 ± 0.01775[b]	2.13 ± 0.04465[c]	2.07 ± 0.04075[b]	1.99 ± 0.03500[c]	1.92 ± 0.04590[b]	1.49 ± 0.03240[c]
MX5	2.41 ± 0.05770[a]	2.25 ± 0.01205[c]	2.24 ± 0.02425[b]	2.05 ± 0.02510[b]	2.02 ± 0.01460[c]	1.61 ± 0.00290[c]	1.47 ± 0.01125[c]

Values with same superscript are not significantly different; SD = 0.05. Keys: *Saccharomyces cerevisiae,* MX1; *Saccharomyces cerevisiae,* MX2; *Saccharomyces uvarum,* MX3; *Saccharomyces cerevisiae,* MX4; *Saccharomyces cerevisiae,* MX5.

to withstand stress has been amply demonstrated by the fact that the yeast strains were able to grow in media containing relatively high degree of ethanol concentrations. This observation is in agreement with the suggestion of Nsikak et al. (2018), who stated that ethanoltolerant yeasts tend to be sugar-tolerant and further asserted that the combination of sugar tolerance and alcohol tolerance is an advantage when a yeast is being considered for industrial use especially where ethanol is being produced. Tolerance to ethanol is one of main characteristics to decide whether an

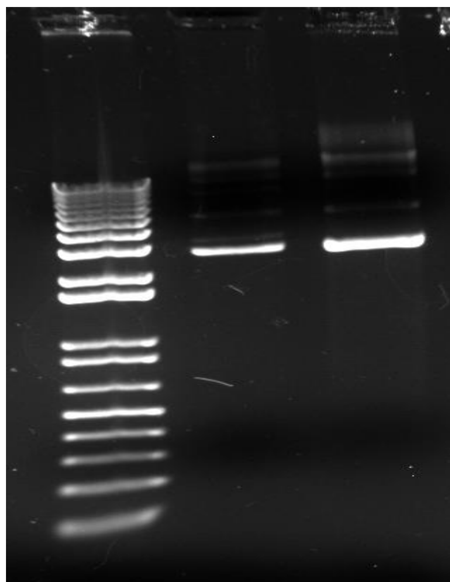

Figure 1. Genomic DNA of MX1 yeast in 0.8% agarose gel electrophoresis.

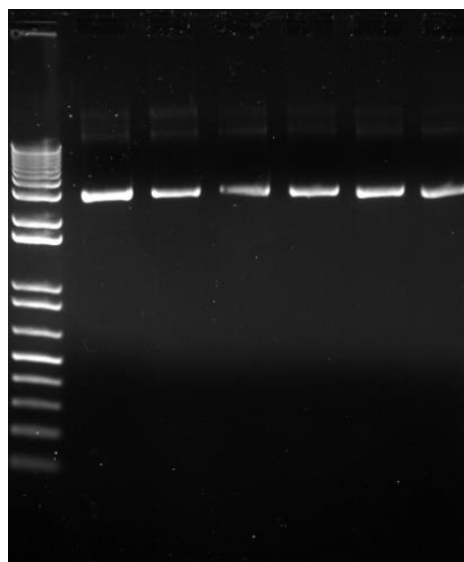

Figure 2. Genomic DNA of MX2 yeast in 0.8% agarose gel electrophoresis.

organism can be used in bio-fermentation process since it can hardly be avoided during fermentation. Ethanol tolerance has yet to be clearly define, although it has been reported to reproducible under defined conditions, and appears to be under complex genetic control. Ethanol has three major effects on yeast, it decreases the rates of growth and of fermentation and it cell viability. The range of ethanol tolerance obtained in the present study was 6-10 %(v/v) which correlates with the previous reports by (Tikka et al., 2013) who screened seven strains of *S. cerevisiae* obtained from different fruit sources for ethanol tolerance. The results obtained in this study show a range of tolerance levels between 7-12% in all the stains.

Ethanol is well known as an inhibitor of growth of microorganisms however, yeast strain MX1 and MX2 were able to tolerate ethanol concentration at a higher

level of 10%(v/v) compared to the other strains, this findings is in agreement with similar results that has been reported by some researchers that some strains of the yeast *S. cerevisiae* show tolerance and can adapt to high concentrations of ethanol (Ghareib et al., 1988; Alexandre et al., 1994). High ethanol production capability of ethanologenic yeasts under the presence of high ethanol is one of the most important factors for ethanol production. The development of such strains is of great economic value to industries involved in fermenting, distilling and refining ethanol. In the ethanol industry, ethanol production is usually among 10-14% (v/v) and the theoretical yield has to be as high as 90-93% of the fermentation efficiency for the conversion of glucose into ethanol (Bai et al., 2008).

The MX3 yeast shows a drastic decrease in growth at 6% concentration of ethanol. Yeast growth is acknowledged to be highly inhibited by ethanol even at relatively low concentrations as it interferes with cell division, decreases cell volume and specific growth rate. Some researchers has also reported that in some yeasts strains where the concentration of ethanol reaches 4 and 5% *v/v*. At that point, between alcohol and the exhaustion of dissolved oxygen, their growth is inhibited (Sergi, 2020). At high ethanol concentrations, cell vitality reduces while cell death increases (Siti et al., 2017). Among the factors, ethanol is considered to be the major stress responsible for decreased ethanol production and stuck fermentation (Gibson et al., 2007). At concentrations in excess of 8% (v/v) ethanol cause the phospholipid of the lipid bilayer of cell membranes and organelles, such as the inner membrane of mitochondria, to become hyperpolarized thereby increasing membrane fluidity and consequentially decreasing membrane integrity (Lloyd et al., 1993; Ly et al., 2002; Mishra and Prasad, 1989).

Yeast isolates MX4 and MX5 were still able to tolerate ethanol at 6% concentration beyond which growth decreases. Ethanol has been reported to inhibit glycolytic enzymes and biological processes associated with lipid synthesis in the cellular membrane. In addition, ethanol could remove hydrate layers around yeast cells and decrease water activity of the medium (Hoang et al., 2015). As a result, ethanol reduced the growth of yeast cells. At high ethanol concentrations, cell vitality reduces while cell death increases (Siti et al., 2017). Ethanol has been reported to damage mitochondrial DNA in yeast cells (Ibeas and Jimenez, 1997) and to cause inactivation of some enzymes, such as hexokinase and dehydrogenase (Nagodawithana and Steinkraus, 1976).

Conclusion

The result obtained from this study reveal a strong indication of isolating tolerant yeasts strains from natural adapted environment. The selected yeast shows high degree of ethanol tolerant which is a physiological character highly considered in yeast utilized as fermentation starters in ethanol industry. The performance of the two high ethanol-tolerant yeast strains MX1 and MX2 has revealed the need for further studies to genetically modify the isolates for bioethanol production.

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