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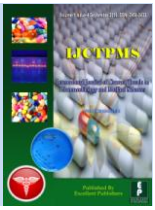

Original Research Article

Fermenter Yeast Identified 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. Three hundred Kocho samples were collected from Angacha District. Serially diluted samples of 0.1ml were Streaked on yeast peptone dextrose agar and incubated at 28°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 hrs at 28°C and micro plate reading were carried out using MicroLog 3 Software version 4.20.05. Seventeen yeast species were read by Micro station. Biolog Micro station 100% probability and ≥0.5 similarity read identity was *Cryptococcus albidus* var. *aerus, Guilliermondella selenospora, Rhodotorula acheniorum* and *Trichosporon beigelii*, 99% *Cryptococcus terreus* A, 98% *Candida zylandase*, 86% *Kluyveramyces delphensis* respectively, and ≤75% probablity, ≤0.5 similarity index recorded *Filobasidilla neoformans, Hyphopichia burtoni, Galactomyces geotrichum, Hanseniaspora valbyensis* Kloecker, *Candida albidus* var. *albidus, Sporobolomyces roseus* and *Debaryomes hanseni* var. *hanesenii.* 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.

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Keywords

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Ensete ventricosum
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Introduction

Enset is one of the potential indigenous crops for food production. Almost 20 million people in Ethiopia are dependent on Enset (*Ensete ventricosum*) (Pijls et al., 1995). It is grown on 67,000 sq.km and 60 mature plants are estimated to provide sufficient food for 5-6 people per year (Demeke, 1986). Pseudo stem and corm are the source of Kocho and Bulla. The pseudo stem is also excellent source of fiber used for making ropes, gunny bags, carpets and Kocho squeezing fiber. Enset leaves are used for many purposes, for lining fermentation pits and wrapping Kocho during baking; for making mattress and cushion; for animal feed and fuel (Mehtzun and Yewelsew, 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, meat 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 (Mehtzun and Yewelsew, 1994). Microorganisms are active in Kocho fermentation for starch hydrolysis, proteolysis and lipolysis in determining of kocho product, odor, color, flavor and spoilage at all. Berhanu (1987a) studied and described the microbiology of Kocho fermentation. He reported that *Leuconostoc mesenteroide s*ituated the fermentation and dominated the lactic flora with counts of 10^7 cfu/g 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 50 days. Spore formers were present at level of ($<10^3$cfu/g). During the first 15 days. Generally, the population of Clostridium species was two to five times more abundant than Bacillus spp. Yeasts reached their highest counts 10^3 cfu/g between 22 and 43 days and the yeast flora consisted of the *Trichosporon, Torulopsis, Rhodotorula* and *Candida* species. Mogessie and Yewelsew (1996) studied the microbial load of market Kocho 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 Kocho (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, *Rhodotorula glutinis, 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 cerevisiae, Rhodotorula* spp., *Pichia* and *Lactobacillus* species were found to dominate fermenter in Tella, Injera, Milk product and other fermented foods and beverage in Ethiopia. However the yeast species involved in Kocho and Bulla fermentation in Earthen pits are not studied well using standard Biolog Micro station 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.

Materials and methods

Study area

Angacha is one of the six Districts in Kambata Tambaro Zone, Southern Nations, Nationalities and Peoples' Region (SNNPR). It is located about 260 km 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 2100 m.a.s.l. and it is a potential Enset production in the area.

Morphological characterization

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 Kambata Tambaro Zone from Angacha district in different Keble, Amberchi wasera, Gubena chafa, Shamimba, Amberchi future, Bucha, Keelema, Kerekicho shino, Gerba fandedae and Ashena. The samples were obtained from different sites within 2100-2553m altitude ranges and different stage of fermentation, 20, 40 and 60 days. The sample was immediately transferred into sterile sample tube and transported to the microbial Directorate laboratory at Ethiopian Biodiversity Institute (Fig. 1).

