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Exploring the Yeast Diversity of Common Alcoholic Beverages of Assam

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A B S T R A C T

Rice is a major crop of Assam. Using rice as a substrate, different communities in the state produces peculiar fermented and non-fermented products like rice beer, sweets and snacks etc. Production of rice beer involves two steps; starter culture preparation and brewing. The starter culture is made of rice and herbal combinations and believed to act as inoculants for brewing. These products often contain mixed microbial population due to allowance of natural fermentation. Some amylolytic fungi plays important role in saccharification, whereas yeast is the dominant fermenting agent of glucose to produce ethanol. Besides, these products often contain wild yeast and other microbial population due to allowance of natural fermentation which have a significant impact on food quality. A study was undertaken to systematically study the fungi associated with rice beer starter culture. Thirty two yeasts were isolated from twelve rice beer starter cultures collected from six different districts *viz*., Jorhat, Sivasagar, Golaghat, Lakhimpur, Dhemaji and Majuli. Morphological identification was carried out with the help of standard literature. Macro- and micromorphological studies revealed the most frequently associated yeasts species viz., *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera* and *Candida* spp.

Introduction

Assam is one of the states in North East India, situated south of the Eastern Himalayas. The climate is temperate to sub-tropical with summer maximum temperature 35-38 °C and winter minimum 6-8 °C, high relative humidity and annual rainfall of average 2500 mm to 4500 mm per annum. Assam is traditionally a rice growing state. Out of total 4 million hectare cultivated area 25 lakh ha is under rice cultivation with a production of 2093 kg/ha where national average is 104.32 mt, area 43.87 mha and productivity of 2381 kg/ha (Anon, 2016). Rice plays a pivotal role in the socio-cultural life of the people of the state. As a traditional practice, rice is processed into rice beer, which is of both ethnic and possible commercial importance (Ahmed *et al.*, 2010; Dutta and Mahanta, 2014).

Assam is inhabited by many indigenous tribes and as part of their socio-cultural lives; most of the tribes prepare their own household alcoholic beverages using rice as a substrate. The alcoholic beverage is used in various traditional ceremonies, religious rituals and also considered to posses medicinal and therapeutic properties. These products are

similar to *shaosingiju* of China; *sake* of Japan; *chongju* and *takju* of Korea; *brem bali, tape-ketan* and *tapuy* of Indonesia, *khaomak* of Thailand and *tapai pulul* of Malaysia (Xie *et al.,* 2007). Moreover, different tribes of Assam like; *Ahom, Deori, Mishing*, etc. have their own unique rice based alcoholic beverages known as *haj, suzen, apong,* respectively. These three major tribal beverages were focused in this study.

Basically, rice wine manufacturing consist of saccharification of steamed rice starch by fungal enzymes under aerobic solid state fermentation and the product is mixed with water and is allowed to undergo submerged alcoholic fermentation by yeasts using traditional starter cakes (Sujaya *et al.,* 2004; Dung *et al.,* 2006).

Variety of microorganisms naturally ferment majority of global fermented foods and beverages. Bacteria, yeast and mycelial fungi are mainly associated with fermentation. Yeast can be present alone or in a stable mixed population with mycelial fungi or bacteria and have a significant impact on food quality. Modern breweries employ closed brewing system that reduces the risk of contamination by 'wild' yeast. However, traditionally fermented products contain mixed microbial populations because of lack of sterility and the use of natural fermentation or mix culture fermentation starters (Robert and Kofi, 2015). Wild yeasts can therefore be non-Saccharomyces yeast, Saccharomyces yeast species other than brewing production yeast, or even production yeast strains other than those intended for a specific fermentation. In other words, yeast not deliberately used and under full control (Gilliland, 1971). Beer spoilage due to the presence of wild yeast contaminants includes production of off-flavours, competition with brewing yeasts for nutrients. Therefore, understanding of yeast mycoflora present in the traditional rice beer is necessary to take decisions regarding the optimization of processes to eliminate unwanted yeast contaminants and thus prevent unnecessary beer spoilage.

