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Original Research Article

Isolation, Identification and Characterization of Yeast Species from Kocho and Bulla Collected from Gedeo Zone, South Nation Nationality People Regional States

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Abstract

Kocho is the bulk of fermented starch food obtained from the mixture of decorticated leaf sheaths and grated corm. The aim of this study was to isolate, identify and characterize yeast species that participate in Kocho and Bulla fermentation by using morphology and Omni log identification techniques where equivalent to molecular techniques. Three hundred samples were collected from Gedeo zone at 1800 - 2400m altitude ranges. Streak plate techniques were done on potato dextrose agar (YDA) by taking 0.1ml of serially diluted samples and incubated at 28°C. Single colony of morphologically identified yeast species were transferred in to micro plate containing different types of nutrients and biochemicals. Incubated for 24, 48 and 72 hrs at 28°C and micro plate reading were carried out using MicroLog 3 Software. Seven yeast species were accurately identified from Kocho and Bulla samples. Yeast species (*Trichosporon beigelii* B, *Candida zylandase, Rhodotorula achenionum, Kluyveramyces delphensis, Guilliermondella selenospora*, *Cryptococcus terreus* A, and *Cryptococcus albidus* var. *aerus*, were accurately identified and two species were not accurately identified using Biolog microbial identification system. Identification and characterization of yeast species found in fermented products are very important to improve, standardize and modernize traditional Kocho and Bulla fermentation process and help to minimize time and energy needed to enhance quality and quantity of food product and also minimize public health problems.

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Introduction

Enset provides year-round food, fiber, animal feed and medicine (Tesfaye, 2002; Tariku and Ashenafi, 2011). The main food types obtained from enset are kocho, bulla and amicho (Spring et al., 1996). Kocho is the fermented starch that is obtained from decorticated (scraped) leaf sheaths and grated corms. Bulla, a starchy liquid, is obtained during scraping of leaf sheaths and grating of corms. The thick liquid is allowed to dry and this produces a white powder rich in starch.

Fermentation is the oldest form of biotechnology. It is essentially transforming simple raw materials in to range of value added products by utilizing the growth phenomenon of microorganisms or their activities on

various substrates (Soni and Sandhu, 1999; Achi, 2005).The importance of fermentation in modern-day life is underlined by wide range of fermented foods marketed both in developing and industrialized countries, not only for the benefit of preservation and safety, but also for their highly appreciated sensory attributes (Holzapfel, 2002).Ethiopia is one of the countries where a variety of fermented foods and beverages are produced and consumed from a wide range of raw materials using traditional techniques. The most known are: breads: injera, koho, bulla, tella (local beer), areki (liquor) and tej.

In Ethiopia, knowledge of the fermentation process is of particular interest for proper utilization of the crop. The fermented kocho is often stored in pits that are lined with enset leaves. The kocho must be left in a storage pit for a minimum of a month, but it can be stored for many months and even for several years (Tariku and Ashenafi, 2011). Kocho needs a lengthy period of processing and preparation, which is carried out by women. The first stage involves removing leaf stalks and grading of the corm. Then the fibers are separated out and 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 one year.

The quality of kocho depends on the age of the harvested enset plant, the type of clone (variety), processing techniques harvesting season and fermentation time. In the cooler regions, it is kept in a pit for years, and the quality is said to increase with increasing fermentation time. In warmer regions, fermentation is rapid and is therefore, terminated within 15 to one month's (Gash, 1987). However, there are many constraints on kocho which influence quality attributes due to the variation in variety selection, duration of fermentation, and method of processing. Moreover, within one plant, quality is influenced by the part of leaf sheath and corm processed.

Bulla is the small amount of water-insoluble starchy product that may be separated from kocho during processing by squeezing and decanting liquid. After decanting, 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. Kocho and other enset food products are basically popular and staple foods among the Gedeo and other ethnic groups in southern Ethiopia. Nowadays, enset foods are becoming increasingly popular among all ethnic groups in urban settings. It is now customary to serve Kitfo (spiced ground meat) with kocho at holidays, weddings and in specialty restaurants.

