Assessment of microbial quality and health risks associated with traditional rice wine starter *Xaj-pitha* of Assam, India: a step towards defined and controlled fermentation

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Abstract

This study reports the microbial quality of ethnic starter culture *Xaj-pitha* used for rice wine fermentation in Assam. Here, we collected 60 *Xaj-pitha* samples belonging to *Ahom* community of the state and enumerated the microorganisms using spread plate technique. Illumina-based whole genome shotgun sequencing detected the presence of microbial contaminants like *Acidovorax*, *Herbaspirillum*, *Methylobacterium*, *Pantoea*, *Pseudomonas*, *Stenotrophomonas*, *Staphylococcus*, *Micrococcus*, *Acinetobacter*, etc. Presence of major health hazards associated with spontaneous rice wine fermentation necessitated method optimization through the development of a defined mixed starter culture. For this, functionally important α -amylase producers viz., *Penicillium* sp. ABTSJ23, *Rhizopus oryzae* ABTSJ63, *Mucor guilliermondii* ABTSJ72 and *Amylomyces rouxii* ABTSJ82 and eight yeasts viz., *Saccharomyces cerevisiae* ABTY1J, ABTY1S, ADJ5 & ADJ1, *Wickerhamomyces anomalus* ADJ2, *Saccharomycopsis malanga* ADJ3, *Saccharomycopsis fibuligera* ADJ4 and *Saccharomycopsis malanga* ADJ6 were retrieved using appropriate media. All the mould cultures tested negative for aflatoxins production. Among the yeasts, *Saccharomyces cerevisiae* ABTY1S and ADJ1 decarboxylated lysine HCl and tyramine HCl, respectively, indicating their biogenic amine production ability. For defined mixed starter culture, *Amylomyces rouxii* ABT82 with α -amylase (5.92 U/ml) and glucoamylase (7.50 U/ml) activities was selected as fungal partner; while *Saccharomycopsis fibuligera* ADJ4 and *Saccharomyces cerevisiae* ABT-Y1J with high ethanol production (up to 10.11% and 9.88% v/v, respectively) were selected as yeast partners. The mixed culture was able to produce high amount of glucose, ethanol and liquid (glucose 10.91% w/v; ethanol 7.5% w/v; liquid 51.0% w/v). Therefore, this study demonstrated the efficiency of mixed starter cultures for safe and controlled rice wine production.

Keywords Mould · Yeast · Contaminant · Aflatoxins · Biogenic amine

Introduction

Since time immemorial, rice-based alcoholic beverages like *Xaj-pani* or *Lao-pani* have been produced by the various indigenous communities of Assam, a northeastern state of

India. *Xaj-pani* or *Xaj* is intricately associated with the social and religious belief system of the *Ahoms* or *Tai-Ahoms*, an ethnic community of Assam (Saikia et al. 2007). The traditional fermentation process of *Xaj* involves parboiling rice followed by mixing with the fermentation starter culture, *Xaj pitha*. The starter cultures involved in the fermentation of the cereal-based alcoholic beverages are the consortia of starch-degrading moulds (fungi), alcohol-producing yeasts and lactic acid bacteria (Lim 1991; Saono et al. 1996) maintained on substrates like rice powder supplemented with various herbs. These prevalent microbes carry out the traditional fermentation of alcoholic beverages through a two-step process; enzymatic breakdown and hydrolysis of rice starch to glucose by fungi and subsequent sugar fermentation to ethanol by yeasts (Nout and Aidoo 2002).

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During the initial stage of fermentation, i.e. saccharification and liquefaction, rice starch is broken down into dextrins by the action of α -amylase and subsequently into glucose by the action of glucoamylase (Dung et al. 2005). Subsequently, fermentable glucose produced in the previous step is metabolized by yeasts to produce ethanol (Limtong et al. 2002).

