Isolation and identification of non-saccharomyces ethanol and thermo-tolerant yeasts strains from fermented carbohydrate wastes

Mohammed Sani Abdulsalami*[1], Nkechi Eucharia Egbe[1], Ugochukwu Okechukwu Ozojiofor[1], Abba Umar Hassan[1]

[1]Department of Biotechnology, Nigerian Defence Academy, Kaduna, Nigeria.

Submitted: 2nd June, 2023; Accepted: 4th June, 2023; Published: 30th June, 2023
DOI: https://doi.org/10.54117/jcbr.v3i3.2
**Corresponding author: Ugochukwu Okechukwu Ozojiofor; ozojiofor.uo@nda.edu.ng*

Abstract
Ethanol is an important chemical with its potential as a biofuel to displace the environmentally unfriendly fossil fuels. Isolation and identification of ethanol and thermo-tolerant yeast capable of producing ethanol were carried out in this study. Seventy (70) samples from different fermented foods and agricultural wastes from millets, sorghum, maize, rice and guinea corn were obtained from local sellers in Panteka, Farin-Gida, Barkallahu and Barnawa in Kaduna State (North West), Nigeria were used in this research. The samples were collected using a sterile plastic container and were transported immediately to the Biotechnology laboratory of the Nigerian Defence Academy in iced chamber. They were serially diluted in distilled water and inoculated on yeast extract peptone dextrose agar (YEPD) media plates supplemented with 0.2% chloramphenicol. The plates were incubated at 30°C for 72 hours and developed colonies were sub-cultured to obtain pure culture which was observed for their morphological and microscopic characteristics. After examination, ten (10) yeast isolates were obtained from the seventy (70) samples collected. Flocculation test, Hydrogen sulphide test, preliminary alcohol production and sugar fermentation test were carried out on the isolates and their tolerance to ethanol at varying concentration 5%-20% (v/v) and temperature at 37ºC-45ºC were studied. Using the internal transcribed spacer (ITS) 1 and 2 regions primer, the DNA were sequenced and the isolates identified. Four isolates FG 17, FG 27, P1 and BN50 showed ethanol tolerance potentials at 20% ethanol and were identified as strains of *Diutina rugosa*, *Candida sp, Candida ethanolica* and *Candida tropicalis* while one isolate FG 35 showed good thermo-tolerance ability at 45ºC and was identified as *Trichosporon sp*.

Keywords: Panteka, Ethanol, Tolerance, Isolates, *Candida*.

Introduction

There has been an increased demand of ethanol globally, which has made it one of the most widely used solvent in the chemical industry (Ocloo, 2010). Ethanol has become an important alternative source of bioenergy today, due to the environmental impact of fossil fuels such as petroleum and coal (Dien *et al.*, 2006; Nghiem *et al.*, 2010). The global interest in ethanol as a biofuel is also due to the increase in global oil prices, and the desire for energy independence and security (Drapcho *et al.*, 2008). Ethanol as a biofuel offers more than fossil fuel because they are renewable and sustainable sources of bioenergy (Ahmad *et al.*, 2011). In some developed countries of the world, bioethanol has been produced in commercial quantity where it's used as an octane rating enhancer when mixed with gasoline in various ratio to form gasohol as fuel. The SAR-COV-2 (COVID-19) pandemic which affected several countries of the world also led to an increase in the demand of ethanol, as a solvent in the diagnostic molecular biology laboratories and its use in the production of sanitizers and other disinfectants in fighting the global pandemic. The primary source of bioethanol production is from carbohydrates such as lignocelluloses material, plant crops, organic wastes and algae. These sources are degraded using microbes and their enzymes producing bioethanol in the process. The microorganisms and enzymes employed in bioethanol production are very critical to the overall efficiency and output of the process. One of the major challenges to improved bioethanol production is the lack of industrially suitable microbes for converting biomass into fuel ethanol (Dien *et al.*, 2003). Yeasts especially strains of *Saccharomyces cerevisiae* are known to produce ethanol through sugar fermentation. Some non-saccharomyces yeasts have also been shown to be good producers too (Ukwuru, 2013). There is the need to explore more yeast strains for industrial processes and this requires a holistic knowledge of their biochemical properties. Certain desirable traits which are inadequate or lacking in this yeasts can be improved on either by genetic engineering or other gene improvement techniques (Ukwuru, 2013). Substrate/Product and temperature inhibition of yeast is a possibility and identifying ethanol tolerant and thermotolerant yeasts can help circumvent these challenges which will lead to improved ethanol production on a large scale. This study was designed to identify some ethanol and thermotolerant yeasts from the different fermented foods and agricultural wastes collected.

Materials and Methods

Study area

This study was carried out in Kaduna, Kaduna state. Kaduna is the state capital of Kaduna State in North-Western Nigeria.

Sample collection

The traditionally fermented foods, agricultural waste and alcoholic beverages were obtained from local sellers from

Panteka, Farin-Gida, Barkallahu and Barnawa in Kaduna State (North West), Nigeria, using sterile plastic bags and the samples were immediately transferred to the laboratory for storage.

Isolation of Yeast

The samples were serially diluted with sterile distilled water and inoculated on yeast extract peptone dextrose agar (YEPD) plates by spread plate method. The plates were incubated at 30°C for 72 hours and developed colonies were observed for their morphology and microscopic characteristic. Colonies obtained from the isolation step were sub-cultured by streaking on YEPD supplemented with 0.2 mg/ml chloramphenicol to inhibit bacterial growth. Colonies with morphologically distinct characters were observed and purified by repeat streaking on YEPD medium. Pure culture of each strain was preserved on YEPD agar slants at 4°C until needed. The pure isolates were subjected to morphological identification using the methods of Barnett *et al.*(2000). Wet mounts of isolated cell samples were prepared in distilled water and examined using 40X objective magnification.

