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Full Length Research Paper

Genetic Profiling of Osmotolerant *Saccharomyces* Yeasts from Tanzania for High Gravity Fermentation in Industry

D. Sumari [1,2]*, K. M. M. Hosea[1] and F. S. S. Magingo[1]

[1]Department of Molecular Biology and Biotechnology, University of Dar es Salaam, Tanzania.
[2]Ifakara Health Institute, Morogoro, Tanzania.

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Naturally occurring yeasts were sought from diverse Tanzanian environments and screened for industrial application. The yeasts were isolated from environments such as traditional brews and wines from various parts of Tanzania. In this regard, a total of thirty yeast isolates were screened for their suitability in Industrial Very High Gravity Fermentation (VHGF). Five of these isolates were able to grow and ferment medium with 40% initial sucrose concentration. Whereby, three were able to grow and ferment medium with 700 g/Litre (70% w/w) initial sucrose concentration. One of the three isolates coded LB2 isolated from a traditional Makonde sorghum brew was able to ferment a medium with initial sucrose concentration of 1000 g/Litre (100% w/w) at 30°C. On the basis of PCR-RFLP of the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA), all the three most osmotolerant isolates were identified to belong to the *Saccharomyces sensu stricto clade*. Phylogenetic analysis of the 650 bp D1D2 domain of the large subunit 26S rDNA of the isolate LB2 clustered this isolate away from the so far known typical osmotolerant yeasts. The fermentation by LB2 isolate of 100% gravity substrate observed in this work is higher than any other encountered in the literatures reviewed.

Key words: Osmotolerant, PCR-RFLP, *Saccharomyces*, traditional brews, very high gravity fermentation (VHGF).

INTRODUCTION

The increasing costs of gasoline and adverse effects of lead gasoline on human and environmental health are causing renewed interest in fermentations to produce ethanol as a renewable energy source (Thomas et al., 2001). The environmental deterioration resulting from the over- consumption of petroleum-derived products, especially transportation fuels, is threatening the sustainability of human society. Ethanol, both renewable and environmentally friendly, is believed to be one of the best alternatives, leading to a dramatic increase in its production capacity (Bai et al., 2008).

Economic of the fermentation process may be improved in various techniques, such as among them, very high gravity (VHG) fermentation has been proposed. It was proposed that VHG ethanol fermentation using medium containing sugar with more than 250 g/l, leads to achieve over 15% (v) ethanol (Wang et al., 1999; Bai et al., 2004a,b). Among many ethanol fermentation technologies, the VHG fermentation is very promising for its industrial application for the increase in ethanol concentration, reduction in the level of contaminating (yield–reducing) bacteria and energy cost (Bafrncova et al., 1999; Bai et al., 2008).

A long-term goal of the brewing industry is to identify yeast strains with increased tolerance to the stresses experienced during the brewing process (James et al., 2008). The dynamic behavior of the continuous ethanol fermentation under high gravity or very high gravity conditions has been neglected, which needs to be addressed in order to further increase the final ethanol concentration and save the energy consumption (Bai et al., 2008).

Ethanol is a typical primary metabolite whose production is tightly coupled with the growth of yeast cells,

*Corresponding author. E-mail: dsumari@ihi.or.tz.

indicating yeast must be produced as a co-product. Conventional industrial ethanol fermentation is often performed at comparatively low carbohydrate concentration of fermentable sugar (Holoberg and Margalith, 1981; Pigeau et al., 2007). Whereas in order to reduce distillation costs, it is desirable to obtain high ethanol concentration during fermentation, starting with high sugar concentrations.

Previously, very few types of yeasts were known to tolerate sugar concentrations of above 40% and normally at such concentrations, yeasts grew very slowly (Benitez et al., 1983). There were also few other yeasts which were reported to grow and ferment media with up to 50% sucrose (Blieck et al., 2007). Due to the advantages of VHGF, this work aimed at isolating and characterising local yeast strains with such characteristics basing on their genetic features from different traditional brews and wild fruits obtained in Tanzania. Previously, yeast investigation studies carried in this area (Rutaindurwa, 1996; Hosea, 1996) used conventional means to identify yeasts isolates.

To avoid ambiguities associated with conventional taxonomy, molecular genetic approaches were used to characterize yeast isolates in this work. Polymorphisms within specific regions of the best osmotolerant yeasts ribosomal DNA (rDNA) were analysed using a combination of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) techniques. Furthermore, we described optimal parameters for the industrial VHG fermentation using one of the yeast isolates.