Chakrabarty (2017) examined the yeast flora present in *nduiyi*, a traditional amylolytic starter used to produce alcoholic beverage called *nduijao* from Dima Hasao district of Assam. Based on cell morphology and phenotypic characterization isolates were identified as *Candida glabrata* and *Saccharomyces cerevisiae.* A metagenomic study revealed the microbial community associated with *haj*, a traditional alcoholic beverage of Ahom tribe of Assam. The existence of ethanol producers *viz*., *Meyerozyma guilliermondii, Wickerhamomyces ciferrii, Saccharomyces cerevisiae, Candida glabrata, Debaryomyces hansenii, Ogataea parapolymorpha* and *Dekkera bruxellensis*, were found associated with the starter culture along with a diverse range of opportunistic contaminants (Bora *et al.,* 2016). Literature is meagre with respect to yeast taxonomical study in north east India with special reference to state of Assam.

This investigation was aimed at the morphological identification of indigenous yeasts isolates from rice beer starter culture samples. The samples were collected from Ahom, Mishing and Deori community residing in seven different districts of Assam, India.

Materials and Methods

Collection of samples

Indigenously prepared rice beer starter cultures *viz*., *xaj, apong, suzen* were collected in sterilized plastic bags and brought to laboratory for isolation of associated mycoflora.

Isolation of yeasts and fungi from starter culture

Rice starter cultures were ground to fine powder with the help of electric grinder (Bhuyan and Baishya, 2013) and 10g of sample is suspended in 90 ml sterilized water and mixed thoroughly. 1ml solution was serially diluted to 10^{-4} and 10^{-5}. Each dilution was spread onto potato dextrose agar (PDA) supplemented with 200ppm streptomycin sulphate and incubated at 25±1°c for 48 hours. Individual yeasts colonies with distinct colony and morphological characters were picked up and repeatedly streaked in yeast extract peptone dextrose (YEPD) agar media (Jeyaram *et al.,* 2008) and maintained.

Cultural and morphological identification of yeasts

Each of the purified colonies of yeasts was grown on yeast extract peptone dextrose (YEPD) agar for assessing their colony characteristics mostly shape, colour, margin, texture (Chavan *et al.,* 2009; Goralska, 2011; Spencer *et al.,* 2011). To induce ascospore formation yeasts isolates were cultured on V8 juice agar (Yarrow, 1998; Barnett *et al.,* 2000).

Microscopic characteristics of yeasts *viz.*, cell shape, size, presence/absence of budding and pseudohyphae, sexual stage, shape and size of ascus and ascospore if present was studied by lacto phenol cotton blue and basic fuchsin staining under 40 x &100x light microscope. Whole preparation of ascus was done by moderately heating and tapping the structures, released ascospores were examined and measured. Microphotographs were taken to show the typical morphology of the fungi.

Identification and characterization of yeasts were carried out with the help of relevant keys, monograph, standard literature (Lodder, 1971; Barnett, 1990; Barnett, 2000) and CBS yeast database.

Results and Discussion

Twenty eight yeasts were identified from all the starter culture samples collected during the investigation.

Morphological identification

Saccharomyces cerevisiae isolates were found to be dominant in all the samples. It showed white to creamy colonies with smooth and butyrous growth on yeast extract peptone dextrose agar. After three days at 25 ± 1° C cells are spheroidal, sub- globose, ovoid and occur singly, in pairs or sometimes in small clusters. Cell size of different isolates ranging from 1.3-3.2 µm to 4.0-5.8 µm. Ascus with two to four round ascospores were typical of *S. cerevisiae* (Fig. 1D). Table 1 shows macro and micomorphology of *S. cerevisiae* isolates. Pseudohyphae or true hyphae were not observed in any of the isolates. S. cerevisiae is the major fermenting agent of all the globally fermented products.

