DR-1829
[1-7]

RESEARCH ARTICLE Asian Journal of Dairy and Food Research, Volume Issue : ()

Isolation and Characterization of the Mycofloral Diversity in Traditional Assamese Alcoholic Fermentation from India

Bandana Saikia, M.S. Ali, Shankar Hemanta Gogoi, P.D. Nath

10.18805/ajdfr.DR-1829

ABSTRACT

Background: Different communities in the state of Assam produces particular fermented and non-fermented products like rice beer, sweets and snacks *etc.* using rice as a substrate. Traditionally, important alcoholic beverages harbour a myriad of microbes including contaminants. The starter culture is made of rice and herbal combinations and believed to act as inoculants for brewing.

Methods: Seven districts of Assam *viz.,* Jorhat, Sivasagar, Golaghat, Lakhimpur, Dhemaji, Majuli and Karbi Anglong were surveyed and starter culture samples were collected during 2019-20. Yeasts and fungal isolates were isolated on culture media and systematically studied to identify by both morphological and molecular means. Study was carried out at Department of Plant Pathology, Assam Agricultural University.

Result: Sixteen yeast isolates and two filamentous fungi from rice beer and starter cultures were isolated. Morphological and molecular identification of the isolates revealed that they belong to four different genera and five species of filamentours saccharifying agents *Aspergillus oryzae* and *A. niger* and yeasts *Saccharomyces cerevisiae, Saccharomycopsis fibuligera* and *Candida tropicalis*.

Key words: *Aspergillus, Candida*, Fermentation, *Saccharomyces, Saccharomycopsis*, Taxonomy.

INTRODUCTION

Fermentation is a spontaneous method of producing alcoholic beverages around the world. The process is dominated by strains of *Saccharomyces cerevisiae* and related species as well as non-*saccharomyces* yeasts from the genera *Candida*, *Pichia, Kluyveromyces* and *Torulaspora*, among others (Qi *et al*., 2015). Initially, the non-*Saccharomyces* yeasts are the dominant partners kick-starting the fermentation process generating various flavor compounds like terpenoids, higher alcohols, esters, acetic acid, glycerol, acetaldehyde and succinic acid (Jolly *et al*., 2014). The liquification and saccharification of rice based fermentation results from moulds that produce α-amylase that converts starch into dextrin, maltose and mostly glucose (Pervez *et al*., 2014). The fermentation is carried out in a time dependent manner and can take upto 3-10 days with varing conditions of residual sugars and sweetness. The process can further be regulated by using standardized starter cultures and to achieve better utilization of the fermentable sugars. Recently, various studies have applied monocultures or co-cultures of *S. cerevisiae* and non-*Saccharomyces* yeast to produce wine (Padilla *et al*., 2016; Zhang *et al*., 2018), sugarcane and pineapple beverage (Ribeiro *et al*., 2015), mango wine (Sadineni *et al*., 2012), *etc*.

Almost the entire human world has followed traditional methods of fermentation and alcoholic beverage production. Few well known beverages are *shaosingiju* and *laochao* of China (Ghosh *et al*., 2016), *tapuy*, *brem bali* and *tape-ketan* of Indonesia (Tamang, 2016), *khaomak* of Thailand, *tapai pulul* of Malaysia, *chongju* and *takju* of Korea and *sake* of Japan (Kitamura *et al*., 2016). Such beverages form an integral part of the producing communities and are used in various traditional ceremonies, religious rituals and also considered to possess medicinal and therapeutic properties. The fermentation process, in almost all cases has the same protocol with variations in the starter culture composition and fermentation period. Previous studies point out to the fact that *Saccharomyces* and non- *Saccharomyces* cultures may act synergistically making it important that the starter inoculants are explored for their mycoflora. Selection of the appropriate strains is generally based on criteria such as fermentative power, tolerance to ethanol, ethanol yield, killer phenotype and low acetic acid production (Suárez-Lepe and Morata, 2012).

The aim of the present work was to isolate native strains of yeast from the alcoholic fermentation of rice leading to the production of rice beer. A comprehensive sampling survey was carried out in six different districts of Assam, India and a total of nine different starter inoculum samples were collected for the study. A detailed morphological and molecular characterization was done to unravel the yeast and other fungal communities present in the starter inoculums.

Department of Plant Pathology, Assam Agricultural University, Jorhat-785 013, Assam, India.

Corresponding Author: Bandana Saikia, Department of Plant Pathology, Assam Agricultural University, Jorhat-785 013, Assam, India. Email: bandanasaikia91@gmail.com

How to cite this article: Saikia, B., Ali, M.S., Gogoi, S.H. and Nath, P.D. (2022). Isolation and Characterization of the Mycofloral Diversity in Traditional Assamese Alcoholic Fermentation from India. Asian Journal of Dairy and Food Research. DOI: 10.18805/ajdfr.DR-1829.

