Isolation and Characterisation of Yeasts and Bacteria From *Mbege*- An Opaque Beer Made From Millet Malt and Banana Juice

[1]Shayo*, N.B., S.A.M. Nnko[1], and A.B. Gidamis[1]

[1]Department of Food Science and Technology, Faculty of Agriculture, Sokoine University of Agriculture, P.O.Box 3006, Chuo Kikuu, Morogoro, Tanzania

Abstract

*The micro-organisms involved in the traditional fermentation process of "mbege", an indigenous Tanzanian alcoholic beverage prepared from malted millet and banana juice were isolated and identified. **Saccharomyces cerevisiae** and **Lactobacillus plantarum** were identified as the yeast and bacteria responsible for the fermentation process respectively. The main spoilage micro-organism of the mbege was identified as of **Acetobacter species**. A diverse range of micro-organisms were also identified in the millet malt. The coliforms were the predominant ones followed by pseudomonads, lactic acid bacteria (LAB) and yeasts. The presence of high numbers of coliforms ($1.98 \pm 0.20 \times 10^7$ c.f.u. g^{-1}) in millet malt poses no health risk as they are destroyed during porridge preparation. In addition, the low pH (pH 4.5) of "mbege" inhibits the growth of coliforms and other pathogenic bacteria. The diverse range of micro-organisms found in millet malt was an indication of unhygienic handling of the millet malt.*

Keywords: Characterisation of micro-organisms, Fermentation and spoilage micro-organisms, Isolation, Opaque beer, Traditional brewing

Introduction

Traditional processing of cereal based alcoholic beverages is wide spread in Tanzania. One of such product is *mbege* which is produced by farmers in the north eastern part of the country, from malted millet and ripe banana fruit (Shayo, 1993). The product is of immense nutritional, social and economic significance to the people in the areas, but its production has not been commercialised widely.

The qualities demanded of lager beers and their production methods are well known and have been a subject of much investigation (Haggblade and Holzapfel, 1989; Novellie, 1982; Priest and Campbell, 1988). However, the same cannot be said of traditional opaque beers whose production is uncontrolled and involves a spontaneous process resulting in quality variations from batch to batch. The main stages carried out in the controlled brewing process of conventional beer such as wort boiling, deliberate inoculation with yeast strains (pitching) and subsequent maturation are not practised in the production of traditional opaque beers.

Most of the traditionally made cereal based beers have poor keeping qualities. The shelf life of these beers does not exceed 2-3 days (Dirar, 1978; Iwuagwu and Izuagbe, 1985 and Tisekwa, 1986). This has been cited as the major problem limiting their commercial exploitation. The brewing process of *mbege* is based on empirical knowledge gained after many years of experience and very little information exists in the literature on the production and microbiology of this type of beer.

The present study was therefore undertaken to isolate and identify micro-organisms from the millet malt and characterise those involved in the fermentation process of *mbege*.

*Corresponding author

Material and Methods

Preparation of the sample

Millet malt and *mbege* were purchased from a local maltster and brewer respectively, in Moshi Town, Tanzania. *Mbege* was aseptically put into sample bottles, packaged in cool boxes (4 °C) and air freighted to U.K. for microbiological analysis.

Isolation of the micro-organisms

Samples of 50 g millet malt or 50 ml. *mbege* were placed in 450 ml. of quarter strength Ringer's solution (¼-R) in a stomacher bag and homogenised for 60 seconds with a stomacher (Lab Blender 400, Seward Medical, London, England) at room temperature. Serial dilutions of samples of millet malt and *mbege* were made in quarter strength Ringer's solution (¼-R). Following the serial dilutions, total viable counts of the samples were determined by spreading 0.1ml of appropriate dilutions on Plate Count Agar (PCA) and incubated at 30 °C for 24-48 hours. Lactic acid bacteria (LAB) were enumerated by spreading 0.1ml of the appropriate dilutions on deMan Rogasa Sharpe (MRS) agar and incubated anaerobically at 30 C for 48 hours. Total coliforms were determined using Violet Red Bile Agar (VRBA) incubated at 30 °C for 24 hours. Pseudomonad were determined using Pseudomonas Base Agar (PsBA) incubated at 30 °C for 48 hours. Yeasts were determined using acidified Malt Extract agar (MEA), Rose Bengal Chloromphenicol agar (RBC) and Oxytetracycline Glucose Yeast Extract agar (OGYA) incubated at 25 °C for 5 days. Yeast isolates were purified by repeated replating on Yeast extract glucose (YG) medium and incubated at 25 °C for 5 days. Stock cultures were maintained at 4 °C on chalk agar slopes. Working cultures were prepared by growing at 25 °C in CBS broth (10 ml) which comprised (g/l): dextrose, 40; bacteriological peptone, 5; yeast extract, 5; adjusted to pH 5.5 with 1 mol/l HCl. All the culture media were purchased from Oxoid.