Three isolates showed typical tough, raised and farinose, partly or entirely hairy colonies on YEPD agar (Table 2.). Based on the colony characters (Fig. 2), it was identified as *Saccharomycopsis* (Lindner) Klocker. The identification was further confirmed based on the micromorphological observations. The yeast was characterized by the formation of septate hyphae and multipolar budding cells. Presence of spherical to oval asci, situated at the ends of the mycelia hyphae or alongside them; bearing two to four hat shaped ascospores confirms the yeast upto species level as *S. fibuligera*. Clear zone formation was also observed when subjected to starch hydrolysis test, indicating the production of amylase enzyme by the yeast (Wickerham *et al.,* 1944). *S. fibuligera* was reported to be the

principal amylolytic yeasts found in Indonesian cassava-tape and ragi tape (alcoholic beverages made from cassava and ragi); that produces large amount of amylases, acid protease and β-glucosidase which have highly potential applications in fermentation industry (Chi *et al.*, 2009). It is also used to produce ethanol from starch, especially cassava starch by co-cultures of *Saccharomyces cerevisiae* (Chi *et al.*, 2009). The presence of *S. fibuligera* is thus beneficial for the beer production process.

Four *Candida* spp. were identified based on presence of pseudomycellium and true mycelium and absence of sexual stage. All the four isolate**s** produced white to creamy, smooth and butyrous colonies on YEPD agar. However, the cell size and shape varies among all the isolates. The cells of one isolate were short-ovoid to ovoid 4.2 -5.4 x 6.5-8.5 µm. Pseudomycelium is abundantly formed and consist of long-stretched, branched pseudohyphae bearing blastoconidia and verticils of blastospores in branched or simple chains. True mycelium occurs. Based on these morphological characters and comparison with standard literature the yeast was identified as *C. tropicalis* (Fig. 3).

The other three isolates have cells of ovoid to cylindrical shape, pseudomycelium present, true mycelium not observed, reproduce by budding. The cell sizes varied between the three isolates, ranging from 2.2-3.5 x 2.8- 4.5 µm, 2.5-3.6 x 2.5- 4.8 µm and 10.2 -11.4 x 10.4-15.5 µm (Table 3).

The study shows that different kinds of yeasts were associated with rice beer starter culture of Assam (Fig. 1–3).

Table.1 Macromorphology and micromorphology of *Saccharomyces cerevisiae*

Location	Macromorphology			Micromorphology			
	Colour	Appreance	Texture	Cell shape	Cell size (µm)	Budding	Sexual stage
Jorhat	White	Smooth	Butyrous	Round, oval to cylindrical	2.4-3.8 x 2.6 - 3.6	Present	Ascus with 1-4 thin walled, smooth, spherical to sub-spherical ascospore
	White	Smooth	Butyrous	do	1.5-2.9 x 2.0 -3.6	Do	do
	White to Creamy	Smooth	Butyrous	do	3.2 x 4.5-4.9	do	Do
	White to Creamy	Smooth	Butyrous	do	1.9-3.1 x 3.1-4.5	do	Do
Golaghat	White	Smooth and raised	Butyrous	do	3.0-4.2 x 4.1- 5.2	do	Do
	White to off white	Smooth	Butyrous	do	2.3 x 2.5-2.6	do	Do
Sivasagar	White	Smooth	Butyrous	do	3.2 x 4.5-4.9	do	Do
	White to Creamy	Smooth	Butyrous	do	1.9-3.1 x 3.1-4.5	do	Do
Lakhimpur	White to Creamy	Smooth	Butyrous	do	2.0-2.9 x 2.8 -4.5	do	Do
	White to Creamy	Smooth	Butyrous	do	3.8-3.9 x 5.9-6.0	do	Do
	White	Smooth	Butyrous	do	2.9-3.0 x 5.1-5.2	do	Do
Dhemaji	White to Creamy	Smooth	Butyrous	do	3.2-3.6 x 5.0-5.2	do	Do
Majuli	White to Creamy	Smooth	Butyrous	do	4.0-4.7 x 5.5-5.8	do	Do
Karbi Anglong	White to Creamy	Smooth	Butyrous	do	1.9-3.1 x 3.1-4.5	do	do