Hydrogen sulfide production and flocculation test

Hydrogen sulfide production were carried out on the isolates growing the yeast isolates on lead acetate medium as described by Ono *et al.*(1991) and incubated 30ºC for 10 days.

For the flocculation test, the isolates were inoculated in 10 ml of YEPD broth and incubated at 30ºC for 3 days. After incubation, tubes were agitated to observe the flocculation formed (Thais *et al*., 2006).

Identification by fermentation assessment

Biochemical identification was carried using Phenol red broth (yeast fermentation base) (Barnett *et al.,* 2000). The isolates were studied for their ability to utilize sugars like glucose, fructose, maltose and sucrose, which also served as a method of biochemical identification using Phenol red broth (yeast fermentation base). Two (2 g) grammes of each sugar type was introduced into a test tube containing 5ml of Phenol Red broth medium and inoculated with the isolates and 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 (Barnett *et al.,* 2000).

Screening for ethanol tolerance

The yeast isolates were inoculated unto 10ml of YEPD broth (yeast extract, peptone, dextrose) supplemented with different concentrations of ethanol (0-20% v/v) differing by 5% (v/v) from one flask to another. The tubes were incubated for 96 hours at 30°C. Samples were drawn every 24 hours and growth was measured using a UV-VIS spectrophotometer at 600nm. Viability were measured following 48 hours of incubation at 30°C by serially diluting with sterile distilled water and plated on YPD agar medium. The numbers of CFU/ml were calculated (Barnett *et al.,* 2000).

Screening and selection of thermotolerant ethanol-fermenting yeasts

The isolates were grown on YEPD broth containing 4% (v/v) ethanol and were incubated at 37, 39, 41, 43 and 45°C for 24 h. Samples were drawn after 24 hours and growth was measured using a UV-VIS spectrophotometer at 600nm (Nuanpeng *et al.*,2016).

Carbohydrate fermentation and ethanol production by isolates

The ethanol production capabilities of the isolates were evaluated at high temperatures using a YEPD medium containing 200g/l of carbohydrate source. The isolates were inoculated into 250 mL of YEPD medium and then incubated on a rotary shaker (150 rpm) at the temperatures of 37ºC and left for 7days. The fermentation broth was distilled for ethanol and quantified. After 60 h of fermentation, culture broths were withdrawn and centrifuged, and the ethanol concentration in the clear supernatant were determined by gas chromatography (GC) as described by Nuanpeng *et al*. (2016).

Classification and identification of ethanol tolerant isolates

Genomic DNA was separated and purified by using DNA extraction kit (Takara, Japan). The sequencing of the D1/D2 domain of the yeast 26S rDNA and the internal transcribed spacer (ITS) 1 and 2 regions were carried out from PCR products of genomic DNA fragment that were extracted from the isolates. The D1/D2 domain and the ITS regions of the rDNA were amplified by PCR with forward primer NL-1 and NL-4 forward primer NL-1: 5'-GCATATCAATAAGCGGAGGAAAAG3'. Reverse primer NL-4: 5'-GGTCCGTGTTTCAAGACGG-3' and primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3′) (White *et al.,* 1990). The PCR product was checked by Agarose gel electrophoresis, purified and sequenced. The sequence compared pair-wise using the basic local alignment search tool (BLAST) homology searchand aligned using CLUSTALW, and a phylogenetic tree were constructed using the maximum-likelihood method with MEGA version 6.0 with a bootstrap number of 1000 (Tamura *et al.*,2013). The sequences obtained were deposited at Genbank under accession numbers OQ230285 (FG17), OQ238688 (FG27) OQ238820 (BN50), OQ238818 (P1) and OQ238858 (FG35).

Results

Sample collection and isolation of yeast

A total of 70 samples from local carbohydrates waste from maize, rice, millet, sorghum, guinea corn were collected from different parts of Kaduna state. A total of 10 isolates of yeast were derived after isolationusing the yeast extract peptone dextrose agar medium.

Table 1: Sample collected for isolation of yeast and number of isolated yeast from different sources at different locations.

Location	Number of samples	Number of yeasts isolated
FARIN GIDA	42	6
PANTEKA	13	3
BARNAWA	10	1
BARKALLAHU	5	-

Table 2: Morphological and microscopic identification of the isolates.

S/N	SAMPLES	MICROSCOPIC COLONY CHARACTERISTICS ON YEPD	COMMENT
1	**P1**	Oval shape,white, medium size,	Yeast
2	**P3**	Oval shape, white, medium size, spongy growth	Yeast
3	**FG3**	Irregular, white centred, rough and dry surface.	Yeast
4	**BN50**	Round shape, white thick spongy growth	Yeast
5	**P4**	Oval shape, white, medium size, spongy growth	Yeast
6	**FG17**	white thick spongy growth, colonies of yeast-like bud with oval shape	Yeast
7	**FG27**	Irregular shape, thick creamy colonies with buds	Yeast
8	**FG29**	Whitish, round, raised, smooth.	Yeast
9	**FG35**	Slimy, round, regular, small, whitish. with buds	Yeast
10	**FG41**	Irregular shape, thick creamy colonies with buds	Yeast

FG-FARIN GIDA, BN-BARNAWA, P-PANTEKA

Table 3: Flocculation test, Hydrogen sulfide test and colony count of the isolates on YEPD.