Fig. 1: Left to right 1. Enset (*Ensete ventricosum*), 2. Kocho processing, 3. Kocho in the earthen pits during fermentation, 4. Kocho ready for food, 5. Kocho bread and 6. Researcher during Kocho sample collection.

Pure culture isolation

Three hundred collected Kocho and Bulla samples were merged in to 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 48 hrs at 28°C. A single yeast colony was sub cultured on growth media until the purified cultures were maintained and kept at 4ºC until further analysis.

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.

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 Biolog Microlog 3 software version 4.20.05. Yeasts were subculture to Biolog Universal Yeast Buy agar (BUY; Biolog Inc, Hayward, Calif., U.S.A.) and incubated at 26°C for 24 to 72 hrs. Pure colony of yeast suspension was prepared in 9ml sterile distilled water and adjusted to49% +/-2 T using Biolog YT turbidiameter. Inoculum of 100 µl 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 (Biolog Inc.) at 24, 48 and 72 hrs 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).

Results

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) (Table 1).

Percentage frequency of yeast on growth media

From similar morphology cluster the percentage occurrence on culture media recorded for *Trichosporon beigelii* B, *Candida zeylanoides, Rhodotorula acheniorum, Kluyveramyces delphensis, Guilliermondella selenospora*, *Cryptococcus terreus* A, *Cryptococcus albidus* var. *aerus*, *Filobasidilla neoformans, Hyphopichia burtoni, Galactomyces geotrichum, Hanseniaspora valbyensis, Candida albidus* var. *albidus*, *Sporobolomyces roseus* and *Debaryomes hanseni* var. *hanesenii* were 16.6%, 14.7%, 13.3%, 13.3%, 10%, 6.67%, 6.67%, 6.67%, 2.32%, 4.35% 3.11%, 3.56%, 1.92% and 2% respectively. The highest percentage occurrence on culture media was *Trichosporon beigelii* B (16.6%) and the lowest occurrence was *Sporobolomyces roseus* (1.92%).

Table 1. Morphological characteristics of the isolated yeasts.

Name of organisms	Pigmentation	Colony color, texture, elevation	Cell size
Trichosporon beigelii B	White	Raised, circular Smooth	Medium
Candida zeylanoides	White	Raised , circular, smooth, shiny	Large
Rhodotorula acheniorum	Orange red	Raised, Smooth, mucoid to butyrous colonies	Medium
Kluyveromyces delphensis	Creamy	Flat, fury	Medium
Guilliermondella selenospora	Brown	Raised, circular, smooth	Medium
Cryptococcus terreus A	White	Globose to slightly oval with mucous capsules raised, mucoid	Medium
Cryptococcus albidus var. *albidus*	Yellowish creamy	Raised, furrowed, Mucoid	Large

Biolog Micro station identification and characterization

Yeast cell containing YT Micro Plate was read by the Biolog Micro Station Reader (Biolog Inc) at 24, 48 and 72 hrs at a single wavelength of 590 nm. The Biolog software Micro Log 3 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. Acceptable species identification must have similarity index value ≥0.5 or probability ≥75% was chosen only for species identification and characterization (Biolog, 1993). Therefore Biolog Micro station 100% probability and ≥0.5 similarity read identified *Cryptococcus albidus* var. *aerus, Guilliermondella selenospora, Rhodotorula acheniorum* and *Trichosporon beigelii;* 99% *Cryptococcus terreus* A, 98% *Candida zylandase* and 86% *Kluyveramyces delphensis* respectively (Table 2).

Table 2. Biolog Micro station identification result.