Gashe (1987) studied and described the microbiology of kotcho fermentation (Table 11). He reported that *Leuconostoc mesenteroides* initiated 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 levels of $\leq 10^3$ cfu/g during the first 15 days. Generally the population of *Clostridium* spp. was two to five times more abundant than *Bacillus* spp. *Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium sticklandi*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus licheniformis* and *Bacillus cereus* were among the spore-formers which appeared to show active growth in fermenting *kocho*. Yeasts reached highest counts (10^3 cfu/g) between 22 and 43 days and the yeast flora consisted of the *Trichosporon*, *Torulopsis*, *Rhodotorula* and *Candida*.

Ashenafi and Abebe (1996) studied the microbial load of market kocho and bulla and found out that products brought to the Awassa open market for sale did not undergo appropriate fermentation and had pH values around neutral. Kocho and bulla had high counts of aerobic mesophilic bacteria and yeasts (>106 cfu/g). Coliform counts were markedly higher in bulla (105 cfu/g) than in kotcho (103 cfu/g). Counts of enterococci, in both products, ranged between 104 and 105 cfu/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.

As kocho and bulla appeared to be processed in unhygienic conditions, unfermented products are likely to spoil easily. When these products were stored at room temperature in a loosely wrapped condition, both products had undesirable odor, slimy surface and dark discoloration after eight days (Ashenafi and Abebe, 1996). Spoiled kocho and bulla had very high counts of aerobic mesophilic bacteria (about 1010 cfu/g) and Micrococcus and Bacillus species dominated the spoilage flora. Psychrophilic microorganisms consisting

of bacteria and molds were isolated at levels of >104 cfu/g and mold spores caused dark discoloration.

Microorganisms active in starch hydrolysis, proteolysis and lipolysis were encountered in the products at varying frequencies. Tightly wrapped samples did not show any detectable spoilage in terms of odor, consistency or color. Negatu and Gashe (1994) showed the antagonistic potential of aqueous extract of fermented kocho against *Salmonella* spp., *Pseudomonas aeruginosa*, *Klebsiella* spp., *Bacillus cereus* and *Staphylococcus aureus*. Metabolites from the fermenting lactic acid bacteria were believed to prevent the survival and growth of the test organisms. In another study on the effect of heat treatment on the antimicrobial property of fermented kocho and baked products, Nigatu and Gashe (1998) observed that the test pathogens were more inhibited in high temperature-treated fermented dough than that treated at lower temperatures. They, thus, concluded that if post-baking contamination was minimized or prevented, products would be microbiologically safe, with respect to asporogenous pathogens, when served fresh. This was due to the increased inhibitory property of the baked products obtained through high temperature baking.

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 view of the fact that a large part of the earth's population is malnourished, due to poverty and inadequate of food, scientists are concerned whether the food supply can keep up with the world population increase (Zheng et al., 2005). There for the present study was focused on isolation, identification and characterization of yeast species that participate in kocho and bulla fermentation to improve its nutritional quality.

Materials and methods

Study area

The study was conducted in Gedeo zone. Gedeo zone is one of the 13 zones in south Nation, Nationalities, and People Regional States (SNNPR) in Ethiopia. The zone is located 355kms south to the capital city of the country (Addis Ababa). The zones cover land area of 1,347 Square kilometers and it lies at an altitudinal ranging from 1,350 to 3,000 m.a.s.1. It shares borders in the north with Sidama zone, in south, east and west with Oromia region. The zone is sub divide in to six districts namely, Wonago, Kocher, Dilla Zura, Bule, Gedeb and Yirgacheffe, its latitude: 6° 7' 38 .17" and Longitude: 38° 16' 37.78".

