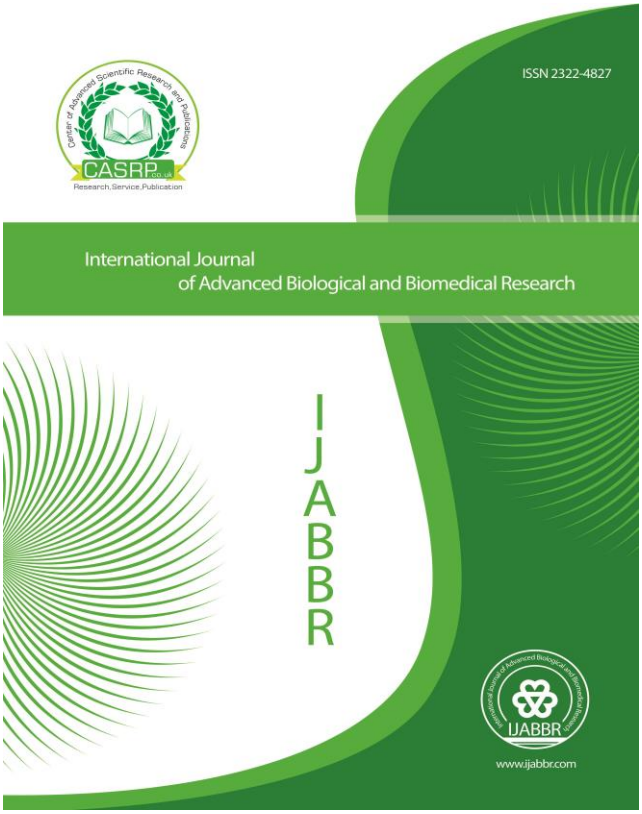

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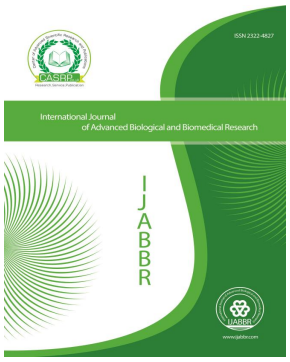
International Journal of Advanced Biological and Biomedical Research 4(3) (2016) 246–252



Original Article Open Access

Isolation and characterization of yeast species from ensete ventricosum product; Kocho and Bulla collected from Angacha district

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Abstract

Kocho and Bula are fermented product of Enset (*Ensete ventricosum*). It is the staple food for 20 million people in Ethiopia. The aim of study was to isolate, identify and characterize yeast species from fermented kocho and bulla by using Biolog Micro station. 300 Kocho samples were collected from Angacha District. 0.1ml of serially diluted samples were Streaked on yeast pepton dextrose agar and incubated at 28^{0}C. Pure yeast colony inoculum were prepared at 9ml distilled water at 49% ±2 turbidometer and transferred in to YT micro plate. Incubated for 24-72 hours at 28^{o}C and micro plate reading were carried out using MicroLog 3 Software version. 4.20.05. Seven yeast species were identified from study samples. Biolog Micro station 100% probability and ≥0.5 Similarity read identify *Cryptococcus albidus Var aerus, Guilliermondella selenospora, Rhodotorula acheniorum and Trichosporon beigelii*. 99% *Cryptococcus terreus* A, 98% *Candida zylandase,* 86% *Kluyveramyces delphensis* respectively. Characterization of yeast involved in kocho fermentation is very important for formulation of starter culture, improving, standardizing and modernizing quality of traditional Enset fermentation and preparation.

Keywords: Bulla, Enseteventricosum, Kocho, Fermentation, Yeast.