Table.2 Macromorphology and micromorphology of *Saccharomycopsis fibuligera*

Location	Macromorphology			Micromorphology			
	Colour	Appreance	Texture	Cell shape	Cell size(um)	Sexual stage	True hyphae
Jorhat	Creamy	Farinose	Membranous	Oval to cylindrical	3.0-3.5 x 3.5-4.5	Spherical to ovoidal asci formed on hyphal structure, 2-4 hat shaped ascospore	Septate mycelium present, originating from spore. Sexual and asexual stage both present together
	White to creamy	Farinose	Membranous	Oval to cylindrical	2.5 -3.4X 4.0 -4.5	do	do
Golaghat	White to creamy	Farinose	Membranous	Oval to cylindrical	2.4 -3.2X 3.9 -4.6	do	do

Table.3 Macromorphology and micromorphology of *Candida tropicalis* and *Candida* spp.

	Colour	Appearance	Texture	Cell shape	Cell size(µm)	Budding	Sexual stage	
a) *Candida tropicalis*								
Sivasagar	White to Creamy	Smooth	Butyrous	Oval to cylindrical	4.2 -5.4 x 6.5-8.5	Present	Absent	Present
b) *Candida* spp.								
Jorhat	White	Smooth	Butyrous	Ovoid forming short chains by budding	2.5-3.5 x 2.5- 4.7	Present	Absent	Present
Sivasagar	White to Creamy	Smooth	Butyrous	Oval to cylindrical	10.2 -11.3 x 10.3-15.5	Present	Absent	Present
Dhemaji	White to Creamy	Smooth	Butyrous	Oval to cylindrical	2.1-3.2 x 2.6- 4.2	Present	Absent	Present

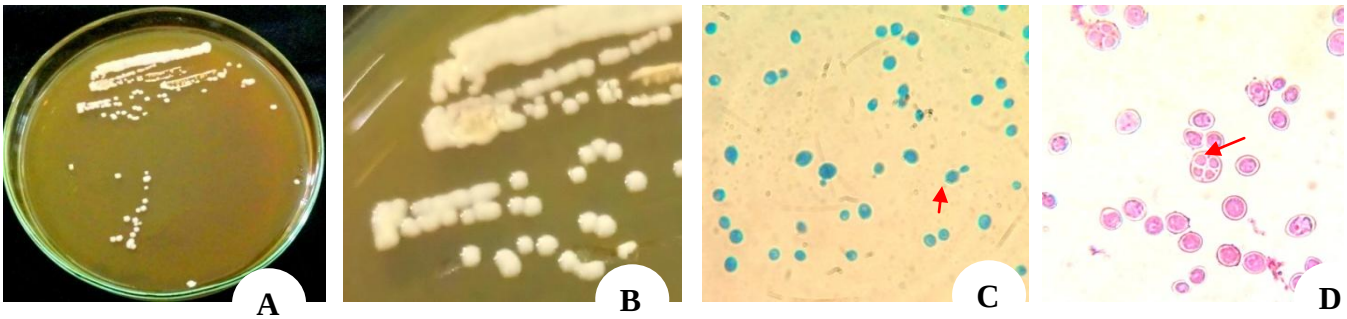

Fig.1 Macro and micromorphology of *Saccharomyces cerevisiae* (A-B) pure culture on YEPD agar (C) *S. Cerevisiae* cells, budding (D) ascus with ascospore/ tetrads