Sample source	Flocculation test	Hydrogen Sulfide test	Yeast count (CFU/ml × 10^2)
FG 41	+	-	57
FG 17	+	-	36
FG 3	+	-	36
FG 27	+	-	44
P1	+	-	40
FG 29	+	-	53
FG 35	+	-	60
BN 50	+	-	61
P3	+	-	36
P4	+	-	33

Key: Response (+), no response (-)

Table 4: Sugar fermentation test of the isolates.

SAMPLES	FG 41	FG 17	FG 3	FG 27	P 1	FG 29	FG 35	BN 50	P 3	P 4
SUGARS	**SUGAR FERMENTATION**									
LACTOSE	-	-	-	+	+	-	-	-	+	+
MALTOSE	+	-	-	+	-	-	+	+	-	-
GLUCOSE	+	+	+	+	+	+	+	+	+	+
FRUCTOSE	+	+	+	+	+	+	-	+	+	+

+: Change in color and gas production, -: no change.

Table 5: Preliminary alcohol production test

SAMPLES	DEGREE OF ALCOHOL PRODUCTION					
FG 41	+	+	+			
FG 17	+					
FG 3	+	+	+	+	+	
FG 27	+	+	+	+	+	
P 1	+					
FG 29	+					
FG 35	+	+	+			
BN 50	+	+	+	+	+	
P 3	+	+				
P 4	+					

Table 6: Carbohydrate fermentation and ethanol production.

Isolates	Concentration of ethanol produced (g/l)	% Ethanol yield
FG 41	1.50	7.5
FG 17	2.40	12.0
FG 3	1.70	8.5
FG 27	1.40	7.0
P 1	2.74	13.7
FG 29	1.70	8.5
FG 35	1.30	6.5
BN 50	2.20	11.0
P 3	1.63	8.2
P 4	3.00	15.0