Submitted: 26-10-2021 **Accepted:** 01-02-2022 **Online:** 28-03-2022

MATERIALS AND METHODS

Collection of samples

Indigenously prepared rice beer starter cultures (in the form

of dry cake) *viz.,* Xaaj pitha, Apong, Suzen (mod pitha) were collected during 2019-20 in sterilized high density polypropylene bags and brought to laboratory of Department of Plant Pathology, Assam Agricultural University, Jorhat for isolation of the associated mycoflora.

Isolation of yeasts and fungi from starter culture

Rice starter cultures were ground to powder in pestle and morter and 10 g of sample is suspended in 90 ml sterilized water. One ml solution was serially diluted to 10^{-4} and 10^{-5} dilutions. Each dilution was spread in different media namely yeast extract peptone dextrose (YEPD) agar, potato dextrose agar (PDA) and V8 juice agar supplemented with 200 ppm streptomycin sulphate to avoid bacterial growth and incubated at 25±1°C for 48 hours (Jeyaram *et al.*, 2008). Individual yeasts and fungal colonies with distinct colony and morphological characters were picked up, purified, sub-cultured and stored in mineral oil for future use.

Cultural and morphological identification

Identification and characterization of yeasts and filamentous fungi were carried out with the help of relevant keys, monograph, standard literature (Barnett, *et al.*, 2000; Barnett *et al.*, 1983; Lodder and Kreger-Van, 1952; Raper and Thom, 1949; Subramanian, 1971; Thom and Church, 1926) and CBS yeast database (http://www.cabri.org/CABRI/srs-doc/cbs_yeast.info.html).

Yeasts

Purified yeasts colonies of was grown on Yeast Extract Peptone Dextrose (YEPD) agar for assessing their colony characteristics mostly shape, colour and margin; texture (Spencer *et al.*, 2011). To induce ascospore formation, yeasts isolates were cultured on V8 juice agar. Microscopic characteristics of yeasts *viz.,* cell shape, size, presence/absence of budding cells and pseudo-hyphae, sexual stage, shape and size of ascus and ascospore if present were studied by lacto phenol cotton blue and basic fuchsin dye staining under 40x and 100x resolution of Magnus USB binocular microscope.

Filamentous fungi

Cultural characteristics of filamentous fungi were analyzed in PDA medium. Petri plates having 20 ml sterile media were inoculated aseptically with 5 mm mycelial disc of the fungus and incubated at 25±1°C. Microscopic observations for head of *Aspergillus*, number of phialides, foot cells, spore characters like spore arrangement, size, colour of different isolates were studied from the pure culture of the isolates. Specimen were mounted with lacto phenol; lacto phenol cotton blue; basic fuchsin. Microphotographs were recorded to show the typical morphology of the fungi.

Starch hydrolysis test

Starch agar medium containing 0.2% w/v of starch, 0.5% peptic digest of animal tissue, 0.15% yeast extract, 0.15% beef extract, 0.5% sodium chloride, 1.5% agar was prepared and pH was adjusted to 7.5. Ten millimetre diameter discs of pure culture were inoculated to three corner of each plate and incubated at 30°C for 48 hours. Visualization of starch degradation was done by flooding with a 0.25% Iodine solution. Clearing of the typically blue coloration of starch with iodine indicate starch degradation.

Phylogenetic analysis, molecular identification and sequencing

PCR amplification of the internally transcribed ribosomal spacer region and the 5.8S rDNA was done by colony PCR method as described by (Jeyaram *et al.*, 2008). Universal primers (ITS1 5′-TCCGTAGGTGAACCTGCGG-3′, ITS4 5′-TCCTCCGCTTAT TGATATGC-3′) were used for amplification (Schoch *et al.*, 2012). The products were visualized on 1.5% (w/v) agarose gel prepared in 1x TAE buffer using gel documentation system. PCR product of samples along with primer pairs were sent to Bioserve Biotechnologies India Pvt. Ltd. for sequencing. Codoncode aligner software was used to assemble the curated sequences into contigs. The sequences were submitted in NCBI and accession numbers were obtained.

Phylogenetic analyses of the sequences were done using MEGA 7 software by maximum likelihood method (Kumar *et al.*, 2016) following Neighbour joining method. ITS sequences of *S. cerevisiae*, *S. fibuligera*, *Candida* sp.were obtained from National Centre for Biological Information to draw a comparative evolutionary profile of the sequenced strains.