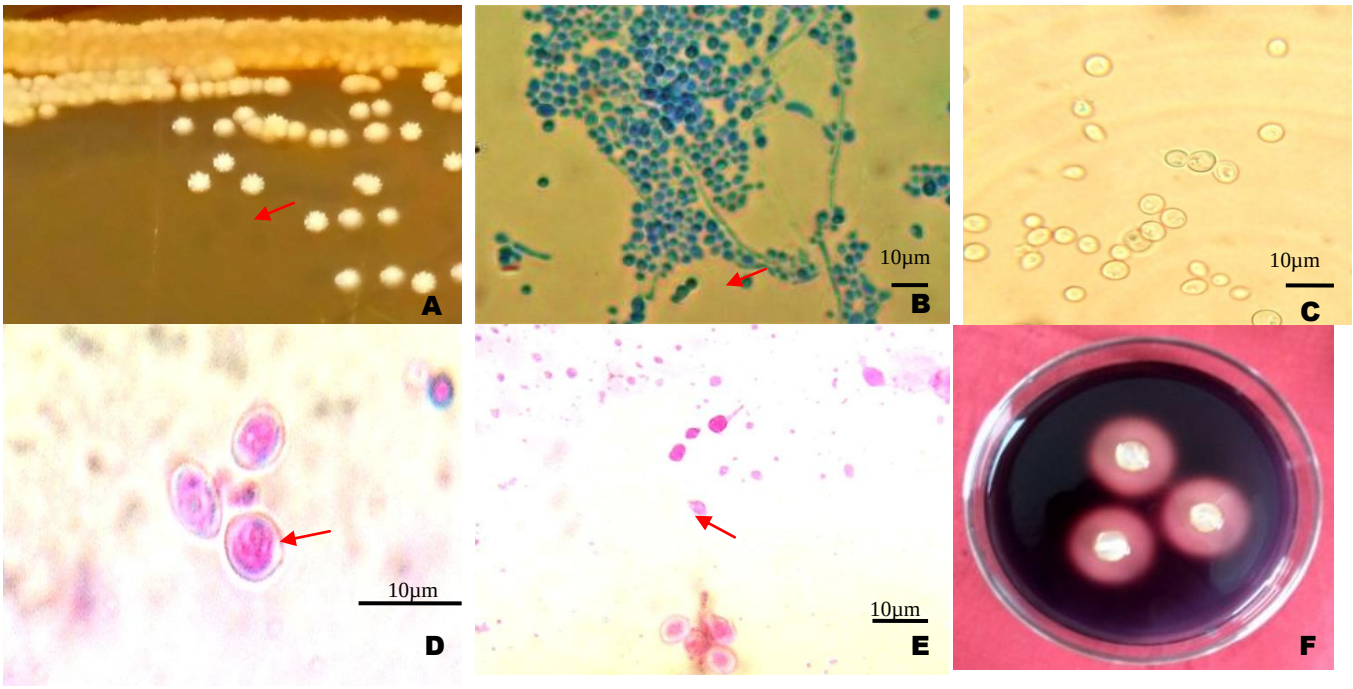

Fig.2 Macro and micro morphology of *Saccharomycopsis fibuligera*
(A) farinose colonies on yepd agar (B) pseudo and true hyphae (C) budding cells
(D) ascus with ascospores (E) hat shaped ascospore (F) clear zone formation on iodine test