MATERIAS AND METHODS

Sources of the yeast strains and isolation medium

Traditional brews, wines and wild fruits were collected from Morogoro, Tanga, Mbeya, Iringa, Dodoma and Dar es Salaam city, where these brews were sources of the yeast strains. The wild fruits and brews from up country were immediately inoculated on malt extract agar slants in the field. Those from Dar es Salaam were collected in small bottles and brought to the laboratory, where they were stored at 4˚C before inoculated on isolation medium. The malt extract agar medium used in this work was prepared according to Van der Walt and Yarrow (1984).

Screening yeast isolates for Osmotolerance

Screening of the ability of fermenting higher sugar concentration, started with an initial medium with 40% sucrose concentration as prepared according to Bertolini et al. (1991). The medium contained the following: 40 Sucrose (400 g/L), 0.3 yeast extract (3 g/L), 0.5 Peptone (5 g/L), 0.1 K_2HPO_4 (1 g/L), 0.025$MgSO_4.7H_2O$ (0.25 g/L) and 0.01% $CaCl_2$ (0.1 g/L).

During screening process, each yeast isolate was cultured overnight in a liquid medium flask having 40% sucrose at 30 ˚C. Fermentation ability of a yeast isolate was monitored in terms of amount of carbon dioxide evolved. An isolate that did the best in terms of osmotolerance under this test, was further studied for optimizing VGH fermentation parameters.

VHG fermentation by the Osmotolerant yeast isolate from "Libeneke" brew under different pH and temperature conditions

Two parameters namely, pH and sugar concentration were studied during the optimization of VHG fermentation parameters. To start with, pH of the culture medium for culturing the osmotolerant yeast was varied from 4.0, 4.5, 5.0, 5.5 and 6.0 while the sucrose concentration was maintained at 20% and temperature was maintained at 30ºC. The culture medium in the fermentation flasks had 20 sucrose (100 g/500 ml), 0.3 Yeast extract (1.5 g/500 ml), and 0.5% peptone (2.5 g/500 ml), and incubation was for up to 96 h. In terms of varying the sugar concentration, this was by progressively increasing the initial sucrose concentration of 40, 50, 80, 90 to 100%, with pH being maintained at 5. Again the cultures in the flasks were incubated at 30ºC for up to 96 h.

Fermentation process was monitored in terms of carbon dioxide evolution and amount of sucrose remaining after every 24 h of incubation. The amount of remained sucrose was measured using laboratory sugar refractometer (Brix) and loss in weight indicated the amount of CO_2 evolved.

Genetic characterization of osmotolerant yeast isolates

DNA extraction

DNA was extracted using a protocol from de Barros Lopez et al., (1998) with some modifications. This method involved physical grinding of yeast biomass and treated with an extraction buffer containing SDS (5%) at 65ºC. DNA was then purified and precipitated using chloroform, isopropanol and 75% ethanol as described in standard protocols (Sambrook et al., 1989). After which DNA was suspended in Tris EDTA (T.E) buffer and stored at –20ºC until use.

Polymerase chain reaction (pcr)

DNA amplification was done by PCR method using two primers (ITS 4 and 5) targeting the ITS region of the DNA template (Figure 1). Primers sequences were:

ITS 4 5'-TCCTCCGCTTATTGATATGC-3' as the sense primer and
ITS 5 3'-GGAAGTAAAAGTCGTAACAAGG-5' as the antisense primer.

Amplification was carried out in a final PCR volume of 25 µl using manufactured master mix. The reaction mixture consisted of 1 µl of yeast DNA template, 1µl ITS 4 and 1 µl ITS 5 primers. The PCR conditions were performed in 35 cycles: 96ºC for 5 min, 96ºC for 1 min. 55ºC for 1 min, 72^{O}C for 2 min and 72ºC for 10 min.

Each PCR amplicon was resolved on 1.5% agarose high resolution, stained with 5 mg/ml ethidium bromide and visualised under UV light and photographed. A 100-bp DNA Ladder was used as a maker (GIBCO BRL) to serve as the size standard.