Identification of yeasts

The procedure of Ison (1987) was followed in identifying the yeasts. A small volume (0.2 ml.) from a 2-3 days working culture was transferred to fresh CBS broth (10 ml.) and incubated for further 15-18 hours. A volume of 3 ml. was filtered through a 0.45 m pore size acetate filter and the cells washed off into 10 ml. of sterile distilled water to give slight turbidity. One ml. of cells was used to seed 50 ml. of the API INOC medium (Oxoid) which had previously been melted and cooled. It was vigorously swirled to give an adequate dispersion of the culture. The API 50 CH galleries were inoculated immediately with the seeded API INOC medium using a sterile Pasteur pipette. The wells were filled to just below the rim. After the medium had set, the cupules were covered with sterile paraffin oil and incubated anaerobically at 25 C for 7-8 days.

The growth response (assimilation of carbohydrates) in the API 50 CH wells was scored as follows: (-) no growth; (+) either growth with no acid production or growth with strong acid production, both within 7 days; (D) as for (+) but after 7 days; (W) either poor growth, no acid production or poor growth and weak acid production (indicator green).

Texture and shape of colonies were recorded after 3 weeks incubation at 25 °C on Malt Extract Agar (MEA) acidified with 10% lactic acid (Dillon et al; 1991). Formation of pseudo-hyphae, true mycelium and arthrospores was determined by direct examination with microscope on Dalmau type plates on Corn Meal Agar (CMA; Oxoid) after 10 days incubation at 25 °C (Van der Walt and Yarrow, 1984). Vegetative production, cell size and shape were examined in Yeast Extract- Malt Extract (YM) broth after 2 days incubation at 25 °C (Anon; 1986) and in 5% MEA cultures after 3 days incubation at 25 °C (Van der Walt and Yarrow, 1984). Water suspensions were made of yeast cultures from CMA, 5% MEA and from slopes of Potassium Acetate agar (Anon; 1986) after 21 and 28 days incubation at 25 °C and examined with microscope for ascospore formation. Heat fixed preparations were stained by steaming with 5% (w/v) safranin (Van der Walt and Yarrow; 1984) to confirm the presence /absence of ascospores.

Identification of Lactic acid bacteria

Typical colonies picked from countable plates of MRS plates were gram stained and presumptive LAB colonies were aseptically transferred into MRS broth. These were incubated at 42 °C for 24 hours after which they were re-streaked on MRS agar. Colonies were examined for their gram and catalase reactions and sporulation behaviour as well as oxygen demand. Pure cultures were preserved in All Purpose Tween (APT) semi solid medium and stored at 4 °C for a maximum of 3 months. Stock cultures in APT semi solid medium were sub-cultured twice in MRS broth and then plated on MRS agar. The plates were incubated anaerobically at 42 °C for 24 hours. Inoculum was prepared from a bacterial culture with a sterile swab that was then suspended in API 50 CHL medium. The CH strip was inoculated by filling the tubes with API 50 medium and overlaying all tests with mineral oil. The strips were placed in plastic incubation tray and incubated anaerobically at 30 °C for 48 hours.

Enrichment and isolation of Acetobacter spp.

Serial dilutions were prepared from *mbege* which had been stored for 21 days and 0.1 ml. of 10^1 to 10^5 dilutions were plated on Glucose Yeast Extract (GYC) standard medium and Frateur medium. These were verified as acetic acid bacteria by gram staining. Colonies were transferred into the enrichment medium using a sterile wire loop. These were incubated at 30 °C for 24 hours after which they were streaked on either GYC or Frateur medium. Colonies were examined to see whether they belonged to the *Acetobacter* group: (gram positive, catalase positive, oxidase negative, non-spore forming and obligately aerobic) (Carr, 1968). Pure cultures of Acetobacter were kept at 4 C in a cold room on the GYC medium and transferred monthly.

Results and Discussions

The two yeast isolates coded A and B from *mbege* were identified as *Saccharomyces cerevisiae* by the AFRC Institute of Food Research Laboratory, Norwich, UK. The isolates were confirmed by comparing them with strains of *Saccharomyces cerevisiae* (NCYC 354, NCYC 609) which were obtained from the National Collection of Yeast Culture- UK. The yeasts were characterised macro- and micro- morphologically and by fermentation and assimilation tests (Table 1 and Plate 1).