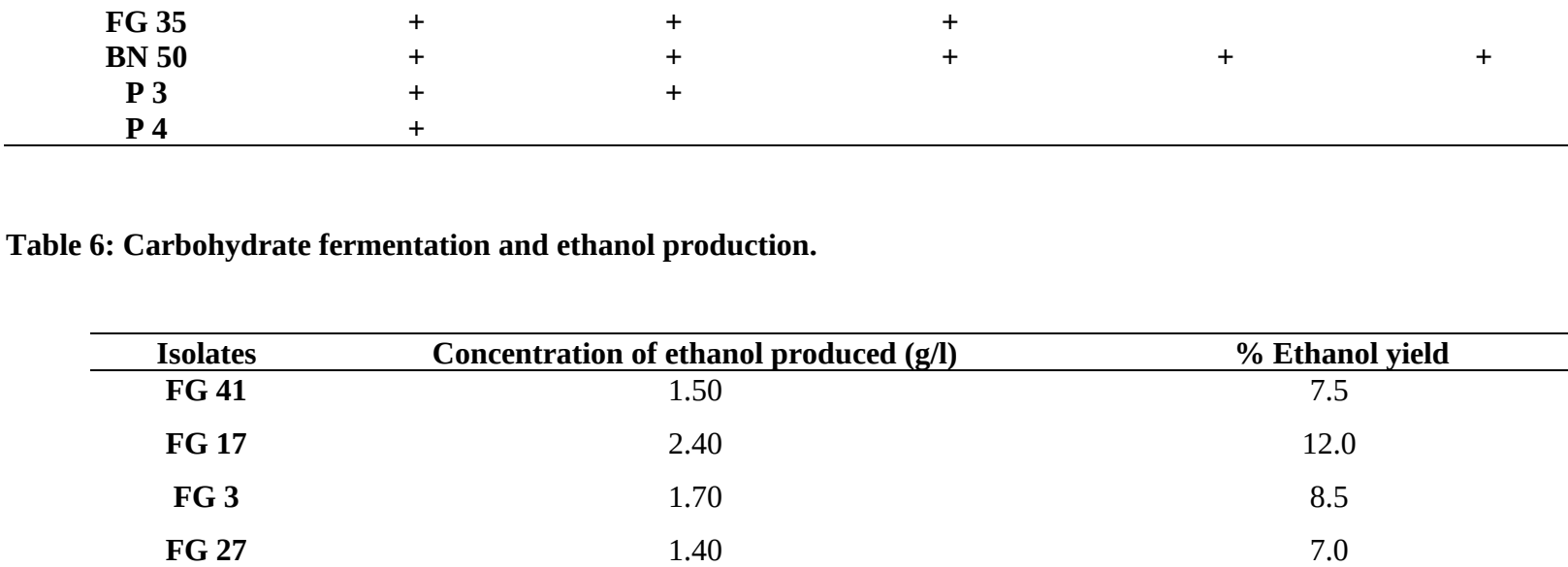


Figure 1: Ethanol tolerance of the isolates after 24 hours

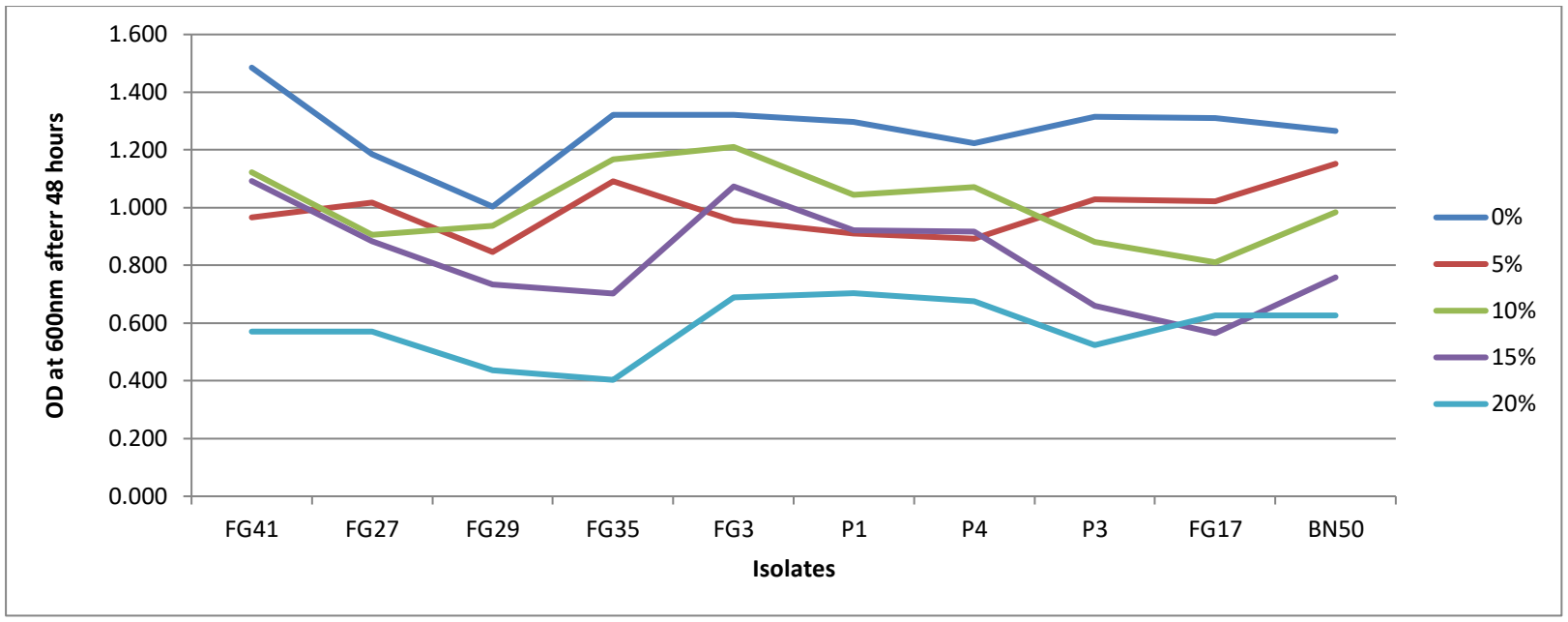


Figure 2: Ethanol tolerance of the isolates after 48 hours

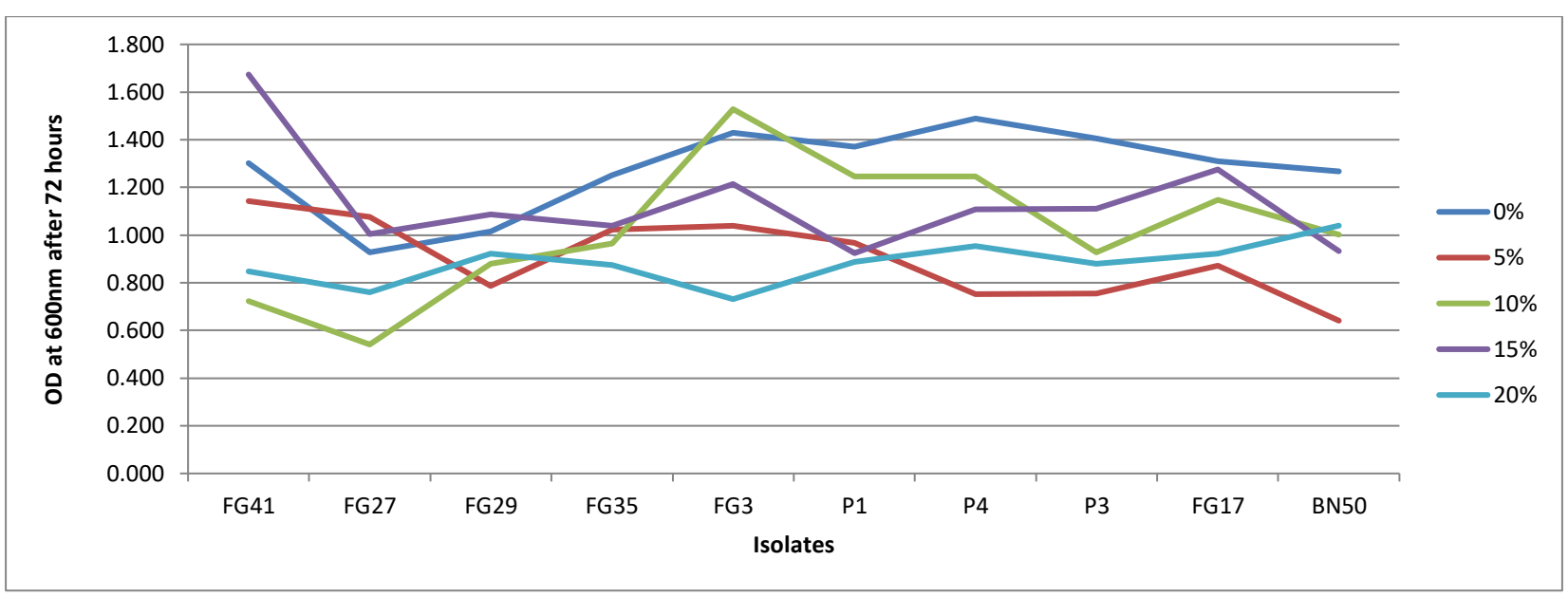


Figure 3: Ethanol tolerance of the isolates after 72 hours

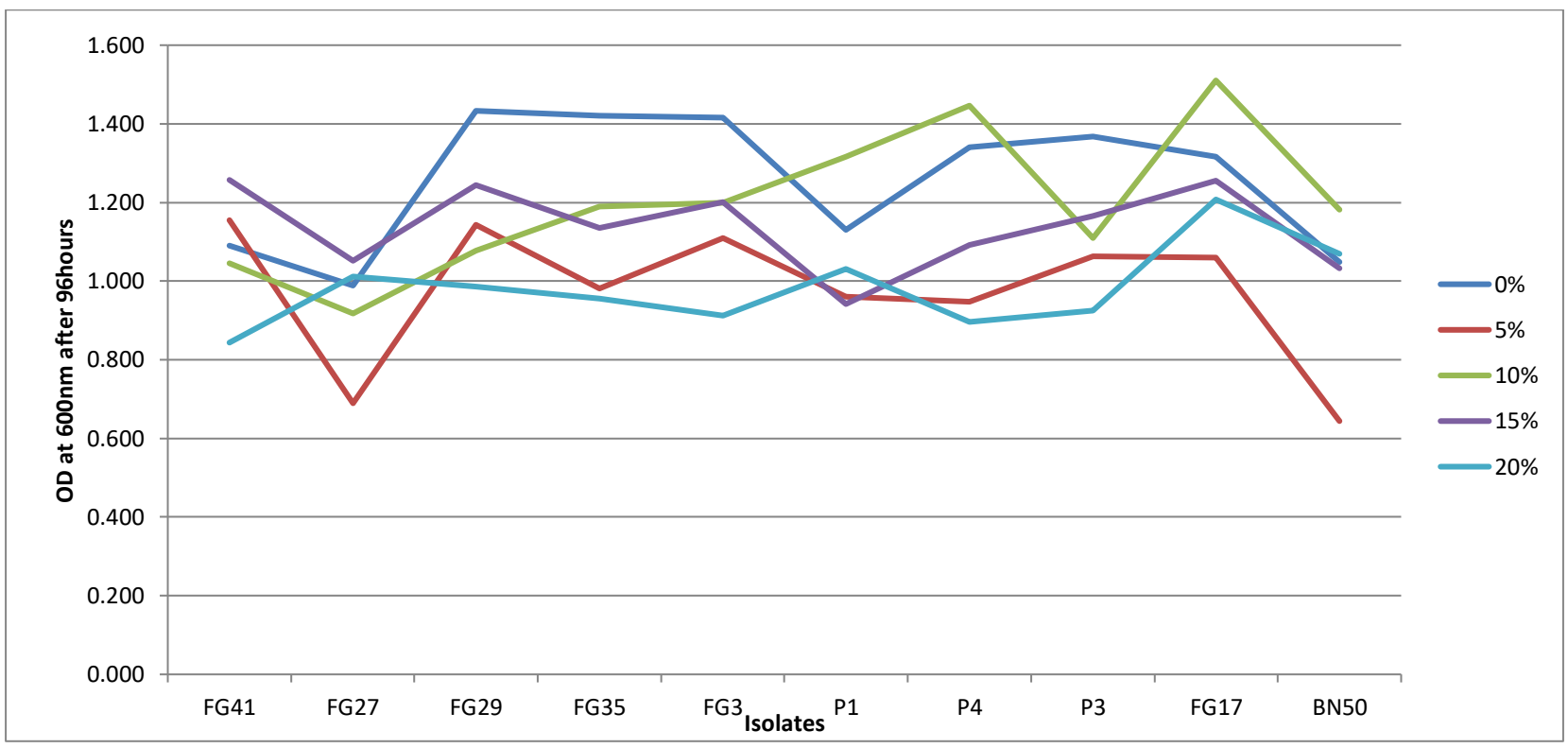


Figure 4: Ethanol tolerance of the isolates after 96 hours