Restriction analysis

For this analysis, PCR products were digested with HAE III (5'GGvCC3') and ALU I (5'AgvCT 3') restriction enzymes for 1 h as described by Jespersen et al. (2000). The restriction reaction set up was for a 50 µl mixture, which contained the following: 5 µl of 10x restriction buffer (this depended on the restriction enzyme, either ALU I or HAE III) in a tube; 5 µl of 10x bovine serum albumin (BSA); 10 µl of PCR products; and 1 µl of restriction enzyme (either ALU I or HAE III) and 29 µl of sterile water. The tubes were mixed well

Table 1. Fermentation ability of a yeast isolates at 40% initial sugar concentration as monitored in terms of carbon dioxide evolution.

Yeast code no.	Area-sample collected	CO_2 evolved (grams)	Comment
1	R	2.16	Vigorous fermentation
2	ML1	1.75	Fermentation noted
3	Soni 5	1.90	Fermentation noted
4	Soni b	1.86	Fermentation noted
5	Saccharo-ref. yeast	1.95	Fermentation noted
6	Mamboleo	1.56	Fermentation noted
7	Ml 11	1.69	Fermentation noted
8	Kilwa (b)	1.64	Fermentation noted
9	MR 11	2.15	Vigorous Fermentation
10	MR1	2.15	Vigorous Fermentation
11	Mbagala	1.82	Fermentation noted
12	Amboni	2.69	Vigorous fermentation
13	Lusanga	1.67	Fermentation noted
14	Mlalakuwa 1	1.31	Fermentation noted
15	Mlalakuwa 2	2.17	Vigorous Fermentation
16	CY1	0.46	Low Fermentation
17	CY2	0.49	Low Fermentation
18	CY3	0.28	Low Fermentation
19	Ulanzi	1.34	Low Fermentation
20	Kimbumu	1.00	Low Fermentation
21	Komoni	0.78	Low Fermentation
22	Dendelua	0.66	Low Fermentation
23	Musabe	0.35	Low Fermentation

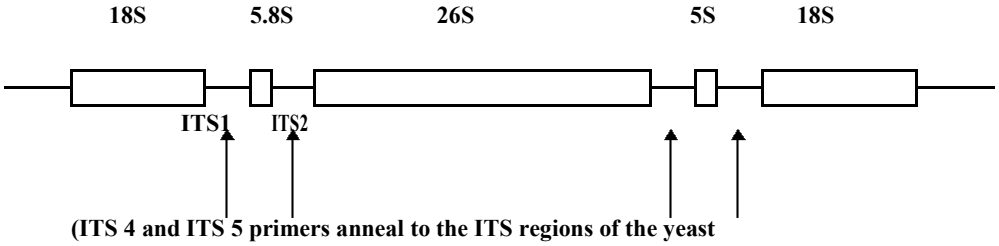

Figure 1. Schematic representation of the fungal rRNA gene ITS (Internal Transcribed Spacer) (Source: Sugita and Nikashiwa, 2003).

and then incubated at 37^{O}C for 1 h for. The restriction fragments were then visualize under UV light and photographed.

RESULTS

Isolation and screening yeast isolates for osmotolerance

A total of 30 yeast isolates from up country obtained from local brews were isolated. These isolates were inoculated in a medium which contained different initial sugar concentrations to assess their fermentation ability. Fermentation ability of different yeast isolates at 40, 55, 60 and 70% initial sugar concentration as monitored in terms of carbon dioxide evolved (Tables 1, 2, 3 and 4, respectively). At all three initial sugar concentrations of 55, 60 and 70%, it was the yeast isolate from the local brew "libeneke" that had the best fermentation ability.

Fermentation capacity of the osmotolerant yeast isolate from "Libeneke" brew under different pH and temperature conditions

With an initial sugar concentration of 20%, the osmotolerant yeast isolate from "Libeneke" brew, had the best fermentation ability at pH 5.0, when the incubation temperature was 30ºC (Figure 2). At the same conditions of pH and temperature, yeast isolates from "Libeneke"

Table 2. Fermentation ability of yeast isolates at 70% initial sugar concentration.

Code	Yeast source	CO_2 evolved (grams)	Sugar consumed (%)
PW1	Kimara	2.33	38
MB2	Kinondoni	1.72	38
UL3	Ulanzi-Iringa	1.71	34.4
LB2	Manzese	2.50	40
UG2	Ugimbi-Songea	0.25	30
PW6	Morogoro	2.34	40

Key: MB – Mbege (Banana wine); PW – Palm wine; LB – Libeneke; UG – Ugimbi UL – Ulanzi.