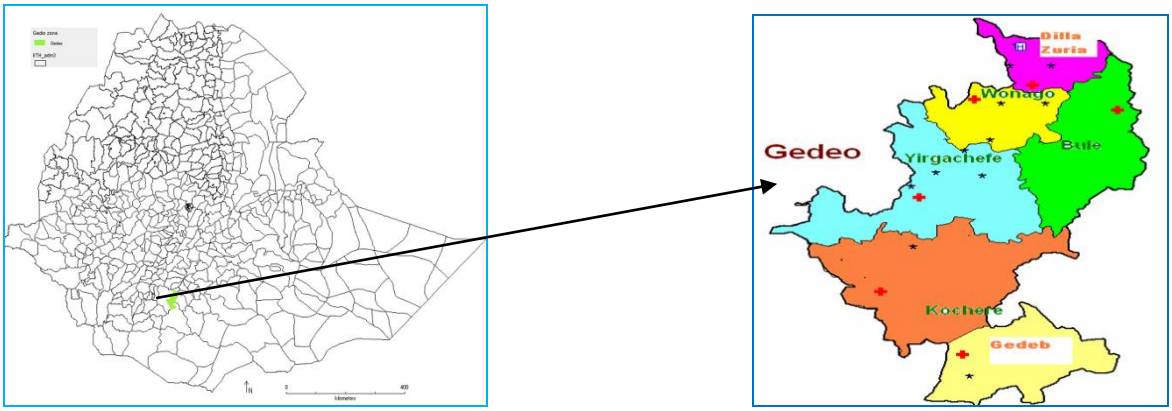

Fig. 1: Study area.

Sample collection

Three hundred kocho and bulla samples were collected aseptically from 5 to 25 cm depths in the earthen pits of bulla and kocho processing area .Samples of actively fermenting opaque kocho and bulla were collected from different household sites in Yirgacheff, Bule and wonago woreda, particularly, Adame, Edido, Orubetela, Molicha, Domariso, Wote, Sokicha, Haro hasse, Banko and Okoto, during 2014 to 2015. Samples were obtained from different sites within 1800 to 2,553m altitudinal ranges and different stage of fermentation. Sample was immediately transferred to sterile sample tube and covered properly. After completion of sample collection the sample was transported to microbial laboratory, Ethiopian Biodiversity Institute.

Pure culture isolation

In the laboratory Kocho and Bulla samples were merged in to thirty samples based on their age and pH similarity. From the merged samples 1g was taken from each merged samples and diluted serially up to10^{-6}. About 0.1ml of serially diluted sample was transferred on potato dextrose agar containing antibiotics (Pons et al., 1986) using streak plate technique. All the plates were incubated at 28^{o}C for 2 to 3 days. The colonies were transferred to slant YPDA cultures and preserved at 4^{o}C for further study.

Identification and characterization of yeast species

Morphological and Omnilog identification system of the yeast species was studied using the conventional methods described by Kurtzman and Fell (1998) and conventional methods of the Biolog identification system.

Morphological identification and characterization

According to the method of Kurtzman and Fell (1998), morphology of the yeast cells was observed.

Colonial characterization

Morphology of yeast isolates on solid Medium (YPD) was examined based on their cultural characteristics (Colony shapes, size, pigment, elevation, edge and surface appearance).

Cellular characterization

Micro morphology of yeast isolates was studied by staining with Lacto phenol cotton blue to examine their shape and size of the isolates.

Biolog identification and characterization

Biolog system for yeast identification consisted of the Micro Station and YT micro plate. Micro Station™ is semi automated machines used for reading YT micro plate. It is our most versatile system, with the ability to identify and characterize a wide range of environmental and fastidious organisms across diverse fields of microbiology. Using all six Biolog databases, over 2000 species of bacteria, yeast and filamentous fungi can be identified in as little as 4 hours with minimal effort. The MicroStation ID System brings a high level of accuracy to the rapid identification and characterization of microbial organisms (MicroLogTM System Release 4.2 User Guide 2001, Biolog).

YT Micro Plate are prefilled and dried with all necessary nutrients and biochemicals in to the 96 wells of the plate. YT Micro Plate is configured with both metabolism test and turbidity tests. The first 3 rows of the panel (rows A-C) contain carbon source metabolism tests using tetrazolium violet as a colorimetric indicator. The next five rows of the panel (rows D-H) contain carbon source turbidity tests. The last row of the panel (row H) has wells that contain 2 carbon sources. These wells test for the co-utilization of various carbon sources with D-Xylose.