RESULTS AND DISCUSSION

Morphological identification of filamentous fungi

Two filamentous fungi were isolated and identified as *Aspergillus oryzae* and *A. niger* based on cultural and morphological characters Table 1. Colony growth of *Aspergillus oryzae* on Czapek Dox Agar is moderate attaining 4.5-5.5 cm in 10 days at 25±1°C (Fig 1 C-D). On PDA mycelium white with green sporulation; reverse mustard yellow (154B, RHS), (149A, RHS) (Fig 1 A-B). The fungus produced concentric rings on Malt Etract Agar (Fig 1 E-F). Conidial head radiate with globose to subglobose vesicles, uniseriate (Fig 1 G). Conidia subglobose, rarely ellipsoidal or ovoid, 3.0-5.0 µm wide, with walls smooth to irregularly rough. Thom and Church (1926) described similar characteristics for *Aspergillus oryzae,* based on which the species was identified. However, the identification was confirmed by National Centre for Fungal Taxonomy (NCFT), New Delhi and given ID. No. 2001.17.

A. niger colonies on Czapek Dox agar, consist of a compact white or yellow (10C, RHS) basal felt covered by dense layer of black conidial heads (202 A, RHS) (Fig 2 A-B). On Oat Meal agar dark black sporulation occurred (Fig 2 C). Conidiophores were smooth-walled, hyaline or turning dark towards the vesicle (Fig 2D). Conidial heads were large up to 250-300 µm in diameter, vesicle globose, dark brown in colour, becoming radiate and tending to split into several loose columns with age. Conidial heads were biseriate with the phialides borne on brown metulae. Conidia are globose to subglobose (3.5-5.0 µm in diameter), dark brown to black

Table 1: Morphological characters of filamentous fungi isolated from rice beer starter culture.

Isolate	Colony characters	Microscopic characters		
		Conidiophore	Conidial heads	Conidia
Aspergillus oryzae	Mycelium white with green sporulation; reverse mustard yellow		Globose to subglobose vesicles, uniseriate	Subglobose, ellipsoidal or ovoid, 3.0-5.0 µm, walls smooth to irregularly rough
A. niger	White or yellow basal Dark black sporulation	Smooth-walled, hyaline or turning dark towards the vesicle	Radiate, biseriate, 250-300 µm in diameter, vesicle globose, dark brown	Globose to subglobose, 3.5-5.0 µm in diameter, dark brown to black

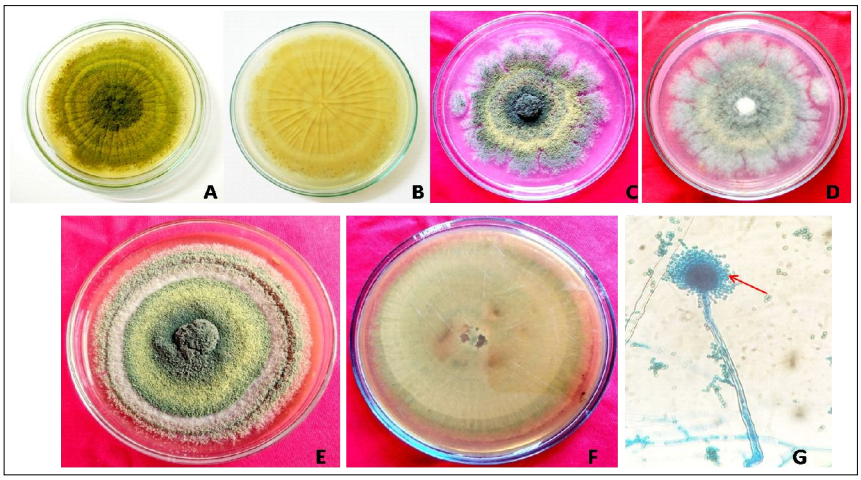

Fig 1: Macro and micromorphology of *Aspergillus oryzae* (A) Front view. (B) Reverse view on PDA. (C) Front view. (D) Reverse view on Czapek Dox Agar. (E) Front view. (F) Reverse view on MEA (G) Radial conidial head.