Although the methodology of *Xaj pitha* and *Xaj* preparation is almost similar among the *Ahoms* of Assam, spatial differences in terms of microbiota, substrate and herbal concoctions can be observed resulting in variations in the quality of the final product. Therefore, the use of improved defined-mixed starter cultures is of increasing interest for controlled production of alcoholic beverages with more complex organoleptic characteristics (Schlander et al. 2017). The criteria for the selection and development of yeasts and moulds for fermentation have evolved over the years and have been discussed by several workers (Bisson 2004; Schuller and Casal 2005). Nevertheless, the major criteria for selecting an amylolytic fungus to be the main fungal flora in a defined mixed starter culture are (i) non-toxicity to human and animal health and (ii) compatibility with the fermenting agent, i.e. the yeast. In case of yeasts, parameters viz., growth characteristics, fermentative efficiency, agglutination capacity, temperature, sugar and ethanol tolerance have been considered as physiological conditions for efficient fermentation (Zott et al. 2008; Soares 2010). Besides ethanol and aroma producing compounds, yeasts are also reported to produce sulphites and biogenic amines in the fermentation medium which adversely affect the health of consumers (Swiegers et al. 2005). Therefore; monitoring, screening and elimination of such contaminating microorganisms are necessary for the development of a defined mixed starter culture.

In the present work, we focused on three major objectives: (i) determining contaminating microbes along with the dominant and functional microflora in *Xaj-pitha*, (ii) isolating and identifying efficient cultivable mould and yeast strains, (iii) screening out moulds and yeasts with food-safety features for potential applications in defined mixed-culture fermentation starter cultures.

Materials and methods

Target community

Root of the *Ahoms* tracks to the *Tai-Shan* or *Tai-Shwan* family of Burma. The community entered Assam through Patkai range under an adventurous leader Siu-Ka-Pha (Kakati 1941). With a population of nearly 1.3 million, *Ahom* people are mainly concentrated in the districts of Sivasagar, Jorhat, Golaghat, Dibrugarh, Tinsukia, Lakhimpur, and Dhemaji.

Sample collection

Samples of *Xaj pitha* were collected from community pockets distributed in the six districts of upper Brahmaputra valley of Assam viz., Sivasagar, Jorhat, Dibrugarh, Tinsukia, Dhemaji and Lakhimpur (Fig. 1) through a random cluster sampling design. For this, 2 clusters from each district were selected (total 12 clusters) and 5 samples (Fig. 2) from each cluster were collected (total 60 samples). The collected samples were carried to the laboratory on ice packs and immediately used for microbial analysis.

Determination of microbial diversity associated with *Xaj-pitha*

Out of the 60 starter cultures collected from the *Ahom* rural households, 5 starters from 1 individual cluster were mixed equally to form a composite sample under sterile laboratory conditions (i.e. 2 composite samples from each district). For microbial diversity study, 1.0 g powdered composite sample was transferred to a test tube containing 9 ml sterile saline (NaCl 0.85%) and homogenized properly for 1 min. Appropriate serially diluted samples were spread on various suitable fastidious and nonfastidious media viz., nutrient agar, potato dextrose agar, yeast extract peptone dextrose agar, MRS *Lactobacillus* agar, plate count agar and *Enterococcus* confirmatory agar, and incubated at an ambient temperature (28–30 °C) for 2–3 days. The colonies that appeared after incubation were enumerated and expressed as colony forming units (CFU) per gram of dry weight samples.

A representative composite sample from the Titabar Subdivision located in the Jorhat district was analyzed for illumina-based metagenomics approach. Briefly, genomic DNA was extracted from 1.0 g sample using Environmental gDNA isolation kit (Xcelgen, India) followed by quantification through a Qubit fluorometer. Starting with fragmentation and ligation, a paired-end sequencing library was prepared using illumina TruSeq DNA Library Preparation Kit. Purified ligated products with a size range of 500–800 bp were further selected for library amplification and 10 pM of the amplified library was loaded onto illumina MiSeq for cluster generation and sequencing. The metagenome reads were assembled using CLC workbench (CLC bio, Denmark) at default parameter (minimum contig length 200) for trimming and de novo assembly (Chan et al. 2012). Taxonomic classification was performed by BLASTN (absolute cutoff: BLAST bitscore 86, relative cutoff: 10% of the top hit) against SILVA SSUref and LSUref databases (release 108) (Urich et al. 2008), followed by annotation of BLAST output files using MEGAN

Fig. 1 Geographical location of Assam, India. Red spots on the actual map showing locations of sample collection—Tinsukia, Dibrugarh, Sivasagar, Jorhat, Dhemaji and Lakhimpur districts of Assam