1. Introduction

Enset is one of the potential indigenous crops for food production. Almost 20 million people in Ethiopia are dependent on Enset (*Enseteventricosum*) (Pijls et al., 1995). It is grown on 67,000sq.km and 60 mature plants are

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Received 19 November 2016 | Accepted 18 December 2016 | Available online 25 December 2016
iThenticate screening 22 November 2016 | English editing 15 December 2016 | Quality control 21 December 2016

estimated to provide sufficient food for 5-6 people per year (Demeke, 1986). Pseudostem and corm are the source of kotcho and bulla. The pseudostem is also excellent source of fiber used for making ropes, gunny bags, carpets and kotcho squeezing fiber. Enset leaves are used for many purposes: for lining fermentation pits and wrapping kotcho during baking; for making mattress and cushion; for animal feed and fuel (Tedla and Abebe, 1994). Kocho is the bulk of the fermented starch obtained from the mixture of the decorticated (scarped) leaf sheaths and the grated corm (underground stem base). Kocho needs a lengthy period of processing and preparation, which is carried out by women. The first stage involves removing the leaf stalks and grading of the corm. Then the fibers are separated out and the pulp is crushed to extract the starch. This is put in a pit about 1.5 m deep and 1 m diameter, wrapped airtight with enset leaves before being packed down with stones. It is then allowed to ferment a process, which may last anything from 4 months to three years. The pit is opened at intervals to allow aeration, and the enset leaves are replaced. This is repeated until the desired fermentation quality is reached or the food is needed. Finally, the fermented starch is dried and treated as flour. This can be used to prepare a pancake − like bread, which is eaten with milk and cabbage. Kocho can be stored for a long period of time without spoiling.

Bulla is the small amount of water-insoluble starchy product that may be separated from kocho during processing by squeezing and decanting the liquid. After decanting, the bulla is left to dry and fermented in a way similar to kocho or can be directly cooked without fermentation. It is considered the best quality enset food and is mainly from fully matured enset plant (Tedla and Abebe, 1994). Microorganisms are active in Kotcho fermentation for starch hydrolysis, proteolysis and lipolysis in determining of kotcho product, odor, color, flavor and spoilage at all. Abegaz Gashe (1987a) studied and described the microbiology of kocho fermentation. He reported that *Leuconostoc mesenteroide s*intiated the fermentation and dominated the lactic flora with counts of 10^7 cfu(g)-1 on day 8. The pH of the fermenting mass dropped from 6.5 to 5.6 in 8 days. *Lactobacillus coryneformis* and *Lactobacillus plantarum* dominated thereafter and further reduced the pH to 4.2 after 50days. Spore formers were present at level of ($<10^3$cfu(g)-1). During the first 15 days. Generally, the population of Clostridium sp. was two to five times more abundant than Bacillus spp. Yeasts reached their highest counts 10^3cfu(g)-1 between 22 and43 days and the yeast flora consisted of the Trichosporon, Torulopsis, Rhodotorula and Candida species. Ashenafi and Abebe (1996a) studied the microbial load of market kotcho and bulla and found out that high counts of aerobic mesophilic bacteria and yeasts ($>10^6$ cfu/g). Coliform counts were markedly higher in bulla (10^5 cfu/g) than in kotcho (10^3 cfu/g). Counts of enterococci, in both products, ranged between 10^4 and 10^5cfu/g. Micrococci and Bacillus spp. dominated the aerobic bacterial flora. Among the yeast species, *Rhodotorul aglutinis, Kluyveromyces marxianus* and *Pichia membranefaciens* were isolated from most samples.