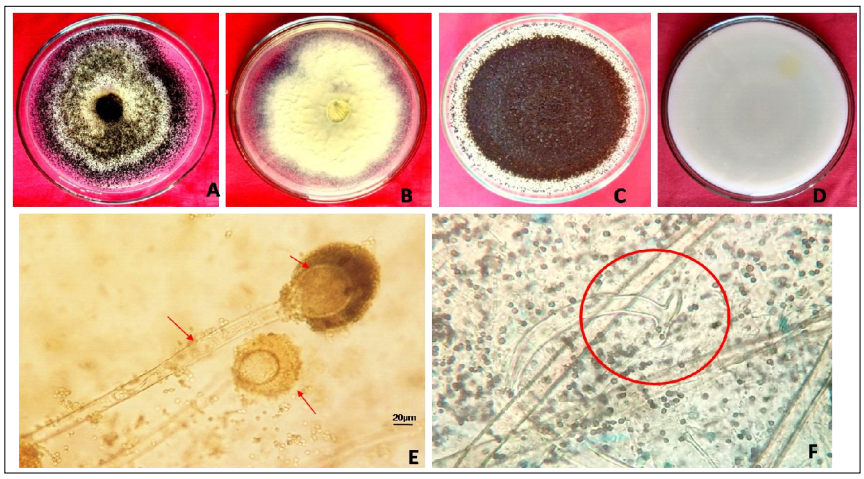

Fig 2: Macro and micromorphology of *Aspergillus niger.* (A) Front view. (B) Reverse view on MEA. (C) Front view. (D) Reverse view on Oat Meal Agar. (E) Hyaline smooth conidiphore, globose vesicle and biseriate conidial head. (F) Foot cell (-20 µm).

(Fig 2E). These characteristics were compared with standard description of Thom and Church (1926) and the species was confirmed as *Aspergillus niger* (Fig 2F).

Morphological identification of yeasts

Sixteen yeast strains were isolated from nine starter cultures and divided into three groups (Group I, II and III) based on cultural and morphological properties Table 2. Phylogenetic, morphological and physiological characterization identified the isolates as *Saccharomyces cerevisiae* (Group I); *Saccharomycopsis fibuligera* (Group II) and *Candida sp.* (Group III). Group I isolates belonging to *Saccharomyces cerevisiae* were found to be dominant in all the nine samples. Colonies white to cream colour, smooth and butyrous growth on YEPD agar (Fig 3 A); cells were spheroidal, sub-globose, ovoid and occurred singly, in pairs or sometimes in small clusters (Fig 3B). 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 3C). Pseudohyphae or true hyphae were not observed in any of the isolates.

Three isolates comprising group II showed typical tough, raised and farinose, partly or entirely hairy colonies on YEPD agar. Based on the colony characters it was suspected to be *Saccharomycopsis fibuligera* (Lindner) Klocker (Fig 4 A). Further, the micro-morphological observations revealed the formation of septate hyphae (Fig 4B) and multi-polar budding cells, presence of spherical to oval asci, situated at the ends of the mycelia hyphae or alongside them; bearing two to

Table 2: Morphological characters of yeasts isolated from rice beer starter culture.

Isolate	Macromorphology			Micromorphology			
	Colour	Appreance	Texture	Cell shape	Cell size (µm)	Special character	Sexual stage
Saccharomyces cerevisiae (GroupI)	White	Smooth	Butyrous	Round, oval to cylindrical	1.3-3.2× 4.0-5.8	Budding present	Ascus with 1-4 ascospore
Saccharomycopsis fibuligera (GroupII)	White to creamy	Farinose	Membrenous	Oval to cylindrical	3.0-3.5× 3.5- 4.5	Septate mycelium present	Ascus with 2-4 hat shaped ascospore
Candida tropicalis (GroupIII)	White to creamy	Smooth	Butyrous	Oval to cylindrical	4.2 - 5.4× 6.5 - 8.5	Budding and pseudohyphae present	Absent

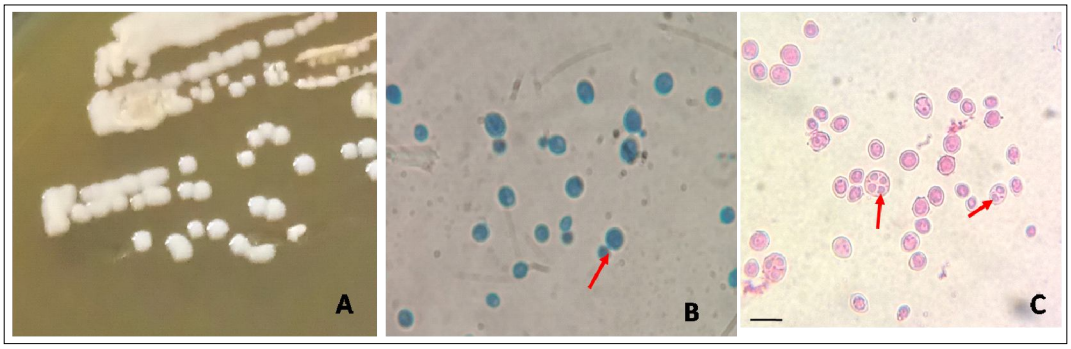

Fig 3: (A) Macro and micromorphology of *Saccharomyces cerevisiae* pure culture on YEPD agar. (B) Budding *S. Cerevisiae* cells. (C) Ascus with ascospore/ tetrads (-10 µm).