Surprisingly, only one species of yeast was isolated from *mbege*. It is possible that the species *Saccharomyces cerevisiae* isolated in this study had become adapted to growth in the special condition of *mbege*. Johansen (1972) who observed that there were varied yeast floras in African traditional beers, with *Saccharomyces cerevisiae* predominating reported similar results. The natural microflora of Tanzanian millet malt consisted of a diverse range of microorganisms (Table 2). The total microbial population recovered on Plate Count Agar (PCA) was 2.85×10^7 c.f.u. g^{-1}. Included in the microflora were bacteria such as coliforms and pseudomonad that are probably of no significance in the fermentation process of *mbege*. However, members of the genus *Zymomonas* of the family Pseudomonadacea are known to carry out an alcoholic fermentation of glucose similar to that of yeast (Salle, 1974).

Coliforms as compared to other micro-organisms were predominant in the microflora (1.98×10^7 c.f.u. g^{-1}). Lactic acid bacteria, which are mainly involved in the fermentation of African traditional beers (Novellie, 1982) were recovered at a level of 1.75×10^5 c.f.u. g^{-1}. The numbers of yeast isolated was 6.0×10^3 c.f.u. g^{-1}. The presence of such high numbers of coliforms in the malt was indicative that proper hygienic conditions were not observed during malt preparation. Traditionally, the grain is spread on open floors and allowed to germinate. Subsequent sun drying is carried out by spreading the grain onto mats placed on bare ground. In this way it is obvious that the malt becomes contaminated with the coliforms that are normally resident in the soil and dust. Similar observations have been reported by Efiuvwevwere and Akoma (1997). In their account cereals are not only contaminated in the field and after harvest but also traditional practices of preparing the malt on the open floor results in

Table 1: Carbohydrates assimilated by yeasts and lactic acid bacteria

Sugar	NCYC354	NCYC 609	Mbege	Malt	LAB
Glycerol	W	-	-	-	-
Erythritol	D	-	+	-	-
D-Arabinose	-	-	-	W	-
L-Arabinose	-	-	-	+	+
Ribose	-	-	-	+	+
D-Xylose	-	-	-	+	-
L-Xylose	-	-	-	W	-
Beta-methly-xyloside	W	-	-	-	-
Galactose	+	+	+	+	+
D-glocose	+	+	+	+	+
D-fructose	+	+	+	-	+
D-mannose	+	+	+	+	+
Rhamnose	-	W	-	-	-
Inositol	-	-	-	W	-
Mannitol	-	-	-	-	+
Sorbitol	-	-	-	-	+
alpha-methly-D-glucoside	+	-	-	-	+
N-Acetyl-Glucosamine	-	-	-	-	+
Amygdaline	+	-	+	-	-
Arbutine	-	+	-	D	-
Esculine	+	+	+	D	-
Salicine	-	D	-	-	-
Celloboise	-	D	-	-	+
Maltose	+	+	-	W	+
Lactose	-	-	-	+	+
Melibiose	+	-	-	W	+
Saccharose	+	-	+	-	-
Trehalose	+	+	+	-	-
Melezitose	+	-	-	-	+
D-raffinose	W	-	-	-	+
Glycogene	-	-	-	W	-
Beta-gentiobiose	-	-	-	+	-
D-turanose	+	W	+	-	-
D-lyxose	-	-	-	+	-
D-fucose	-	-	-	+	-
Gluconate	-	-	-	-	+

Key:
+ Growth with no acid production or growth with strong acid production within 7 days
- No growth
W Poor growth with no acid production or poor growth and weak acid production
D Growth with no acid production or growth with strong acid production after 7 days

higher microbial loads and greater microbial diversity. Despite the high numbers of coliforms in the malt, they do not pose a high health risk. This is because during the subsequent porridge (mash) preparation they are destroyed by boiling. The recontamination during the subsequent processes of *mbege* preparation is normally checked by the acid fermentation. LAB has the ability to exert a wide spectrum of antagonistic effects against gram-negative and gram-positive micro-organisms which is attributed to the production of bacteriocins and simultaneously increasing the acidity (DeVuyst and Vandamme, 1995). At low pH of pH 4.5 as found in *mbege*, growth of undesirable bacteria is inhibited and their spores do not germinate (Nout, 1992;