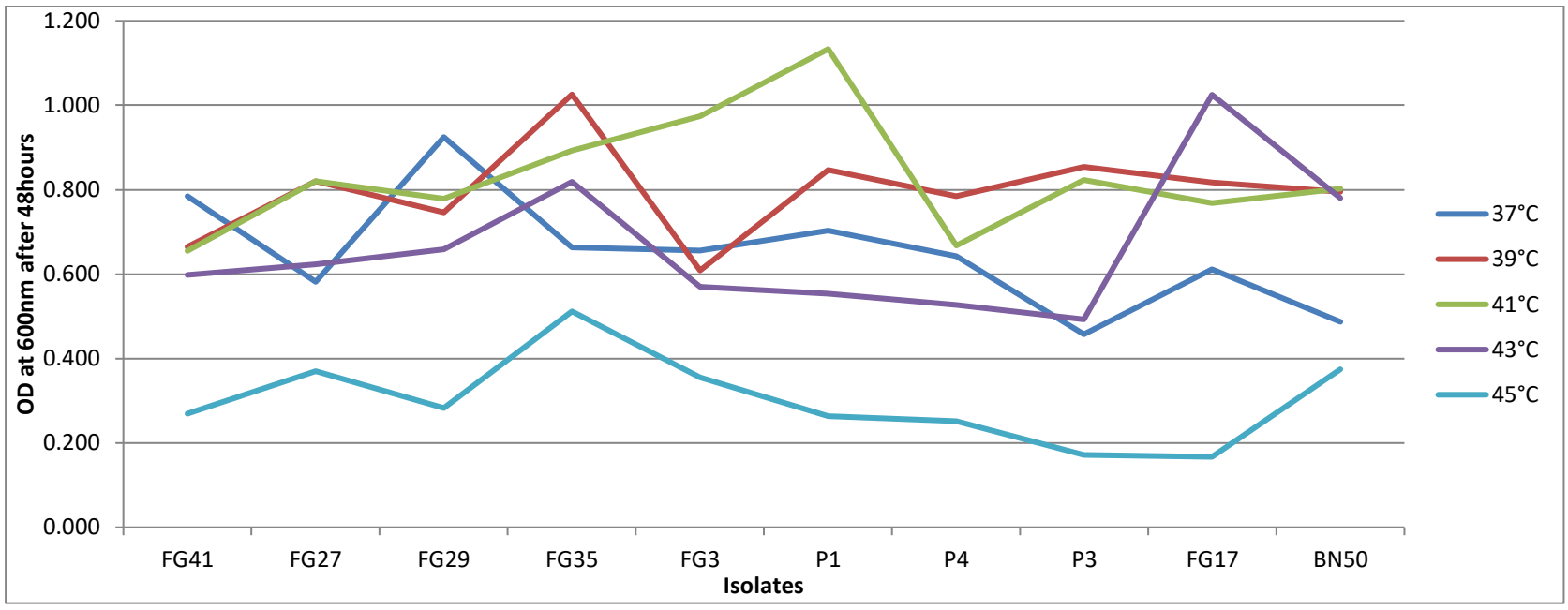
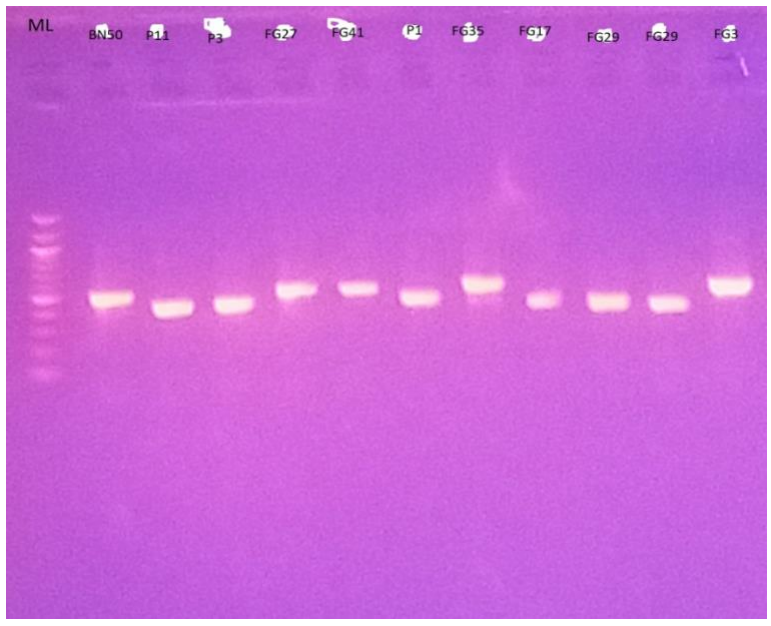


Figure 5: Thermo-tolerance of the isolates



f amplified product



Figure 7: Culture plates of some of the isolates



Figure 8: Phylogenetic reconstruction for the isolate FG17 based on ITS analysis using Maximum-likelihood algorithm method and 1000 replicate bootstraps.



Figure 9: Phylogenetic reconstruction for the isolate BN50 based on ITS analysis