Yeasts are unicellular, eukaryotic and polyphyletic organisms classified in the kingdom fungi. They are ubiquitous, and commonly found on fruits, vegetables, insect and other plant materials. Some yeast is found in association with soil and water. Approximately 100 genera comprising more than 1500 species of yeast have been described (Kurtzman and Fell, 2006). The significance of yeasts in food technology in a world of low agricultural production and rapidly increasing population makes the production of food grade yeasts extremely important (Bekatorou et al., 2006). In Ethiopia there are several fermented foods such as, kocho, bulla, tella, tej, milk product and injera, etc .A lot of research was undertaken on microbial profile of these commodities through conventional methods. In most cases, strain of *Saccharomyces cervisiae, Rhodotorula* spp, pichia, Lactobacillus spp were found to dominate fermenter in Tella, injera, milk product and other fermented foods. However the yeast species involved in Kocho and Bulla fermentation in Earthen pits are not studied well using standard Biolog Microstation identification technology for shortening fermentation time and selecting potential fermenter yeast in future. Through this gap this study is designed for isolation, identification and characterization of yeast species involved in kocho and bulla fermentation which are very important for formulation of starter culture, improving, standardizing and modernizing of traditional Enset fermentation process through selecting potential fermenter yeast that will help to minimize time and energy needed, enhance quality and quantity of food product and also minimize wastage and related public health problems.

2. Materials and methods

2.1. Study area

Angacha district is one of the six woredas in Kambata Tambaro Zone, Southern Nations, Nationalities and Peoples' Region (SNNPR). It is located about 260 kms south west of Addis Ababa. Angacha is bordered on the south

by KachaBira, on the west by Doyogena, on the north by the Hadiya Zone. It is located at 07°12`47`` East and 38°79`00`` North. The area has an average elevation of 2100m.a.s.l. and it is a potential Enset producing in the area.

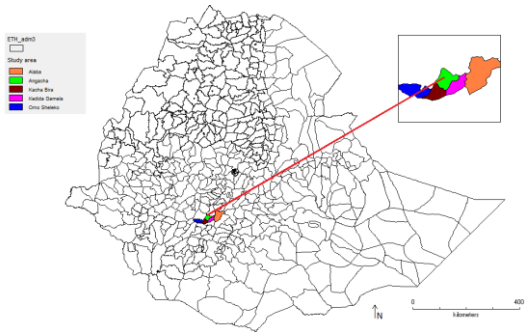

Fig. 1. Map of the study area.

2.2. Sample collection

Three hundred Kocho and bulla samples were collected aseptically from 5 to 25 cm depths in the earthen pits of kocho processing area. Samples of actively fermenting Kocho and bulla were collected from different household sites in KambataTambaro Zone from Angacha district in different Keble, Amberchiwasera,Gubenachafa, Shamimba, Amberchi future,Bucha, keelema, kerekichoshino, Gerbafandedae, Ashena. The samples were obtained from different sites within 2100-25 53m altitude ranges and different stage of fermentation. The sample was immediately transferred into sterile sample tube and transported to the microbial Directorate laboratory at Ethiopian Biodiversity Institute (Fig. 2).

Fig. 2. Left to right 1. Enset (*Enseteventricosum*), 2. Kocho proceesing, 3. Kocho in the earthen pits during fermentation, 4. Kocho ready for food, 5. Kocho Bread, 6. Researcher during kocho sample collection.

2.3. Laboratory work

2.3.1. Pure culture Isolation

Three hundred collected Kocho and Bulla samples were merged into thirty samples according to their fermentation stage in laboratory. From the merged thirty samples 1g was taken from each merged samples and diluted serially up to10^{-6}ml. About 0.1ml of serially diluted sample was transferred by nichrome loop on yeast peptone dextrose agar using streak plate technique. The inoculated plate incubated for 48h at 28 ^{0}C. A single yeast colony was sub cultured on growth media until the Purified cultures were maintained and kept at 4ºC until further analysis.

2.3.2. Identification and characterization of yeast species

Morphological identification and characterization According to the method of Kurtzman and Fell (1998), morphology of the yeast cells was observed based on their cultural characteristics (Colony shapes, size, pigment, elevation, edge and surface appearance were recorded.