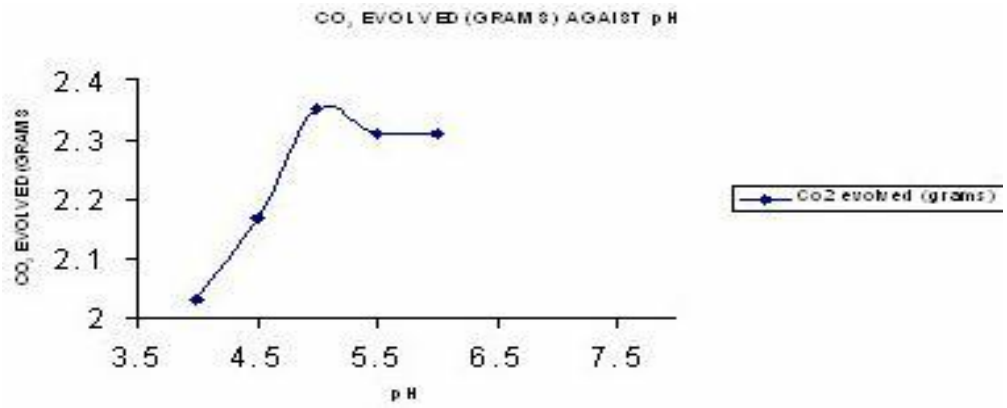

Figure 2. Fermentation capacity of the Osmotolerant yeast isolate from "Libeneke" brew under different pH conditions 20% sugar concentration and 30˚C.

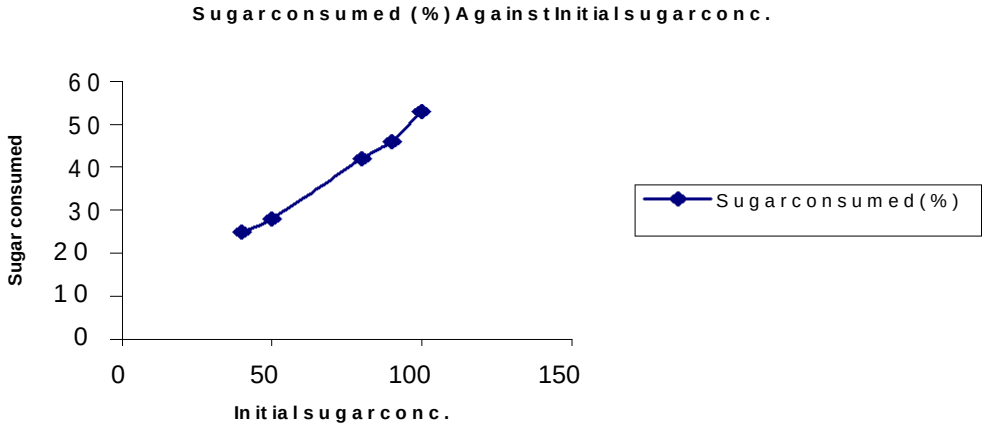

Figure 3. Fermentation capacity of the Osmotolerant yeast isolate from "Libeneke" brew at different initial sugar concentrations at pH 5.0 and 30˚C.

brew increased alcohol production with an increase in initial sugar concentration (Figure 3).

Genetic characterization of the osmotolerant yeast isolates

DNA was successfully isolated from all the three yeast isolates, which showed osmotolerance to high sucrose concentration and DNA amplicon results gave bands of interest with similar size between 800 - 900 base pairs (bp). The bands were clear and appeared to be the same. The size was determined using lane 1 which is 100 bp molecular weight markers (Figure 4). PCR products obtained were digested using endonuclease Hae III for further characterization (Figure 5). Lanes 2, 3 and 4 showed three fragments after digestion with the size ranging from 150 - 400 bp, while that of lane 5 was