Fig. 2 Samples of fermentation starter culture *Xaj pitha* used for microbial analysis

v.5.2.3 (Huson et al. 2007). MEGAN uses the lowest common ancestor (LCA) algorithm for assigning rDNA or rRNA sequences to the lowest common ancestor in the taxonomy from a subset of the best scoring matches in the BLAST result (Urich et al. 2008). The taxonomical approach places reads directly onto the leaves of the NCBI taxonomy representing different species and strains. The MG-RAST (Meta Genome Rapid Annotation using Sub-system Technology, v3.1) server at the Argonne National

Library (<https://metagenomics.nmpdr.org>) was used for functional analyses. A single similarity search entry at this server provides results from several databases like NCBI-nr, KEGG, SEED, egg-NOG, COG, etc.

Microbial population dynamics

Microbial dynamics during fermentation was observed following the method of Dung et al. (2005). For this, 2.0 g of

composite starter samples was mixed with 50.0 g of gelatinized glutinous rice (*Bora* variety). Fermenting samples (1 g/1 ml) were regularly withdrawn from day 1 to day 2 during solid-state fermentation and from day 3 to day 5 from submerged alcoholic fermentation, and were serially diluted. Inoculants of 50 µl at a dilution range of 10^{-3} to 10^{-8} were plated to various aforementioned media and counted for CFU after 2–3 days of incubation. Further, the mould isolates were repeatedly sub-cultured for characterization and applications in the defined starter culture. For yeasts, the morphologically different colonies were picked up from yeast extract peptone dextrose (YPD) agar medium and were repeatedly sub-cultured to obtain pure culture. These strains were further examined under a light compound microscope to confirm their distinctiveness.

Isolation and identification of moulds and yeasts

Screening of starch hydrolyzing moulds was performed following the method described by Williams et al. (1970). The solid-based method for screening of amylase production or secretion was carried out on nutrient agar supplemented with starch in place of dextrose. The mould strain was pinpoint-inoculated onto this media at the centre of the Petri plate. After incubation, the plate was flooded with iodine solution (KI: 2% w/v, I_2 : 1% w/v, dissolved in distilled water) which reveals a clear zone around the inoculum indicating degradation of starch into glucose by the action of microbial amylase. Morphological, biochemical and physiological characterization of the yeast isolates were carried out following standard protocols (Kreger-van Rij 1984; Kurtzman and Robnett 1998). Yeast isolates were preliminarily identified based on carbohydrate utilization profile on the API ID 32C (bioMérieux, Lyon, France) kit. Molecular characterization of the yeast and mould strains was performed by amplifying and sequencing the internal transcribed spacer region (ITS) of 5.8S and 28S rRNA gene.

Functionality and safety tests

Amylase activity was determined for the amylase positive fungal strains using glutinous rice grain as substrate under solid state fermentation (SSF) conditions (Gessesse and Mamo 1999). Glutinous rice (5.0 g) was soaked in 15 ml distilled water (in a 50 ml conical flask covered by a cotton plug) for 1 h at room temperature. The soaked rice was steamed and allowed to cool to 30–35 °C. Sterile 15 ml of nutrient salt solution (NSS) (5.0 g/l KH_2PO_4 , 5.0 g/l NH_4NO_3 , 1.0 g/l NaCl and 0.5 g/l $MgSO_4$) was added to it. The cooked rice was then inoculated with one disc of fungal isolates (a diameter of 5 mm), and subsequently incubated at 30 °C. After 3 days of incubation, 15 ml of distilled water was added. The contents were then crushed and agitated for

10 min in 55 °C on a rotary shaker at 200 rpm. The slurry obtained was squeezed through four layers of muslin cloth. The extract was filtered with Whatman filter paper no. 1 (Whatman, Maidstone, England) and then centrifuged at 5000 rpm for 10 min. The filtrate obtained was treated as crude enzyme and amylase activity was determined by DNS method at 540 nm and reported as U/ml using glucose as a standard. One unit of amylase is defined as the amount of enzyme which releases 1 micromole of reducing sugar per minute (U/mL), with glucose as standard (Miller 1959). Glucoamylase activity was determined by incubating a mixture of 0.5 ml of the enzyme aliquots with soluble starch (1% dissolved in 0.1 M sodium acetate buffer, pH 5) at 55 °C for 15 min. One unit (U) of glucoamylase activity is defined as the amount of enzyme that releases 1 micromole of reducing sugar as glucose, per minute, under the assay conditions and expressed as U/g of dry substrate (gds) (Miller 1959). Activities of amylase and glucoamylase during solid state fermentation (SSF) under different pH (4–7.5), incubation temperature (20 °C to 40 °C) and time (24 h to 120 h) were determined. Production of aflatoxins by the mould isolates was assessed through ELISA analysis according to the instructions in the Neogen Veratox aflatoxin protocol (Kim et al. 1998). The limit of detection of aflatoxin was set at 20 ppb and less than that range was regarded as atoxigenic.