Species	Probability	Similarity	Distance	Remark
Cryptococcus albidus var. *aerus*	100%	0.73	5.98	Hid
Guilliermondella selenospora	100%	0.653	5.33	Hid
Rhodotorula acheniorum	100%	0.623	5.78	Hid
Trichosporon beigelii B	100%	0.615	5.91	Hid
Cryptococcus terreus A	99%	0.693	4.62	Hid
Candida zeylanoides	98%	0.668	4.87	Hid
Kluyveromyces delphensis	86%	0.553	5.47	Hid
Hyphopichia burtoni	0	0.476	8.42	Lid
Filobasidilla neoformans	0	0.186	9.39	Lid
Galactomyces geotrichum,	0	0.001	29.79	Lid
Hanseniaspora valbyensis Kloecker	0	0.001	32.17	Lid
Candida albidus var. *albidus*	0	0.001	32.25	Lid
Debaryomes hanseni var. *hanesenii*	0	0.001	32.46	Lid
Sporobolomyces roseus	0	0.001	37.77	Lid

Hid=High identification; Lid=Low identification;

Discussion

Kocho and Bulla are starchy foods obtained from by fermenting edible part of the leaf sheath and corm of enset plant [*Ensete ventricosum* (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 (Gashe, 1987). 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, for the purpose of formulating starter culture, and selecting potential fermenter yeast for improving, standardizing and modernizing of traditional Enset fermentation process that will help in minimizing time and energy needed for the traditional processing ways. 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). In this study a total of 450 different yeast colonies were screened and those having similar morphology clustered together then the representative colony transferred into Biolog Micro station reading, a total of 14 yeast species were compared with Biolog Micro station data base, only 7species have above 75% probability and ≥0.5 similarity index value read and the rest 7 species were of low probability (Table 2).

Some of this results also corresponds to the report of Gashe (1987) suggest that yeasts reached their highest counts (10^3 cfu/g) between 22 and 43 days and the yeast flora consisted of *Trichosporon*, *Rhodotorula*, *Candida*, *Torulopsis* species. Mogessie and Yewelsew (1996) also reported the yeast species *Rhodotorula*, *Kluyveramyces* and *Pichia* species were isolated from most Kocho and Bulla samples. *Guilliermondella* and *Cryptococcus*, yeast species were newly identified by Biolog Micro station in this study. This might be fermentation stage by different microbial communities, micro-environment, different enset cultivar of the area or the capacity of biolog identification power. However, in Biolog mark 7 species were under low probability and similarity index, which might be a new species out of the Biolog data base, or they do have different metabolic patterns that is unable to assimilate 96 carbon source tagged on Micro plate (Table 2). The identified yeast species were characterized for their assimilation and oxidative potential from the 96 carbon source tagged on microplates. The result confirms that 7 species of yeasts (*G. selerospora*, *Cryptococcus terreus* A, *Kluyveramyces delphensis*, *Cryptococcus albidus* var. *aerus*, *Rhodotorula acheniorum*, *Candida zylandase*, *T. beigelii* B, *F. neoformans* and *H. burtonim* have positive oxidative test for Succinic acid, L-aspartic acid, L-glutamic acid, 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-glucuronic acid+ D-xylose carbon sources.

Conclusion

- Seven yeast species, *Cryptococcus albidus* var. *aerius*, *Guilliermondella selenospora*, *Rhodotorula acheniorum*, *Trichosporon beigelii* B, *Cryptococcus terreus* A, *Candida zylandase* and *Kluyveromyces delphensis* have high similarity index value with Biolog Database and identified from Kocho and Bulla which having fermentation role.
- All yeast species identified from Kocho and Bulla were non-Saccharomyces yeasts.
- Seven yeasts were characterized from Biolog Micro station result that L-glutamic acid, D-gluconic acid, cellobiose, maltose, a-D-glucose, D-galactose+ D-xylose, D-glucuronic acid+ D-xyloses were found to be assimilated by these yeast.
- In order to formulate starter culture or to select potential fermenter yeast for improving, standardizing and modernizing of traditional Enset fermentation process, it is important to isolate, identify and characterize microbial profile from Kocho and Bulla fermentation that will help to minimize time and energy needed, enhance quality and quantity of food product and also to 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 filamentous fungal 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.
- Different fermentation process like earthen pits and surface fermentation must be studied in different area and community,

Conflict of interest statement

Authors declare that they have no conflict of interest.

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