Fermentative capacity test

Fermentative test was conducted as described by Atlas and Parks (1997). Prior before yeast cells grew into yeast fermentation broth (YFB) (Peptone 7.5 g/L, yeast extract 4.5g/L; 1ml of 1.6% (w/v) bromothymo blue as an indicator), 6% (w/v) glucose, sucrose, fructose, maltose and lactose were autoclaved. Yeast cells were grown at 30^{o}C for 3 days. The YFB was added with respective sugar, then yeast cells were examined on the fermentative ability using different carbon source. The Durham tubes were also placed into the media to trap carbon dioxide released. The fermented media were green in color and turn to yellow (acidic) or blue (alkaline) if the yeast cells have the ability to ferment the respective sugar.

Results

Isolation of yeasts

In the present study yeast cultures were isolated from different Kocho and bulla samples as mentioned in the methods and materials. A total of seventeen different yeast colonies were isolated from all collected kocho and bulla samples having different ages and pH ranges. The isolates were identified as yeast based on their colonial and cellular morphology (pigmentation, shape, size, texture, elevation and margin) (Fig. 2).

Morphological identification and characterization of yeast isolates

The primary identification of the yeast isolates from different kocho and bulla samples was done on the basis

of morphological characteristics of colonies on solid media. Their morphology on culture media (BUY) agar was obtained. The percentage occurrence on culture media recorded as, 20%*Trichosporon beigelii* B, 16.6% *Candida zylandase*, 13.3% *Rhodotorula achenionum*, 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*. The highest percentage occurrence on culture media was *Trichosporon beigelii* B (20%), and the lowest occurrence was *Cryptococcus terreus* A, *Cryptococcus albidus* var. *aerus*, *Filobasidilla neoformans* and *Hyphopichia burtoni* (6.7%) (Table 1).

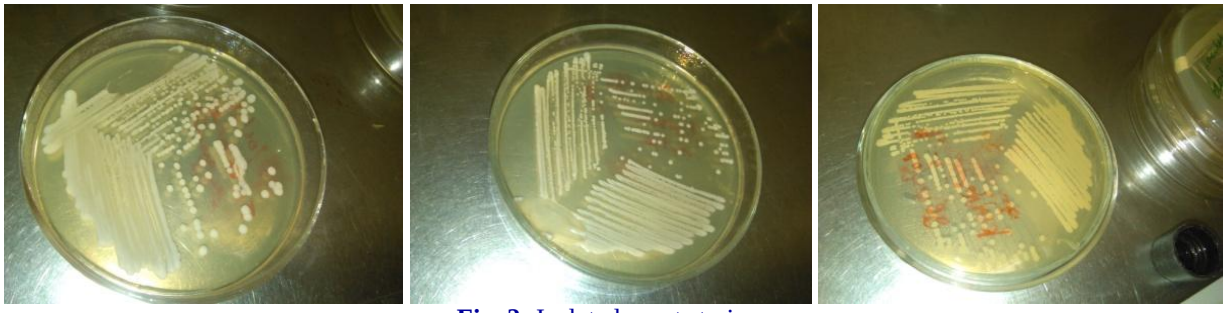

Fig. 2: Isolated yeast strains.

Table 1. Morphological characteristics of the isolated yeasts**.**

Isolated code	Name of organisms	Pigmentation	Colony morphology	Cell size
Y1	*Trichosporon beigelii* B	creamy	Raised, Smooth Wrinkled	medium
Y2	*Candida zylandase*	white	Raised , circular, smooth	Large
Y6	*Rhodotorula achenionum*	red	Raised, Smooth	Medium
Y8	*Kluyveromyces delphensis*	Creamy	Flat, Fury	medium
Y9	*Guilliermondella selenospora*	brown	Raised, circular, smooth	Medium
Y11	*Cryptococcus terreus* A	white	Raised, Smooth	Large
Y13	*Cryptococcus albidus* var. *albidus*	Creamy	Raised, Smooth, Mucoid	Large
Y15	*Filobasidilla neoformans*	Brown	Mucoid, circular	Medium
Y16	*Hyphopichia burtoni*	white	Dry, flat, rough	Large

Biolog identification and characterization

During the present investigation a selective medium that is BUY agar were used for sub culturing yeast isolates that isolated from kocho and bulla samples for Biolog identification and characterization.