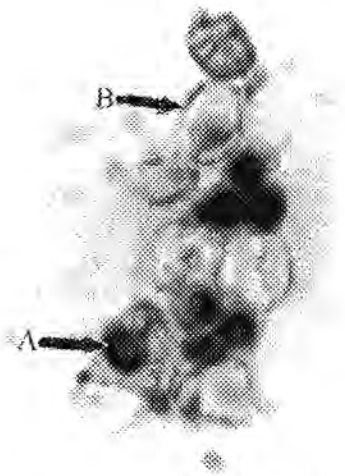

Figure 1: Ascospores and pseudohyphae of yeast

galactose, glucose and mannose whereas fructose was fermented by all the yeast isolates with the exception of the one isolated from millet malt (Table 1). However, according to Dillon and Board (1990) *Saccharomyces cerevisiae* and other wine yeasts assimilate only a few carbohydrates. Additional biochemical tests were set up in conjunction with conventional methods as suggested by Dillon et al., (1991). All the yeasts tested, grew at 37 °C (Table 3) but did not grow on vitamin free medium. All the yeasts tested exhibited growth in 50% glucose. Urease activity and starch production were not observed in any of the yeasts. Extensive and poorly differentiated candida type pseudomycelium was observed on Dalmau type plates of Corn Meal Agar (CMA). Ascospore formation

Table 2: Number of micro-organisms isolated from Tanzanian millet malt

Medium	Type of micro-organism	c.f.u.[a]g^{-1}*
PCA	Total plate count	$2.85 \pm 0.20 \times 10^7$
VRBA	Coliform count	$1.98 \pm 0.47 \times 10^7$
PsBA	Pseudomonad	$4.63 \pm 0.40 \times 10^6$
MRS	Lactic acid bacteria	$1.75 \pm 0.59 \times 10^5$
RBC	Yeasts	$6.74 \pm 0.12 \times 10^3$
OGYA	Yeasts	$7.40 \pm 0.14 \times 10^3$
MEA	Yeasts	$5.73 \pm 0.21 \times 10^3$

[a]c.f.u Colony forming units

*Each result is the mean with mean range of two independent determinations

Table 3: Characteristics of yeast isolates from millet malt and *mbege*

	Yeast	Yeast		
Characteristic	NCYC 354	NCYC 609	Mbege	Malt
Ascospore	(+)	(+)	(+)	(+)
Pseudohyphae	(+)	(+)	(+)	(+)
Growth				
Vitamin free	(-)	(-)	(-)	(-)
50% glucose	(+)	(+)	(+)	(-)
37°C	(+)	(+)	(+)	(+)
Urease activity	(-)	(-)	(-)	(-)
Starch production	(-)	(-)	(-)	(-)

Key:
(+) Presence or positive reaction
(-) Absence or negative reaction

Pickerill, 1986).

The yeasts isolated both from the malt and *mbege* were found to assimilate a diverse range of carbon compounds. All the yeasts fermented was observed on Potassium Acetate Agar with all the yeast isolates.

Lactic acid bacteria colonies were white, round and smooth. On gram staining the colo-

nies were observed to be gram positive, straight rods with rounded ends occurring singly or in short chains of two or three cells. The carbohydrate fermentation profile (Table 1) was in agreement with that for *Lactobacillus plantarum*. Kandler and Weiss (1986) reported that *Lactobacillus plantarum* fermented ribose, gallactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, α-methyl-D-glucoside, cellobiose, maltose, lactose, mellibiose, D-raffinose, melezitose and gluconate.

On identifying acetic acid bacteria according to Carr (1968), it was confirmed that all the tests were in agreement with that for *Acetobacter*. The number of *Acetobacter* was compared to those of other micro-organisms after 21 days of storage of *mbege*. The viable counts of *Acetobacter* as determined on GYC and Frateur media were 3.8 10^6 and 4.2 10^6 c.f.u. g^{-1}, respectively. The yeast count was slightly less than that of *Acetobacter*. Gilliland and Lacey (1964; 1966) reported lethal action by an *Acetobacter* on yeasts. They observed that when no *Acetobacter* was present, the yeast followed the normal growth curve. When the ratio of *Acetobacter* to yeast was 1:1 very little growth occurred. When this ratio was 3:1 no growth took place and the yeast cells started to die.