For yeasts, growth and population dynamics were tested by measuring cell number (CFU counts) in YPD agar up to 48 h at 30 °C. Total viable counts (CFU/ml) were determined and specific growth rate constant for each culture was calculated following Kalscheuer et al. (2006). Further, the isolates were tested for ethanol fermentation (Thammasittirong et al. 2013), ethanol tolerance and osmo-tolerance (Ali and Khan 2014), temperature tolerance, flocculation capacity (Soares and Mota 1996). A reference *Saccharomyces cerevisiae* (ATCC culture 9763) was kept in all experiments for identification and characterization of the yeast isolates under study. Production of biogenic amines by yeast isolates was qualitatively assessed following standard method with minor modification (Bover-Cid and Holzapfel 1999). For inducing decarboxylase production, the yeast isolates were repeatedly sub-cultured in yeast extract potato dextrose broth medium supplemented individually with tyrosine-free base, histidine monohydrochloride, ornithine monohydrochloride and lysine monohydrochloride. Pyridoxal-5-phosphate (0.005%) which acts as co-factor for decarboxylase enzyme was also added in the media. The screening medium used to detect decarboxylating yeasts contained tryptone (0.5%), yeast extract (0.5%), glucose (0.05%), thiamine (0.001%), NaCl (0.5%), Tween 80 (0.1%), $MgSO_4 \cdot 7H_2O$ (0.02%), $CaCO_3$ (0.01%), $MnSO_4 \cdot 4H_2O$ (0.005%), $FeSO_4 \cdot 7H_2O$ (0.004%), bromocresol purple (pH indicator, 0.006%), agar (2%) and a 2% concentration of a particular precursor amino acid (tyrosine free base, histidine monohydrochloride,

tyramine monohydrochloride and lysine monohydrochloride). The pH of the final medium was adjusted to 5.3 and autoclaved for 10 min at 121 °C. The streaked yeast strains were incubated at 32 °C for 3 days.

Statistical analysis for the selection of efficient yeast

Pearson's correlation statistical analysis was performed to investigate the relationship among the important growth parameters viz., temperature, alcohol tolerance, alcohol production, osmotolerance, biogenic amines and growth rate that are essential for the production of alcohol. This correlation is helpful for identification of favorable parameters for large scale production of alcohol.

Compatibility test

The selected yeast and fungal strains were grown on slants of malt extract agar medium at 30 °C for 2 days and at 30 °C for 5 days, respectively. The mature cultures were tested for their compatibility as follows: glutinous rice (50 g) was soaked in 60 ml distilled water (in a 250 ml conical flask covered by a cotton plug) for 4 h at 22 °C. The soaked rice was steamed and allowed to cool to 35–40 °C, after which it was inoculated with pure cultures as well as a control

without inoculum. These were incubated for 3 days at 30 °C (Dung et al. 2005).

Results and discussion

Microbial load and population dynamics of *Xaj-pitha*

Microbial diversity and dynamics of *Xaj-pitha* was assessed through the culture-dependent approach. The starters contained high counts of fungi, lactic acid bacteria (LAB) and aerobic bacteria (Table 1). Changes in different microbial populations viz., fungus, yeast, lactic acid bacteria and enterobacteria were noted during *Xaj* fermentation. During the 7 days of fermentation period, the counts for yeast and lactic acid bacteria increased to 4.06 log₁₀ CFU/g and 5.52 log₁₀ CFU/g, respectively, from the initial 3.24 log₁₀ CFU/g and 3.63 log₁₀ CFU/g with the highest counts observed on the 4th and 5th days of fermentation (Fig. 3).