Yeast suspension was prepared in 15ml sterile distilled water and adjusted to 49% +/-2 T using Biolog YT turbidity standard. One hundred microliters of inoculums was added to each well of YT Micro Plate (BiologInc) and incubated at 26°C for 24, 48 or 72 hrs until a sufficient metabolic pattern is formed. During incubation, yeast respiration in wells containing compounds that can be utilized will either reduce the tetrazolium dye forming a formazon purple color or initiate growth leading to an increase in turbidity. Each metabolic pattern was read by a Micro Station (BiologInc)) at a single wavelength of 590 nm and interpreted by micro log software ver. 4.20.05 (Biolog, Hayward, CA).

Interpretation of Results: The color density or turbidity increase in each well is referenced against the negative control wells, A-1 and D-1. All wells optically resembling the negative control wells are scored as "negative" (-) and all wells with a noticeable increase in absorbance at 590 nm are scored as "positive" (+). Wells with an extremely slight increase in absorbance at 590 nm are scored as "borderline" {\}. Reports that indicated a probability and similarity were chosen only for species identity. For Micro Plates read at 24 hours of incubation, the similarity index must be at least 0.75 to be considered as acceptable species identification. At 48 or 72 hrs of incubation, the similarity index must be at least 0.50 to be considered as acceptable ranges.

Seven yeast species were accurately identified which is involved in kocho and bulla fermentation process. Among these yeast species Omni log 100% probability and $\geq$0.615 similarity results read was observed in yeast species such as. *Cryptococcus albidus* var. *aerus*, *Guilliermondella selenospora*, *Rhodotorula achenionum*

and *Trichosporon beigelii* and followed by 99% probability *Cryptococcus terreus* A, *Candida zylandase*, *Kluyveramyces delphensis* respectively. Low probability Biolog identification and 0.186 and 0.476 similarity was recorded by *Filobasidilla neoformans* A, *Hyphopichia burtoni* respectively (Table 2).

Table 2. Biolog identification result yeasts.

Species	Probability	Similarity	Distance	Status
Cryptococcus. albidus var. *aerus*	100%	0.73	5.98	Identified
Guilliermondella selenospora	100%	0.653	5.33	>>
Rhodotorula achenionum	100%	0.623	5.78	>>
Trichosporon beigelii B	100%	0.615	5.91	>>
Cryptococcus terreus A	99%	0.693	4.62	>>
Candida zylandase	98%	0.668	4.87	>>
Kluyveromyces delphensis	86%	0.553	5.47	>>
Hyphopichia burtoni	0	0.476	8.42	Unidentified
Filobasidilla neoformans	0	0.186	9.39	Unidentified

Fermentative capacity test

Fermentative capacity of the identified yeast species was conducted using different carbohydrate and bromothymo blue on the basis of the color change within YPD broth. The fermented media were green in color and turn to yellow (acidic) or blue (alkaline) if the yeast cells have the ability to ferment the respective sugar. Yeast species (*Trichosporon beigelii* B, *Rhodotorula achenionum, Kluyveromyces delphensis* and *Hyphopichia burtoni*) ferment dextrose and sucrose, *Candida zylandase* ferment dextrose, *Guilliermondella selenospora* ferment sucrose, *Trichosporon beigelii B* and *Cryptococcus terreus* A ferment fructose. All identified yeast species do not ferment lactose (Table 3).

Table 3. Fermentative capacity test for yeast isolates from local fruits.