using Maximum-likelihood algorithm method and 1000 replicate bootstraps.

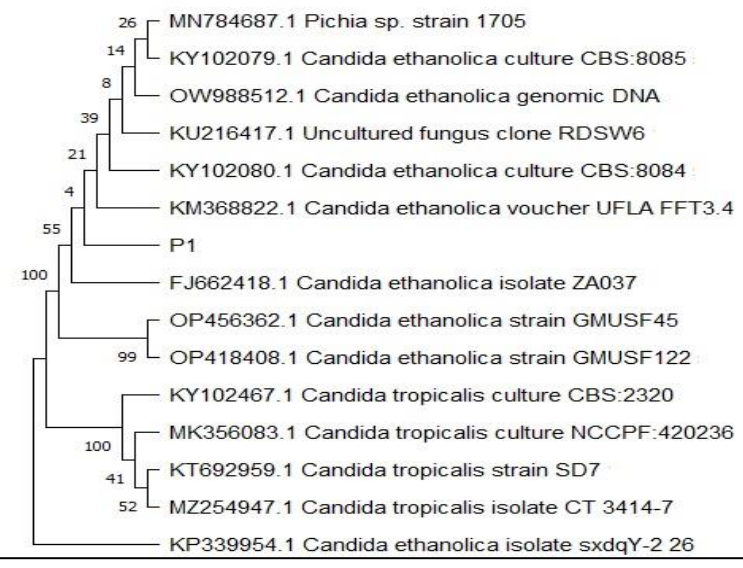


Figure 10: Phylogenetic reconstruction for the isolate P1 based on ITS analysis using Maximum-likelihood algorithm method and 1000 replicate bootstraps.



Figure 11: Phylogenetic reconstruction for the isolate FG27 based on ITS analysis using Maximum-likelihood algorithm method and 1000 replicate bootstraps.