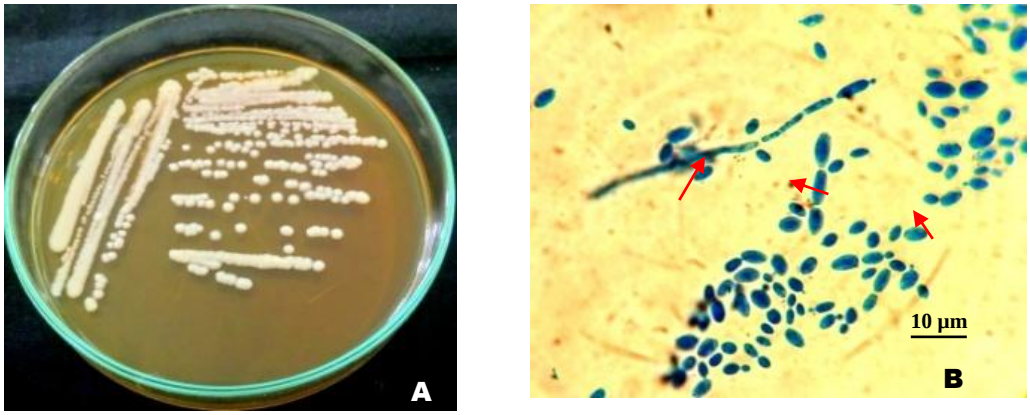

Fig.3 Macro and micromorphology of *Candida tropicalis* (A) pure culture on YEPD agar (B) budding cells, pseudohyphae and true hyphae

Isolates derived from all the seven districts revealed the presence of *S. cerevisiae*. Many worker reported *S. cerevisiae* as the dominant alcohol producing yeast in fermented products worldwide (Sujaya *et al.*, 2004; Thapa and Tamang, 2004; Xie *et al.*, 2007;do Amaral Santos *et al.*, 2012; Jimoh *et al.*, 2012; Miguel *et al.*, 2013; Chakrabarty, 2017).

In the present study *S. fibuligera* was found to be associated with starter cultures from Jorhat, Golaghat and Sivasagar districts. *S. fibuligera* was the principal amylolytic yeasts found in Indonesian cassava-tape and ragi tape (Kuriyama *et al.*, 1997). Many workers reported *S. fibuligera* from various fermented drinks (Tamang and Sarkar, 1995; Tamang, 2003; Thapa and Tamang, 2004; Tsuyoshi *et*

al., 2005; Soka and Irene, 2013; Thakur *et al.,* 2015). *S. fibuligera* produces large amount of amylases, acid protease and β-glucosidase which have high potential of applications in fermentation industry (Chi *et al.,* 2009). It is also used to produce ethanol from starch, especially cassava starch by co-cultures of *S. cerevisiae*. It is probable that high yield of alcohol could be exploited by combining both the yeast. However, this is the first report of *S. fibuligera* from rice beer of Assam.

One *Candida* sp. was identified upto species level as *C. tropicalis*. Several workers also retrieved *Candida* spp. from fermented products (Hancioglu and Karapinar, 1997; Sujaya *et al.,* 2004; Thapa and Tamang, 2004; Xie *et al.,* 2007; Soka and Irene, 2013; Thakur *et al.,* 2015; Chakrabarty, 2017).

The dominant yeast species associated with another Indian starter for rice wine from Manipur called 'Hamei' were identified as *S. cerevisiae, Pichia anomala, Trichosporon sp., Candida tropicalis, Pichia guilliermondi, Candida parapsilosis, Torulaspora delbrueckii, Pichia fabianii* and *Candida montana* (Jeyaram *et al.,* 2008) which is in agreement with Balinese rice wine starter 'ragi tape' and Vietnamese rice wine starter 'mem' (Dung *et al.,* 2006; Sujaya *et al.,* 2004). In the present study domination of *S. cerevisiae, S. fibuligera* and *Candida* spp. in rice beer starter culture of Assam is in agreement with Sikkimish rice wine starter 'Marcha' (Tsuyoshi *et al.,* 2005). From this, it is inferred that the yeast species associated with rice wine starter used in Himalaya regions (Assam, Sikkim) are distinctly differ from the starter used in Indo-Burma Biodiversity hotspot (includes Manipur, Vietnam and Indonesia of south eastern Asia) (http://www.biodiversityhotspots.org).

The study also revealed that the region within Himalayan-biodiversity hotspot, a consortium of *S. cerevisiae* and *S. fibuligera* may be a possible way of exploitation of yeasts species of the region to produce local wine of interest avoiding unwanted contaminants of the fermented products.

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