2.3.3. Biolog Micro station identification and characterization

The Biolog Micro station system for yeast identification consists of micro plates each containing a 96 well with a range of dehydrated carbon, a multichannel pipetter, a turbidiameter, a computer linked micro plate reader and BiologMicrolog3 software version. 4.20.05. Yeasts were subculture to Biolog Universal Yeast Buy agar (BUY; BiologInc, Hayward, Calif., U.S.A.) and incubated at 26 °C for 24 to 72 h. Pure colony of yeast suspension was prepared in 9ml sterile distilled water and adjusted to49% +/-2 T using Biolog YT turbidiameter. 100 µ Lof inoculums was dispensed to each well of the biolog yeast (YT) Micro Plate and incubated at 26 °C. The YT Micro Plate measures both metabolic reactions as well as turbidity growth to produce identifications. A YT Micro Plate was read by the Biolog Micro Station Reader (BiologInc) at 24 h, 48 h, and 72 h at a single wavelength of 590 nm. The Biolog software micro log3 ver. 4.20.05 compared the results obtained with the test strain to the database and provided identification based on distance value of match and separation score produces similarity index value and probability. An acceptable species identification must have similarity index value≥0.5 or probability ≥75% were Chosen only for species identification and characterization (Biolog, 1993).

3. Results and discussion

3.1. Isolation of yeasts

A total of 450 different yeast colonies were isolated from all collected kocho and bulla samples having different fermentation ages. All yeast colonies having similar morphology were clustered and read by micro station. The yeast isolates were identified based on their colony morphology (pigmentation, shape, size, texture, elevation and margin) were summarized in Table 1.

3.2. Percentage frequency of yeast on growth media

From similar morphology cluster the percentage occurrence on culture media recorded as, 20% *Trichosporon beigelii* B, 16.6% *Candida zeylanoides,* 13.3% *Rhodotorulaacheniorum,* 13.3% *Kluyveramyces delphensis,* 10% *Guilliermondella selenospora*, 6.67% *Cryptococcus terreus* A, 6.67% *Cryptococcus albidus Var aerus*, 6.67% *Filobasidilla neoformans* and 6.67% *Hyphopichia burtoni*. (1) The highest percentage occurrence on culture media was *Trichosporonbeigelii* B (20%), and the lowest occurrence was *Cryptococcus terreus* A, *Cryptococcusalbidus Var aerus, Filobasidilla neoformans,* and *Hyphopichiaburtoni (*6.7%).

Table 1
Morphological characteristics of the isolated yeasts.

Name of organisms	Pigmentation	Colony color, texture, elevation	Cell size
Trichosporonbeigelii B	White	Raised, circular, smooth	Medium
Candida zeylanoides	White	Raised, circular, smooth, shiny	Large
Rhodotorulaacheniorum	Orange red	Raised, Smooth, mucoid to butyrous colonies	Medium
Kluyveromycesdelphensis	Creamy	Flat, Fury	Medium
Guilliermondellaselenospora	Brown	Raised, circular, smooth	Medium
Cryptococcus terreus A	White	Globose to slightly oval with mucous capsules raised, mucoid	Medium
Cryptococcus albidusvaralbidus	Yellowish Creamy	Raised, furrowed, mucoid	Large

3.3. Biolog Microstation identification and characterization

Yeast cell containing YT Micro Plate was read by the Biolog Micro Station Reader (BiologInc) at 24 h, 48 h, and 72 h at a single wavelength of 590 nm. The Biolog software micro log3 ver. 4.20.05 compared the results obtained with the test strain to the database and provided identification based on distance value of match and separation score produces similarity index value and probability. An acceptable species identification must have similarity index value≥0.5 or probability≥75% were chosen only for species identification and characterization. (Biolog, 1993). Therefore Biolog Micro station 100% probability and ≥0.5 similarity read identify *Cryptococcus albidusVar*

aerus, Guilliermondella selenospora, Rhodotorula acheniorum and *Trichosporon beigelii*. 99% *Cryptococcus terreus* A, 98% *Candida zylandase,* 86% *Kluyveramyces delphensis* respectively (Table 2).