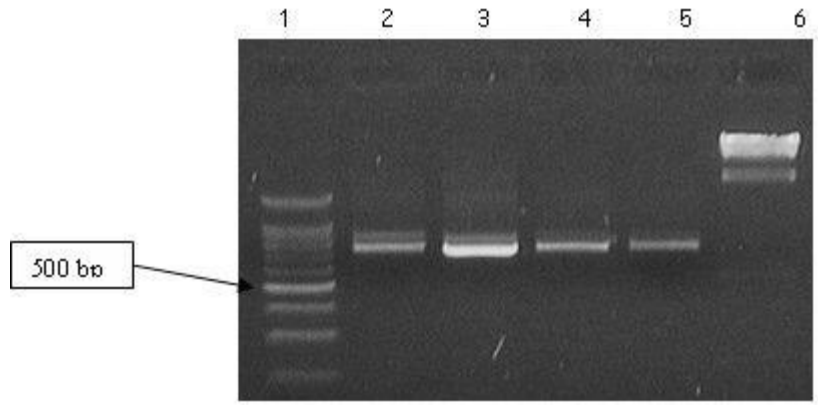

Figure 4. Amplification of the ITS region of the DNA of the Osmotolerant isolates LB1, LB2, and LB3 from "Libeneke" brew. Key: Lane 1: 100 bp DNA marker, Lane 2: Palm wine yeast- Kimara (LB1), Lane 3: Libeneke yeast (LB2), Lane 4: MR1 Palm wine yeast-Morogoro (LB3), Lane 5: Control S*accharomyces cerevisiae* (LB4), Lane 6: Lambda marker.

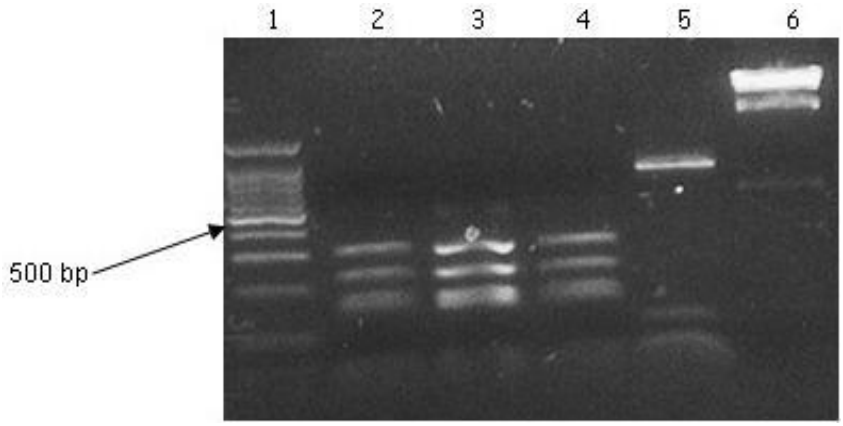

Figure 5. Restriction digestion results of PCR products with Hae III endonuclease of the DNA of the Osmotolerant yeasts from "Libeneke" brew. Lane 1: 100 bp DNA molecular weight marker, Lane 2: Palm wine yeast- Kimara (LB1), Lane 3: Libeneke yeast (LB2), Lane 4: MR1 Palm wine yeast-Morogoro (LB3), Lane 5: Control S*accharomyces cerevisiae* (LB4), Lane 6: Lambda marker

not digested. Furthermore, another restriction enzyme Alu I was used to clearly have specific molecular characterization of anonymous yeast isolates from local brews. After digestion, two fragments as seen in lanes 2 and 3 which appeared to be seen clearly than in lane 4 and 5 (Figure 6).

DISCUSSION

Yeasts from 30 different sources have been isolated in this study. Fermentation ability of yeast isolates from local brews was tested starting with an initial high sugar concentration of 40%. Out of 30 isolates, only five strains (Table 1) proved to have higher fermentation yield than others. When these and other strains tested for ability to carry out fermentation of cultures with even higher initial sugar concentrations (100% v/v), it was only isolates from "Libeneke" brew that could give the highest fermentation results, followed by yeast isolates from palm wine from Morogoro and Kimara (Table 2). Fermentation process of local brews in Tanzania is done locally and in actual fact it utilizes wild yeast from the environment which in particular the ingredients and brewing process of "Libeneke" brew, naturally selected for the osmotolerant yeast:

(i) Code number 1 - 13 - yeast strains from palm wine obtained from Morogoro and Tanga.
(ii) Code number 14 – yeast strains from Mbege local