The amylolytic fungi that play an important role in saccharification of the rice-starch were active during the initial phase of fermentation. Fungal population decreased after the 5th day of fermentation with the highest CFU value of 5.3 log₁₀ CFU/g on the 4th day. The enterobacterial population increased slightly (from 0.33 to 1.3 log₁₀ CFU/g) during the

Table 1 District-wise colony forming units (CFU) counts (average of counts from starter cultures) of microbes

District (state of Assam)	Cluster name	CFU count for fungi (log ₁₀ CFU/g)	CFU count for yeasts (log ₁₀ CFU/g)	CFU count for LAB (log ₁₀ CFU/g)	CFU count for aerobic mesophiles (log ₁₀ CFU/g)	CFU count for enterobacteria (log ₁₀ CFU/g)
Tinsukia	Margherita	2.3	6.8	8.1	3.1	1.1
	Sadiya	2.5	7	7.6	1.2	0.83
	District average	2.4	7.9	7.85	2.15	0.965
Dibrugarh	Moran	2.9	7.8	7.5	2.4	1.2
	Naharkatiya	3.9	7.3	8.1	2.5	0.97
	District average	3.4	7.55	7.8	2.45	1.085
Sivasagar	Bokota	3.2	8.2	8.1	1.5	0.56
	Sonari	4.2	8.6	7.9	2.3	0.11
	District average	3.7	8.4	8	1.9	0.335
Jorhat	Titabar	2.9	7.6	9	1.3	1.1
	Nimati	3.5	7.9	8.4	1.7	1.2
	District average	3.2	7.75	8.7	1.5	1.15
Lakhimpur	Bihpuria	3.8	7.3	8	2.1	1.4
	Dhakuakhana	3.1	7.89	7.7	1.9	1.2
	District average	3.45	7.595	7.85	2	1.3
Dhemaji	Bordoloni	3.7	7.6	8.1	2.4	0.98
	Silapathar	2.9	8	7.9	3	1.2
	District average	3.3	7.8	8	2.7	1.09
Range of CFU		2.4–3.7 (log ₁₀ CFU/g)	7.5–8.4 (log ₁₀ CFU/g)	7.8–8.7 (log ₁₀ CFU/g)	1.5–2.7 (log ₁₀ CFU/g)	0.33–1.3 (log ₁₀ CFU/g)

Fig. 3 Microbial dynamics during *Xaj* fermentation

initial period of fermentation (up to day 3) and then finally disappeared on day 4. The decrease in pH from 6.0 to 3.3 might be due to the increased lactic acid production by lactic acid bacteria until the end of fermentation and that might have inhibited the growth of enterobacterial population. The temperature remained relatively constant at 25–30 °C.

In case of *Bhaati Jaanr*, a mild alcoholic drink of Sikkim and Himalayan region, an exponential increase (from 10^6 to 10^7 CFU/g) in the load of LAB was observed till day 2 and a decrease to 10^5 CFU/g onwards up to day 10. The fungal population continually decreased and disappeared completely on the 5th day of fermentation (Tamang and Thapa 2006). In case of traditional fermented rice-based beverage *Haria* of West Bengal, the population of aerobic bacteria was higher ($10.51 \log_{10}$ CFU/g) at the beginning of fermentation and dominated up to 3rd day. Similarly, the number of yeast and fungi was $2.38 \log_{10}$ CFU/g and $3.34 \log_{10}$ CFU/g at the beginning, and reached a maximum level ($7.64 \log_{10}$ CFU/g and $9.38 \log_{10}$ CFU/g) on the 2nd day, and thereafter

declined significantly. The abundance of fungi and yeast in the initial phase of the fermentation is due to the presence of aerobic conditions (Ghosh et al. 2015).

Major microbial contaminants associated with *Xaj-pitha*

Illumina platform-based WGS of the composite sample from one cluster produced a total of 278,231 sequences (178,540,333 base-pairs) with an average read length of 641 bp. Out of these sequences, 5302 sequences (about 1.9%) failed to qualify the CLC quality control procedure. After removing 1240 numbers of artificial duplicate reads (ADRs), a total of 272,929 sequences (143,119,277 base pairs) with sequence length of 524 ± 397 were considered further for downstream analysis. A total of 590 curated sequences harbored ribosomal RNA genes useful for taxonomic classification; 119,194 sequences (47.30%) contained predicted proteins with known functions, and 132,223