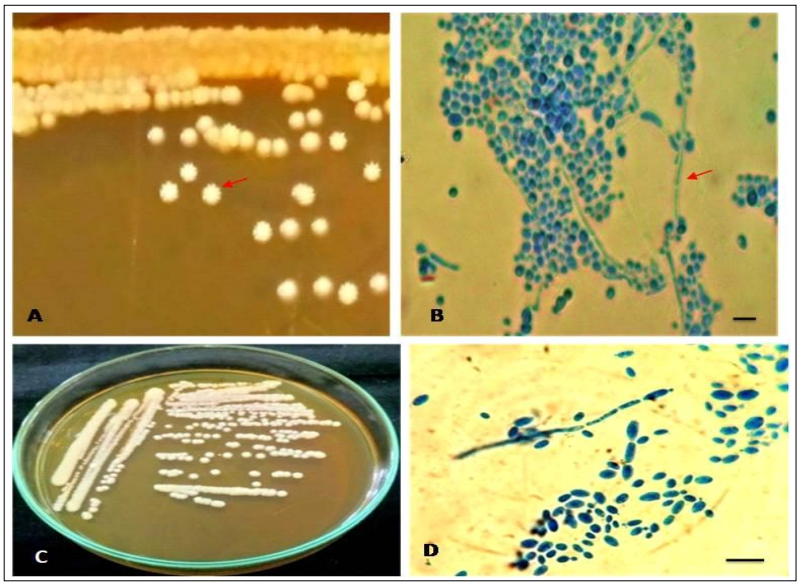

Fig 4: Macro and micro morphology of *Saccharomycopsis fibuligera.* (A) farinose colonies on YEPD agar. (B) Pseudo and true hyphae (C) Macro and micromorphology of *Candida tropicalis* pure culture on YEPD agar. (D) Budding cells, pseudohyphae and true hyphae (-10 µm).

four hat shaped ascospores confirming the yeast upto species level. Clear zone formation was also observed when subjected to starch hydrolysis test which indicated the production of amylase enzyme by the yeast (Wickerham *et al.*, 1944).

Group III containing *Candida* sp. were identified based on presence of pseudo-mycelium and true mycelium and absence of sexual stage. The isolates produced white to creamy, smooth and butyrous colonies on YEPD agar (Fig 4 C-D). The cells were short-ovoid to ovoid 4.2-5.4× 6.5-8.5 µm; pseudo-mycelium is abundantly formed and consists of long-stretched, branched pseudo-hyphae 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.*

Phylogenetics and molecular characterization of yeasts

Representative strains from each group were randomly selected (strain Y-2 and Y-3 from group I; strain Y-6 from group II; strain Y-21 from group III) and their phylogenetic positions were examined based on evolutionary phylogenetic analysis. Fig 5 represents the phylogenetic position of the yeast strains based on the almost full-length 18S rDNA curated sequences. All the analyzed strains were in the class hemiascomycetes of division ascomycotina.

Molecular characterization of selected strains was carried out in support of the detailed morphological identification performed. Fig 6 depicts the gel electrophoresis results of the amplification of ITS in the isolated cultures. The sequenced strains *viz.*, Y-32 (group I), Y-6 (group II) and Y-21 (group III) were highly homologous to the genera *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera* and *Candida tropicalis,* respectively. The sequences were submitted in NCBI under the accession numbers MK110643 (*Saccharomyces cerevisiae*), MK110642 (*Saccharomycopsis fibuligera*) and MK110644 (*Candida tropicalis*).

Morphological, phylogenetic and molecular analysis revealed the presence of diverse mycoflora associated with

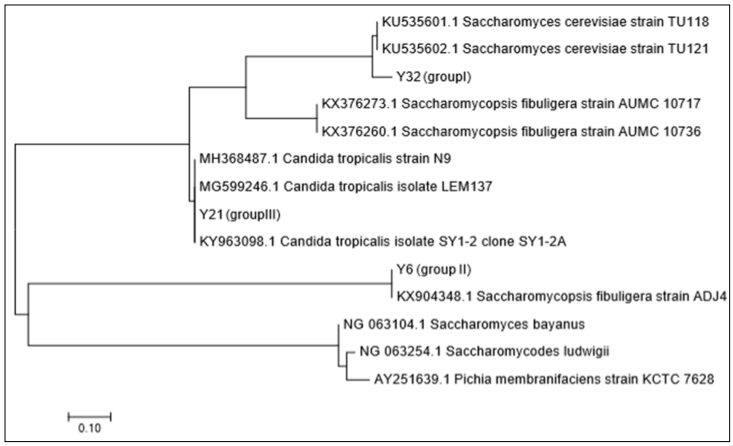

Fig 5: The morphological and phylogenetic characteristics identified the isolates as *S. cerevisiae* (nine strains of Group I), *S. fibuligera* (three strains of Group II), *Candida* spp. (four isolates of Group III).