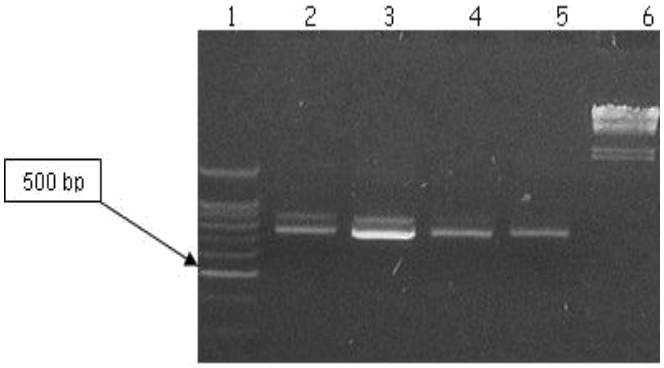

Figure 6. Restriction digestion results of PCR products with Alu 1 endonuclease of the DNA of the osmotolerant yeasts from "Libeneke" brew. Lane 1: 100 bp DNA maker, Lanes 2-5: LB1, LB2, LB3, LB4 and Lane 6: Lambda maker.

banana wine) from Mlalakuwa Dar es Salaam.
(iii) Code number 15 - yeast strains from palm wine obtained from Mlalakuwa Dar es Salaam.
(iv) Code number 16 - yeast strain from choya with honey from Dodoma.
(v) Code number 17 - yeast strain from choya without honey from Dodoma.
(vi) Code number 18 - yeast strain from choya flower from Dodoma.
(vii) Code numbers 19 - 23 - Yeasts trains from Mbeya and Iringa.

Various yeast isolates were reported to have the capacity of performing VHG ethanol fermentation of solutions having only up to 50% initial sugar concentration (Backhus et al., 2001; Blieck et al., 2007). The yeast isolate LB 2 from "Libeneke" brew of Manzese in Dar es Salaam city which surpassed all yeast isolates so far, as it could ferment very well, solutions with 100% initial sugar concentration. Based on this fact, the time this study was conducted, to the best of my knowledge there was no published work reporting on this finding. In this regard, the yeast isolate LB2 characterized in this work, tolerated much higher sugar concentration than yeast isolates reported by other studies at optimal pH of 5.

Results from PCR and RFLP of genomic DNA from the yeast isolated from the local brews indicate a closer genetic relationship with the most common industrial brewing yeast *S. cerevisiae*. The amplification of the 800-900 bp DNA fragment indicated that the isolates just like *S. cerevisiae* belong to the brewing yeast group "*Saccharomyces sensu stricto".* However, further characterization of the PCR products by using two restriction enzymes Hae III and Alu I revealed that the isolates were a bit different from *S. cerevisiae* since one enzyme (Hae III) failed to recognize its specific area to digest as it occurred in the other isolates (Figure 5).

The approach used in this study has been used before in a taxonomic study carried out for isolates of yeast strains identified as contaminants (wild yeast) in 24 different breweries, with reference to the current taxonomy. All isolates were found to belong to the *Saccharomyces cerevisiae stricto* complex although some of these isolates were previously identified as *S. cerevisiae* by Hae III restriction digest of PCR - amplified intergenic transcribed space (ITS) regions (Jespersen et al., 2000).

The suitability of the primers used in this study support the theory put in the beginning that, a combination of PCR and Restriction Fragment Length Polymorphism (RFLP) of the ITS region can be used to rapidly and accurately associate and discriminate yeasts from the *genus* Saccharomyces that could not be differentiated on the basis of phenotypic characteristics (Masneuf et al., 1998; De Barros lopes et al., 1998; Yamagishi et al., 1999).

The Osmotolerant yeast strains isolated from the traditional brews, which were very similar to the typical brewing yeast *S. cerevisiae,* had the potential for use in VHG industrial fermentations to produce substantial amount of alcohol. In having more than one types of yeast strains that can show similar or even more fermentation ability than the typical industrial brewing yeast *S. cerevisiae*, this will give opportunity to the brewing industries and other energy production industries to have an alternative source of energy production in low developed countries.

In this study, only few Tanzanian local brews and wines have been evaluated as sources of new yeasts. It is envisaged that, more comprehensive studies on the same line can be of paramount importance in identification of more osmotolerant yeasts capable of VHG fermentation for local and industrial use in the rest of the Tanzanian local brews and wines which were not screened in this study. Therefore the role of fermenting yeasts from other local brews that can be of a great importance in VHG industrial fermentation remains to be confirmed since these findings provide important baseline for further studies.

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