Figure 12: Phylogenetic reconstruction for the isolate P1 based on ITS analysis using Maximum-likelihood algorithm method and 1000 replicate bootstraps

Discussion

All the isolates gave negatives Hydrogen sulphide test and positive flocculation test. Flocculation is an important characteristic that enables the easy separation of the final product of fermentation at the end of the process without the need for further filtration steps and it also allows the utilization of immobilized yeasts on fermentation processes (Stratford, 1992).Yeasts with a elevated levels of hydrogen sulphide are not suitable for ethanol production because it confers some flavor and taste that compromise the quality of the ethanol obtained (Ribeiro and Horii, 1999). Sample collected from Barnawa had the highest yeast count of 61.0 CFU/mL, while the lowest value of 33.0 CFU/mL was obtained from the sample collected from Panteka. The samples were rich in carbohydrates and sugars which is an excellent substrate for the growth and proliferation of microorganisms such as yeasts (Karamoko *et al*., 2012; Santiago-Urbina *et al*., 2013). Gidado *et al*. (2014) had earlier reported varied yeast populations and the isolation of yeast *S cerevisiae* from palm wine and other carbohydrates sources. The variation observed in the yeast isolated from the samples can be attributed to the processing, human activities and the different types of carbohydrates used.

The morphological and microscopic observation of the yeast isolates under the microscope showed spherically or ellipsoid-shaped cell/bud cell of ascospore. The morphologic features of the yeast isolate was confirmed by mounting on glass slide and stained with crystal violet.

Preliminary biochemical investigation revealed that all ten isolates possessed the ability to utilize carbon sugars like fructose, glucose, galactose and sucrose using Phenol red broth (yeast fermentation base). The colour change observed (red to yellow) was indicative of the presence of an array of organic acids, and this agreed with the report of Kumar *et al* (2011). Mukherjee *et al*. (2014) reported that this acid may include phytic acid, a compound produced by *Pichia* species and a number of other non-Saccharomyces yeasts which aids in the release of phosphorus compounds from organic sources in the soil. This observation suggests that the culture organisms possess good fermentative capabilities. This result is similar to that of Walker *et al*. (2006) who observed that yeast isolates studied could ferment one or more sugar types.

Preliminary alcohol production test based on the ability of the isolates to produce carbondioxide from glucose metabolism, showed that all isolates have variable capacity for ethanol production.

All the isolates fermented carbohydrate to produce ethanol with P4 having the highest yield of 15% and FG35 having the lowest yield of 6.5%. BN50, FG27, FG17 and P1 showed tolerance to ethanol at 20% (v/v) after 96 hours of incubation. FG35 was the only isolate that showed good thermotolerance potential at 45^{o}C after 48hours. The ability of yeasts to tolerate ethanol is important for industrial use in ethanol production (Lei *et al*., 2007).Ethanol tolerance is an essential property of any ethanol producing microbes as ethanol buildup can hardly be avoided during fermentation even though substrate inhibition can be reduced through stepwise addition of substrate (Fakruddin *et al*., 2013).A desirable organism should be tolerant to both alcohol and temperature to be able to withstand industrial conditions.

Molecular identification by the DNA sequencing of the internal transcribed spacer (ITS) 1 and 2 confirmed that the four ethanol tolerant isolates (BN50, FG17, FG27 and P1) were *Candida tropicalis, Diutina rugosa*, *Candida tropicalis and Candida ethanolica respectively* while the only thermo-tolerate isolate (FG35) was identified as *Trichosporon asahii.* Phylogenetic analysis by MEGA 6 revealed that isolate P1 (identified as *C. ethanolica*) was evolutionary distinct from other strain of *Candida ethanolica and* FG 27 (identified as *C. tropicalis*) was also distinct from other strains of *Candida tropicalis.*

C. tropicalis, although an occasional human pathogen, has also been used as industrial scale for the production of ethanol and long chain dicarboxylic acids (Talukder *et al*., 2016). *D. rugosa*

is well known for its biotechnological application in the production of lipases (Leathers and Dien, 2000).

Conclusion

Among all the 10 isolates,BN50, FG17, FG27 and P1 could be useful to producing ethanol at low temperature as they showed good ethanol tolerance ability. FG35 with its thermo-tolerance ability and lower alcohol production capacity can be genetical modified to improve alcohol yield which will make it the most useful for the production of alcohol at industrial scale. The isolates can further be improved on by genetic manipulation via metabolic pathway engineering to direct ethanol production.

Acknowledgement

This research work was funded by the TETFUND Institutional Based Research grant, Nigerian Defense Academy.
(TETF/DR&D/CE/NDA/KADUNA/IBR/2020/VOL. 1).

Competing Interests

Authors have declared that no competing interests exist

References

Ahmad, F., Jameel, A.T., Kamarudin, M.H. (2011): Study of growth kinetic and modeling of ethanol production by Saccharomyces cerevisiae. *Afr J Biotechnol*, 6(81),18842–18846.

Barnett, J.A., Payne, R.W., and Yarrow, D. (2000):*Yeasts: Characteristics and identification*. 3rd edn. Cambridge University Press, UK. ISBN-13: 978-0521573962.

Dien, B.S., Cotta, M.A. and Jeffries, T.W. (2003): Bacteria engineered for fuel ethanol production current status. *Applied Microbial Biotechnology*, 63, 258 – 266.

Dien, B.S., Jung, H.J.G., Vogel, K.P. (2006): Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canary grass and switch grass. *Biomass Bioenergy*, 30,880–891.