Conclusion

The yeast *Saccharomyces cerevisiae* and bacteria *Lactobacillus plantarum* were isolated and identified as the micro-organisms responsible for the fermentation of *mbege*, a Tanzanian traditional opaque beer. A diverse range of micro-organisms were present in the millet malt from Tanzania with the coliforms dominating. This was a result of unhygienic handling of the millet from field, harvest, preparation of the malt and subsequent storage of the malt. *Acetobacter* was identified as the main spoilage micro-organism of *mbege*.

Acknowledgement

The authors are grateful to AFRC Institute of Food Research, UK, for identifying the yeasts isolated from Tanzanian *mbege*.

References

Anon, 1986. NCYC computer service handbook. Norwich, U.K.

Carr, J.G. 1968. Methods for identifying acetic acid bacteria. Page 1-8 in: B.M. Gibbs and D..A. Shapton (Eds.). Identification Methods for Microbiologist. Academic Press, London, New York.

De Vuyst, L. and Vandamme, E.J. 1995. Antimicrobial potential of lactic acid bacteria. Page 91-142 in: L. De Vuyst and E.J. Vandamme (Eds), Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and applications. Blackie Academic & Professional, London.Dillon, V.M. and Board, R.G. 1990. A Review: Yeasts associated with red meats *J. Appl. Bact.* 71:93-108.

Dillon, V.M.; Davenport, R.R. and Board, R.G. 1991. Yeasts associated with lamb. *Mycol. Res.* 95(1): 57 - 63.

Dirar, H.A. 1978. A microbiological study of Sudanese *merissa* brewing. *J. Food Sci.* 43:1683-1686.

Efiuvwevwere, B.J.O. and Akoma, O. 1997. Microbiological studies on a Nigerian maize product, *kwoka*, supplemented with soybean. *Journal of Food Safety* 17: 249-259.

Gilliland, R.B. and Lacey, J.P. 1964. Lethal action by an *Acetobacter* on yeasts. *Nature* 202:727 - 728.

Gilliland, R.B. and Lacey, J.P. 1966. An *Acetobacter* lethal to yeasts in bottled beer. *J. Inst. Brew.* 72:291 - 303.

Haggblade, S. and Holzapfel, W. 1989. Industrialization of African's indigenous beer brewing. Page 191-283 in: K.H.Steinkrans (Ed.). Industrialization of Indigenous Fermented Foods. Marcel Dekker, Inc. NY and Basel.

Ison R.W. 1987. A revised method for application of API 50 CH carbohydrates kits to yeasts. *Letters in Applied Microbiology* 4:9-11.

Iwuagwu, Y.O. and Izuagbe, Y.S. 1985. Studies on the preservation and bottling of *Oyokpo* a Nigerian beer from millet (*Pennisetum typhoideum*). *J. Appl. Bact.* 59:487-492.

Johansen, E. 1972. Yeast flora composition of acceptable Bantu beers with characteristic flavour. Bantu Beer Unit progress Report. National Food Research Institute, CSIR, Pretoria, South Africa.

Kandler, O. and Weiss, N. 1986. Genus *Lactobacillus Beijerinck 1901, 212 AL* Page 1208-1260 in: P.N.A. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology Vol. 2. Williams & Wilkins, Baltimore, MD 21202, USA.

Nout, M.T. 1992. Accelerated natural lactic fermentation of cereal based formulas at reduced water activity. *Int. J. Food Micro.* 14(4):313-221.

Novellie, L. 1982. Fermentation beverages. Pg 113 in: L.W. Rooney,, D.S. Murty and J. V. Mertin (Eds). Proceedings of the International symposium grain quality. Pantacharu, ICRISAT, A.P. India.

Priest, F.F. and Campbell, I. 1988. Brewing Microbiology. Elsevier Applied. Science. London. Pg. 187-204.

Pickerill, A.T. 1986. The influence of free -amino nitrogen in sorghum beer fermentation. *J. Inst. Brew,* 92: 568.

Salle A.J. 1974. Fundamental Principles of Bacteriology, TM-Hill Publ. Co. Ltd. New Delhi 518 p.

Shayo, N.B. 1993. Studies on the preservation of *Mbege* an indigenous fermented beverage in Tanzania. PhD The-

sis, University of Reading, UK.
Tiisekwa, B. 1986. Traditional beer processing in Tanzania: Development Needs. *African Focus,* 2:299.
Van der Walt, J.P. and Yarrow, D. 1984. Methods for the isolation, maintenance, classification and identification of yeasts. Pg 45-104 in: N.J.W. Kreger-van Rij (Ed.). The Yeasts-a Taxonomic Study. Elsevier Science, Amsterdam pp 45-104.