Table 2
Biolog Microstation identification result.

Species	Probability	Similarity	Distance	Status
Cryptococcus. albidusvaraerus	100%	0.73	5.98	Identified
Guilliermondellaselenospora	100%	0.653	5.33	Identified
Rhodotorulaacheniorum	100%	0.623	5.78	Identified
Trichosporonbeigelii B	100%	0.615	5.91	Identified
Cryptococcus terreus A	99%	0.693	4.62	Identified
Candida zylandase	98%	0.668	4.87	Identified
Kluyveromycesdelphensis	86%	0.553	5.47	Identified
Hyphopichiaburtoni	0	0.476	8.42	Unidentified
Filobasidillaneoformans	0	0.186	9.39	Unidentified

Kocho and Bulla are starchy foods obtained from fermenting edible part of the leaf sheath and corm of enset plant (*Enseteventricosum* (Welw.), Cheesman). The plant does not produce edible fruit, but its corm and pseudo stem are scraped to separate the starchy pulp from fiber, and the pulp is made to ferment in earthen pits (Abegaz Gashe, 1987a). The length of fermentation time varies from a few weeks, to several months or years depending on ambient temperature of incubation and microbial species involvement (pijls et al., 1995). Hence, in this research the yeast species were Isolated, Identified and Characterized by using Biolog identification techniques, since different microbial species have great role during Enset processing and fermentation. Therefore Isolation, Identification and Characterization of microbes involved in kocho and bulla fermentation are very important for improving, standardizing and modernizing of traditional Enset fermentation process that will help to formulate starter culture, to select potential fermenter, minimize time and energy needed. So far about 1500 yeast species are identified and they are ubiquitous in their distribution and populations mainly depend on the type and concentration of organic materials. The distribution of species, as well as their numbers and metabolic characteristics were found to be governed by existing environmental conditions (Panneerselvam and Maragatham, 2011).

BUY agar was used to culture yeast species from feremented Kocho and Bulla and 9 species were isolated on culture plate, 20% of culture plate was dominated by *Trichosporonbeigelii B,* followed by 16.6% *Candida zylandase,* 13.3% *Kluyveramycesdelphensis, 13.3% Rhodotorulaacheniorum* 10% *Guilliermondella selerospora, 6.67% Cryptococcus terreus A, 6.67% Cryptococcus albidusVaraerus, 6.67% Filobasidillaneoformans, 6.67% Hyphopichiaburtoni.* Morphological and Biolog identification of the yeast isolate from Kocho and bulla indicates diversity of yeast flora. four species*, Cryptococcus albidusVar aerus, Guilliermondella selenospora, Rhodotorula acheniorum* and *Trichosporonbeigelii,* have 100% Biolog probability and $\geq$0.5 similarity index value read, followed by 99% *Cryptococcus terreus A, 98% Candida zylandase, 86% Kluyveramyces delphens*, two species had zero probability read (Table 2).

This results also corresponds to the report of Abegaz Gashe (1987a) suggest that yeasts reached their highest counts (10^3cfu $(g)^{-1}$ between 22 and 43 days and the yeast flora consisted of *Trichosporon, Rhodotorula, Candida, Torulopsis*species*.* Ashenafi and Abebe (1996a) also reported the yeast species Rhodotorula, Kluyveramyces and Pichia species were isolated from most Kocho and Bulla samples*. Guilliermondella and Cryptococcus,* yeast species are new identified by biology micro station in this study, This might be fermentation stage of microbial community difference, Micro environment, Enset cultivar difference of the area or the capacity of biolog identification power this yeast species at its data base. *Filobasidilla neoformans A* and *Hyphopichia burtoni*, have low percentage occurrence on culture plate and Biolog identification probability were 0%. But have 0.186 and 0.476 similarity with reference organism on data base respectively. This may be due to low ability to utilize tagged carbon source on YT micro plate during incubation time. These small similarity result shown by *Filobasidillaneoformans A* and *Hyphopichia burtoni*, may be due to their positive oxidation test. This idea corresponds to Chaskes and Tyndall, (1975). *Filobasidilla neoformans*is unique in its oxidative activity.