Yeast species isolated from local fruits	Different sugars			
	Dextrose	Sucrose	Fructose	Lactose
Trichosporon beigelii B	+	+	+	-
Candida zylandase	+	-	-	-
Rhodotorula achenionum	+	+	-	-
Kluyveromyces delphensis	+	+	-	-
Guilliermondella selenospora	-	+	-	-
Cryptococcus terreus A	-	-	+	-
Cryptococcus albidus var. *albidus*	-	-		-
Filobasidilla neoformans	-	-	-	-
Hyphopichia burtoni	+	+	-	-

Discussion

Kocho is fermented starch food that obtained from decorticated (scraped) leaf sheaths and grated corms. Bulla, a starchy liquid, obtained during scraping of leaf sheaths and grating of corms. The thick liquid is allowed to dry and this produces a white powder rich in starch. The length of kocho and bulla 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).

In the cooler regions, it is kept in a pit for years, and the quality is said to increase with increasing fermentation time. In warmer regions, fermentation is rapid and is therefore, terminated within 15 to one month's (Gash, 1987). After the fermentation is completed, a portion is removed from the pit and the liquid is squeezed out of it, resulting into a moist fibrous kocho. However, there are many constraints on kocho which influence quality attributes due to the variation in variety selection, duration of fermentation, and method of processing.

Biolog microbial identification system used to identify and characterize yeast species that participate in enset products fermentation system. Nine yeast species were identified from kocho and bulla samples were dominated by 20% *Trichosporon beigelii* B, followed by 16.6% *Candida zylandase*, 13.3% *Kluyveramyces delphensis*, 13.3% *Rhodotorula achenionum*, 10% *Guilliermondella*

selerospora, 6.67% *Cryptococcus terreus* A, 6.67% *Cryptococcus albidus* var. *aerus*, 6.67% *Filobasidilla neoformans* and 6.67% *Hyphopichia burtoni*. This results also corresponds to the report of Gashe (1987) 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 (1996) also reported the yeast species *Rhodotorula, Kluyveramyces* and *Pichia* were isolated from most Kocho and bulla samples. *Filobasidilla neoformans* A and *Hyphopichia burtoni*, have low percentage occurrence in YT micro plates. This may be due to low ability to utilize different chemicals and biomolecules filled in to micro plate during incubation time.

Most of the yeast species found in Kocho and bulla were not photogenic to human but Casadevall and Perfect (1998) reported that *Filobasidilla neoformans* is functioning as the major virulence factor in Cryptococcal infection and disease. However in our study this pathogenic yeast species is not accuracly identified that means has low Biolog probability similarity.

Most Trichosporon species are oxidative; an occasional species is also fermentative. Assimilation of KN03 is generally absent, but present in *Trichosporon pullulans; Trichosporon beigelii and Trichosporon pullulans* are urease-positive species. Trichosporon species can be distinguished from *Guilliermondella selenospora* and *Kluyveramyces delphensis*, by its assimilation of a greater number of the usual sugars.

The genus *Cryptococcus* is strictly oxidative; assimilation of certain sugars and of KN03 is useful for differentiating species. Utilization of inositol and the usual absence of carotenoid pigments distinguish this genus from Rhodotorula. This also suggested by kreger van (1964) the distinctive difference between the two is the assimilation of inositol, which is positive in Cryptococcus. Nine yeast species (*G. selerospora, C. terreus* A, *K. delphensis,C. albidus* var. *aerus, R. achenionum, C. zylandase, T. beigelii* B, *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.

These listed different chemicals and biomolecules that pre-filled in the YT micro plate are very important for growth of those identified yeast species. However, *F. neoformans and H. burtoni* do not assimilate most of chemicals and biomolecules filled in micro plate but assimilate only cellobiose and maltose carbon sources because of this mechanism those two yeast species have no role in fermentation process but also act as contaminants of kocho and bulla due to improper hygiene.

Conclusion and recommendation

Isolation, identification and characterization of yeast species that involved in kocho and bulla fermentation process are very important to improve, standardize and modernized traditional Enset processing systems and help to minimize time and energy needed, enhance quality and quantity of food product and also minimize public health problems. Some of yeast species are pathogenic to human being like *Filobasidilla neoformans*, *Candida* and *Trichosporon* except *Trichosporon beigelii* B, which are cosmopolitan. Therefore aseptic condition and environmental hygiene are recommended during Enset processing time.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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