Drapcho, C.M., Nhuan, N.P., Walker, T.H. (2008):*Biofuels engineering process technology*. USA: The McGraw-Hill Companies, Inc.

Fakruddin, M., Islam, M.A, Quayum, M.A, Ahmed, M.M., Chowdhury, N. (2013): Characterization of Stress Tolerant High Potential Ethanol Producing Yeast from Agro-Industrial Waste. *AmericanJournal of BioScience*. Vol. 1, No. 2, pp. 24-34. doi: 10.11648/j.ajbio.20130102.11

Isteretant, D., Spiegle, K. (2006): The influence of recombinant systems on the protoplast fusion of Saccharomyces cerevisiae. *J Am Soc Brew Chem*, 46(3),60–64.

Karamoko, D.D.,N'guessan, K.F.,Bouatenin, K.M.J., and Dje, K.M.(2012): The biochemical and microbiological quality of palm wine samples produced at different periods during tapping and changes which occurred during their storage. *Food Control*, 26(2),504-511.

Kumar, R.S., Shankar, T., and Anandapandian, K.T.K. (2011):Characterization of alcohol resistant yeast Saccharomyces cerevisiae isolated from Toddy. *Int Res J Microbiol*,2(10),399-405.

Kurtzman, C.P., Fell, J.W. (2006):*Yeast systematics and phylogeny- implications of molecular identification methods for studies in ecology. In: Biodiversity and ecophysiology of yeasts*. The yeast handbook, Springer;. Available from: http://www.ars.usda.gov

Leathers, T.D., Dien, B.S. (2000): Xylitol production from corn fibre hydrolysates by a two-stage fermentation process. *Process Biochem*,35,765–776.

Lei, J.J., Zhao, X.Q., Ge, X.M., and Bai, F.W. (2007): Ethanol tolerance and the variation of plasma membrane composition of yeast floc populations with different size distribution. *Journal of Biotechnology*, 131,270-275.

Lorant, H., Laszlo, M., Laszlo, K. (2006): Production of Trichoderma strains with pesticide poly resistance by mutagenesis and protoplast fusion. *Antonino Van Leeuwenhoek*,89 (4),387–393.

Mukherjee, V., Steensels, J., Lievens, B.(2014): Phenotypic evaluation of natural and industrial *Saccharomyces* yeasts for different traits desirable in industrial bioethanol production. *Applied Microbiology andBiotechnology*, 98,9483–9498.

Nghiem, N.P., Hicks, K.B., Johston, D.B. (2010): Production of ethanol from winter barley by EDGE (Enhanced dry grind enzymatic) process. *Biotechnol Biofuels*,3,8-16.

Nuanpeng, S., Thanonkeo, S., Yamada, M., Thanonkeo, P. (2016): Ethanol production from sweet sorghum juice at high temperatures using a newly isolated thermotolerant yeast Saccharomyces cerevisiae DBKKU Y-53. *Energies* ,9,253-260.

Nwachukwu, I.N., Ibekwe, V.I., Nwabueze, R.N. (2008): Production of high-ethanol-yielding *Saccharomyces cerevisiae* of palm wine origin by protoplast fusion. *Life Sci*, 5(4),64–68.
\

Ocloo, F.C.K., Ayernor, G.S. (2010): Production of alcohol from cassava flour hydrolysate. *J Brew Distil*,1(2),15–21.

Ono, B.I., Ishi, N.,Fujino, S., Aoyama, I. (1991): Role of Hydrogen Sulfide Ions HS- in Methylmercury Resistance in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*,57, 3183-3186

Rajoka, M., Khalid, A., and Ferhan, M. (2005): Kinetic and thermodynamics of ethanol production by a thermotolerant mutant of *Saccharomyces cerevisiae*. *Letters in Applied Microbiology*, 40, 316–21.

Ribeiro, C.A.F., Horii, J. (1999): Potentialities of yeast Saccharomyces cerevisiae for the fermentation of sugarcane juice. *Scien. Agricultural*, 56, 1-9.

Santiago-Urbina, J.A., and Ruíz-Terán, F. (2014): Microbiology and biochemistry of traditional palm wine

produced around the world. *Int Food Resource J*, 21,1261–1269.

Stratford, M. (1992): Lectin-mediated aggregation of yeasts: yeasts flocculation. *Biotechnol. Gen. Engin. Rev*, 10,283-341.

Talukder, A.A., Easmin, F., Mahmud, S.A., and Yamada, M. (2016): Thermotolerant yeasts capable of producing bioethanol: isolation from natural fermented sources, identification and characterization.*Biotechnology & Biotechnological Equipment*, DOI 10.1080/13102818.2016.1228477

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013): MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*, 30,2725–2729.

Thais, M., Guimarães, D.G., Moriel, I.P., Machado, M.T., Cyntia, P. and Tania, M.B. (2006): Isolation and Characterization of Saccharomyces cerevisiae strains of winery interest. *Brazilian Journal of Pharmaceutical Sciences*, 42,119-126.

Ukwuru, M.U., Awah, J.I.(2013): Properties of palm wine yeasts and its performance in wine making. *Afri J Biotechnol*, 12(19),2670–2677.

Walker, L.P., Hii, H., and Wilson, D.B. (2006): Enzymatic hydrolysis of cellulose: An Overview. *BioresourcesTechnology*, 36, 3-14.

White, T.J., Bruns, T.D., Lee, S.B., Taylor, J.W. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: InnisMA,Gelfand DH, SninskyJJ,White TJ (eds) PCR protocols: *a guide to methods and applications.* Academic Press, San Diego, pp315–322.