Most of the yeast species found in Kocho and Bulla were not pathogenic to human but Casadevall and Perfect (1998) reported that *Filobasidillaneoformans* is functioning as the major virulence factor in Cryptococcal infection and disease. However in our study this pathogenic yeast species has low Biolog probability identification that may not be worrying.

Yeast Micro Plate has two different reactions: assimilation or utilization of carbohydrates and oxidation. Nine yeast species (*G.selerospora, Cryptococcus terreus A,* Kluyveramyces*. delphensis, Cryptococcus albidus Var aerus, Rhodotorula acheniorum, Candida zylandase, T. beigeliiB, F. neoformans and H. burtoni*) have positive oxidative test for Succinic acid, L-aspartic acid, L-glutamicacid, D-gluconic acid, dextrin, cellobiose, gentiobiose acid, maltose, maltotriose, sucrose, N-acetyl-D-D glucosamine, a-D-glucose, D-galactose and tween 80 carbon sources and seven accurately identified yeast species shown positive assimilation test for L-glutamic acid, L-glutamic acid, D- gluconic acid, cellobiose, maltose, a-D-glucose, D-galactose+ D-xylose, D-glucuronicacid+ D-xylose carbon sources.

4. Conclusion

- Seven yeasts *Cryptococcus albidus Varaerius, Guilliermondellaselenospora, Rhodotorula acheniorum, Trichosporonbeigelii B, Cryptococcus terreus A, Candida zylandase and Kluyveromycesdelphensis* were identified from Kocho and Bulla which having fermentation Role.
- All yeast identified from Kocho and Bulla are are non-Saccharomyces yeast.
- Seven yeasts were characterized from Biolog micro station result that L-glutamicacid, D- gluconic acid, cellobiose, maltose, a-D-glucose, D-galactose+ D-xylose, D-glucuronicacid+ D-xyloses were found to be assimilated by these yeast.
- In order to formulate starter culture or to select potential fermenter yeast it is important to Isolate, identify and characterize microbial profile involved in kocho and bulla fermentation for improving, standardizing and modernizing of traditional Enset fermentation process will help to minimize time and energy needed, enhance quality and quantity of food product and also minimize wastage.

Recommendation

- So as to formulate and selecting potential fermenter yeast research must carry out in broad all type of Enset cultivar and in different enset growing area at different altitude ranges and fermentation stage.
- Society traditional knowledge on enset processing and utilization must be collected from different area, that could be a clue for understanding yeast diversity study.
- Traditional Kocho fermentation process in earthen pits is not free from microbial contamination and spoilage, to modernize production process, microbial community study is very crucial. Therefore researcher must work on bacterial and other filamentus fungi fermenters.
- Some of yeast species are pathogenic to human being like *Filobasidilla neoformans*, Candida and Trichosporon except *Trichosporon beigelii* B, which are cosmopolitan (Soil, water, air, and human skin). Therefor aseptic condition and environmental hygiene are recommended during Enset processing time.

Acknowledgement

The Author would like to thank and pleasure to acknowledge Dr. Genene Tefera for his unreserved guidance and encouragement and support in providing and facilitating the nessaccery equipment. And extremely grateful to acknowledge regional, zonal, district and Keble leader in the research area who helped us guiding the study area and finally goes to Ethiopian biodiversity institute for financial support, Microbial directorate and its research team for their for technical and unreserved support.

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How to cite this article: Gizaw, B., Tsegay, Z., Tilahun, B., 2016. Isolation and characterization of yeast species from ensete ventricosum product; Kocho and Bulla collected from Angacha district. International Journal of Advanced Biological and Biomedical Research, 4(3), 246-252.