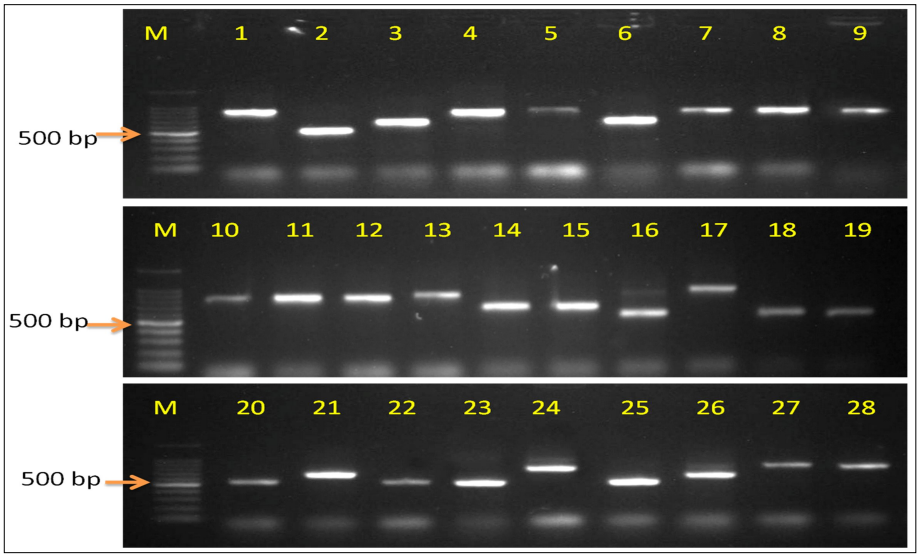

Fig 6: Agarose gel electrophoresis showing amplified PCR product of yeast isolates with primer pairs ITS1/ITS4. M: 100 bp DNA ladder, lanes 1-28: yeast isolates.

rice beer starter cultures (*xaj pitha*, *suzen* and *apong*). The filamentous fungi were identified as *Aspergillus oryzae* and *A. niger* and the dominant yeasts were *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera* and *Candida tropicalis*. *S. cerevisiae* as the dominant alcohol producing yeast in fermented products worldwide (Chakrabarty, 2017; Xie *et al*., 2007). However, the isolates of *Saccharomycopsis fibuligera* had not been identified from starter cultures of Assam. It is significant as there are a handful of reports which suggests an important role of *S. fibuligera* in the fermentation process. Earlier, Piršelová *et al*. (1993) suggested the co-culturing of *S. fibuligera* and *S. cerevisiae* for fermentation since *S. fibuligera* carries out the breakdown of starch into reducing sugars. These reduced sugar are then taken up by *S. cerevisiae* for ethanol production (Piršelová *et al*., 1993). Lee *et al*. (2018) identified and quantified the bio-formation of various volatile and non-volatile metabolites by *S. fibuligera* KJJ81 strain (Mi Lee *et al*., 2018). Pandian *et al*. (2016) carried out the co-cultivation of *S. fibuligera* NCIM 3161 and *Zymomonas mobilis* MTCC 92 and successfully achieved 93.75% of the theoretical yield of ethanol production using cassava peels (Pandian *et al*., 2016).

It was generally assumed that three types of fungi were associated with *xaj pitha, suzen* and *apong*: Amylolytic fungi and yeast; alcohol producing yeasts and other fungi that acts as either flavour and odour enhancers or contaminants. Fermented rice batter itself contains 1.6 to 2.3 log10 cfu/g yeasts count (Angadi et al, 2021). Xie *et al*. (2007) identified species of *A. oryzae* and *A. niger* in wheat Qu, a Chinese alcoholic drink and recorded amylolytic activity of the former. Recently, a report from Korea indicated that the contents of all 18 amino acids detected were the highest in *makgeolli* fermented by *S. fibuligera* CN2601-09 and increased after combining with *A. oryzae* CN1102-08, unlike the contents of most fatty acids (Son *et al*., 2018). The studies clearly indicate that much work is to be done to understand the dynamics of filamentous fungi and metabolites produced by them. Also, the growth kinetics of these fungi have to be explored while in the fermentation systems (Abdul Manan and Webb, 2018). Yeast like *S. fibuligera* degrades starch and produce glucose upon which *S. cerevisiae* readily act to produce ethanol (Tamang and Sarkar, 1995; Tamang, 2016). Considering the presence of *C. tropicalis,* which is widely considered the second most virulen *Candida* species, preceded by *C. albicans* (Zuza-Alves *et al*., 2017) it can inferred that presence of this pathogenic fungus may lead to health hazard of the rural consumers.

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). Interestingly the domination of *S. cerevisiae, S. fibuligera* and *Candida* spp. in rice beer starter culture of Assam is in agreement with Sikkimese rice wine starter *Marcha* (Tsuyoshi *et al*., 2005). Hence it is hypothesized that the yeast species associated with rice wine starter used in Himalaya regions particularly Assam and Sikkim (Himalaya biodiversity hotspot) are distinctly different from the starter used in Indo-Burma Biodiversity hotspot (includes Manipur, Vietnam and Indonesia of south eastern Asia).

CONCLUSION

The controlled fermentation processes is the main technology of fermentation industries. Selection of local effective yeast and filamentous fungal isolates and their maintenance for producing local alcoholic beverages can lead to the development of a small scale industry in the rural North-East states of India. Quality control along with this tradition specific fermentation has a potential to attract the consumers of international market. The current study revealed some good quality fermenting agents of the Assam state of India. Saccharomyces and Saccharomycopsis together in fermentation produce alcohol from sugars and break down complex sugars into simpler form, respectively. The native isolates along with the tribe specific fermentation recipes, their microbiota and herbs used should be explored extensively to develop marketable products.

Conflict of interest: None.

REFERENCES

Abdul Manan, M., and Webb, C. (2018). Estimating fungal growth in submerged fermentation in the presence of solid particles based on colour development. Biotechnology and Biotechnological Equipment. https://doi.org/10.1080/13102818.2018.1440974.

Angadi, V., Ramachandran, B., Puranik, D.B. and Prabha, R. (2021). enumeration of microflora from ingredients and Idli Batter. Asian Journal of Dairy and Food Research. 40(3): 327-331.

Barnett, J.A., Payne, R.W. and Yarrow, D. (2000). Laboratory Methods for Identifying Yeasts. In Yeasts Charact. Identification (3rd ed). Cambridge Univ. Press.

Barnett, J.A., Payne, R.W., and Yarrow, D. (1983). Yeasts: Characteristics and Identification. Cambridge University Press.

Chakrabarty, J. (2017). Identification of Yeast Strain Isolated from Nduiyi of Dima Hasao District of Assam, India. International Journal of Current Microbiology and Applied Sciences. https://doi.org/10.20546/ijcmas.2017.603.068.

Dung, N.T.P., Rombouts, F.M., and Nout, M.J.R. (2006). Functionality of selected strains of moulds and yeasts from Vietnamese rice wine starters. Food Microbiology. https://doi.org/10.1016/j.fm.2005.05.002.

Ghosh, S., Rahaman, L., Kaipeng, D.L., Deb, D., Nath, N., Tribedi, P., and Sharma, B.K. (2016). Community-wise evaluation of rice beer prepared by some ethnic tribes of Tripura. Journal of Ethnic Foods. https://doi.org/10.1016/j.jef.2016.12.001.

Jeyaram, K., Singh, W.M., Capece, A., and Romano, P. (2008). Molecular identification of yeast species associated with

'Hamei'-A traditional starter used for rice wine production in Manipur, India. International Journal of Food Microbiology. 124(2): 115-125.

Jolly, N.P., Varela, C., and Pretorius, I.S. (2014). Not your ordinary yeast: Non-Saccharomyces yeasts in wine production uncovered. In FEMS Yeast Research. https://doi.org/10.1111/1567-1364.12111.

Kitamura, Y., Kusumoto, K.I., Oguma, T., Nagai, T., Furukawa, S., Suzuki, C., Satomi, M., Magariyama, Y., Takamine, K., and Tamaki, H. (2016). Ethnic Fermented Foods and Alcoholic Beverages of Japan. In: Ethnic Fermented Foods and Alcoholic Beverages of Asia. Springer. pp. 193-236.

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis Version 7.0 for Bigger datasets. Molecular Biology and Evolution. 33(7): 1870-1874. https://doi.org/10.1093/molbev/msw054.

Lodder, J., and Kreger-Van, R.I.J. (1952). The yeasts-a taxonomic study. The Yeasts-a Taxonomic Study.

Mi Lee, S., Jung, J.H., Seo, J.A., and Kim, Y.S. (2018). Bioformation of volatile and nonvolatile metabolites by *Saccharomycopsis fibuligera* kjj81 cultivated under different conditions-carbon sources and cultivation times. Molecules. https://doi.org/10.3390/molecules23112762.

Padilla, B., Gil, J.V., and Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: From spoilage micro organisms to biotechnological tools for improving wine aroma complexity. In Frontiers in Microbiology. https://doi.org/10.3389/fmicb.2016.00411.

Pandian, C.A., Suganya, C., Sivamani, S., and Baskar, R. (2016). Saccharification and single step fermentation of cassava peel by mixed culture of *Saccharomycopsis fibuligera* ncim 3161 and zymomonas mobilis MTCC 92. American Journal of Biomass and Bioenergy. https://doi.org/10.7726/ajbb.2016.1005.

Pervez, S., Aman, A., Iqbal, S., Siddiqui, N.N., and Ul Qader, S.A. (2014). Saccharification and liquefaction of cassava starch: An alternative source for the production of bioethanol using amylolytic enzymes by double fermentation process. BMC Biotechnology. https://doi.org/10.1186/1472-6750-14-49.

Piršelová, K., Šmogrovièová, D., and Baláž, S. (1993). Fermentation of starch to ethanol by a co-culture of *Saccharomycopsis fibuligera* and *Saccharomyces cerevisiae*. World Journal of Microbiology and Biotechnology. https://doi.org/10.1007/BF00383075.

Qi, K., Xia, X.X., and Zhong, J.J. (2015). Enhanced anti-oxidative activity and lignocellulosic ethanol production by biotin addition to medium in *Pichia guilliermondii* fermentation. Bioresource Technology. https://doi.org/10.1016/j.biortech.2015.02.089.

Raper, K.B., and Thom, C. (1949). A manual of the Penicillia. A Manual of the Penicillia.

Ribeiro, L.S., Duarte, W.F., Dias, D.R., and Schwan, R.F. (2015). Fermented sugarcane and pineapple beverage produced using *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast. Journal of the Institute of Brewing. https://doi.org/10.1002/jib.218.

Sadineni, V., Kondapalli, N., and Reddy Obulam, V.S. (2012). Effect of co-fermentation with *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* or *Metschnikowia pulcherrima* on the aroma and sensory properties of mango wine. Annals of Microbiology. https://doi.org/10.1007/s13213-011-0383-6.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L. *et al.* (2012). Nuclear Ribosomal Internal Transcribed Spacer (ITS) Region as a Universal DNA Barcode Marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America. https://doi.org/10.1073/pnas.1117018109.

Son, E.Y., Lee, S.M., Kim, M., Seo, J.A., and Kim, Y.S. (2018). Comparison of volatile and non-volatile metabolites in rice wine fermented by Koji inoculated with *Saccharomycopsis fibuligera* and *Aspergillus oryzae*. Food Research International. https://doi.org/10.1016/j.foodres.2018.05.008.

Spencer, J., Rawling, S., Stratford, M., Steels, H., Novodvorska, M., Archer, D.B., and Chandra, S. (2011). Yeast identification: Reassessment of assimilation tests as sole universal identifiers. Letters in Applied Microbiology. https://doi.org/10.1111/j.1472-765X.2011.03130.x.

Suárez-Lepe, J. A., and Morata, A. (2012). New Trends in Yeast Selection for Winemaking. In: Trends in Food Science and Technology. https://doi.org/10.1016/j.tifs.2011.08.005.

Subramanian, C.V. (1971). Hyphomycetes. An account of Indian species, except Cercosporae. Hyphomycetes. An Account of Indian Species, except Cercosporae.

Tamang, J.P., and Sarkar, P.K. (1995). Microflora of murcha: An amylolytic fermentation starter. Microbios. 81(327): 115-122.

Tamang, Jyoti Prakash. (2016). Ethnic fermented foods and alcoholic beverages of Asia. In Ethnic Fermented Foods and Alcoholic Beverages of Asia. https://doi.org/10.1007/978-81-322-2800-4.

Thom, C., and Church, M.B. (1926). The aspergilli. The Aspergilli. Tsuyoshi, N., Fudou, R., Yamanaka, S., Kozaki, M., Tamang, N., Thapa, S., and Tamang, J.P. (2005). Identification of yeast strains isolated from marcha in Sikkim, a microbial starter for amylolytic fermentation. International Journal of Food Microbiology. https://doi.org/10.1016/j.ijfoodmicro.2004.08.011.

Wickerham, L.J., Lockwood, L.B., Pettijohn, O.G. and Ward, G.E. (1944). Starch Hydrolysis and Fermentation by the Yeast Endomycopsis Fibuliger. Journal of Bacteriology.

Xie, G.F., Li, W.J., Lu, J., Cao, Y., Fang, H., Zou, H.J., and Hu, Z.M. (2007). Isolation and identification of representative fungi from shaoxing rice wine wheat Qu using a polyphasic approach of culture-based and molecular-based methods. Journal of the Institute of Brewing. https://doi.org/10.1002/j.2050-0416.2007.tb00287.x.

Zhang, B.Q., Shen, J.Y., Duan, C.Q., and Yan, G.L. (2018). Use of indigenous Hanseniaspora vineae and Metschnikowia pulcherrima Co-fermentation with *Saccharomyces cerevisiae* to improve the aroma diversity of Vidal blanc icewine. Frontiers in Microbiology. https://doi.org/10.3389/fmicb.2018.02303.

Zuza-Alves, D.L., Silva-Rocha, W.P., and Chaves, G.M. (2017). An update on *Candida tropicalis* based on basic and clinical approaches. In Frontiers in Microbiology. https://doi.org/10.3389/fmicb.2017.01927.