sequences (52.47%) contained predicted proteins with unknown function. MG-RAST-based phylotyping revealed that at domain level, 68,913 sequences (64.11%) belonged to eukaryota, 38,287 sequences (35.62%) belonged to bacteria, 255 sequences (0.24%) to viruses and 14 sequences (0.01%) to archaea. The MEGAN taxonomic analysis assigned the maximum contigs to *Rhizopus delemar* (6660 contigs), *Mucor circinelloides* (6082 contigs), *Lactobacillus plantarum* (2194 contigs), *Meyerozyma guilliermondii* (1693 contigs), *Stenotrophomonas maltophilia* (1644 contigs), *Wickerhamomyces ciferrii* (1304 contigs), *Lactobacillus brevis* (1054 contigs). The assembled contigs were subjected to annotation (Fig. 4).

The bacterial microflora was found to be dominated by the genus *Lactobacillus* (7.2%) with members like *Lactobacillus plantarum*, *Lactobacillus brevis*, *Leuconostoc lactis*, *Weissella cibaria*, *Lactococcus lactis*, *Weissella para mesenteroides*, *Leuconostoc pseudomesenteroides*, etc. Among the group of lactic acid bacteria, the genus *Lactobacillus* has the highest representation and consists of a highly diverse group of Gram-positive, microaerophilic bacteria that microscopically appear as long to short rods or even coccobacilli. These are split into three groups based on the carbohydrate fermentation pathways: (1) obligate homofermentative (they produce only lactic acid from sugars through the Embden-Meyerhof pathway); (2) facultative heterofermentative and

(3) obligate heterofermentative lactobacilli (they can produce either alcohol or lactic acid from sugars through the phosphoketolase pathway). Few lactic acid bacteria (LAB) are considered to be main functional contributors in proteolysis, lipolysis, and amino acid/lipid catabolism (De et al. 2016), thereby concentrating and fortifying nutrients (minerals, vitamins, and essential amino acid synthesis) (Holzapfel 1997) and therapeutic components (phenolics, maltooligomers, prebiotics, probiotics, antioxidants, and antimicrobials). In this process, these bacteria also degrade undesirable components (antinutrient, mycotoxin, and other endotoxins), and modify organoleptic qualities (taste, aroma, texture, consistency, and appearance) (Holzapfel 2002). Lactic acid and acetoin generated during fermentation impart a tart taste and a typical flavor, thus influencing sensory qualities of the end product (Aidoo et al. 2006).

Major amylolytic moulds detected were *Rhizopus delemar*, *Mucor circinelloides* and *Aspergillus* sp. Out of these, *Mucor circinelloides* has been identified as the causal pathogen of primary invasive cutaneous and maxillofacial zygomycosis (Khan et al. 2009). Therefore, *Mucor circinelloides* could be a potential human health risk factor associated with ethnic starter culture. Along with the amylolytic moulds, various yeasts such as *Saccharomyces cerevisiae*, *Meyerozyma guilliermondii*, *Wickerhamomyces ciferrii*, *Candida glabrata*, *Debaryomyces hansenii*, *Ogataea parapolyomorpha*

Fig. 4 Microbial genus abundance ordered from the most abundant to least abundant. Only the top 50 most abundant are shown. The Y-axis plots the abundances of annotations in each genus on a log scale

and *Dekkera bruxellensis* were found to be abundant in *Xaj pitha*.

Ethnic fermentation process is carried out at rural household level where hygiene is often compromised. Metagenomics analysis revealed that ethnic *Xaj-pitha* samples harbored several plant pathogens like *Acidovorax avenae*, *Herbaspirillum seropedicae*, *Pantoea*, *Methylobacterium*, *Sphingomonas*, *Xanthomonas*, etc. The plants used for organoleptic augmentation or clinical contribution bear phyloplane microbial loads along with several plant pathogens (Dele'toile et al. 2009). Other environmental contaminants included *Pseudomonas aeruginosa*, *P. fluorescens*, *P. stutzeri*, *Stenotrophomonas maltophilia*, etc. Several opportunistic human skin commensals, such as *Staphylococcus* sp., *Micrococcus* sp., *Microbacterium* sp., *Acinetobacter guillouiae*, etc. were also detected (Fig. 5). *Acinetobacter guillouiae*, an opportunistic pathogen, can cause life-threatening diseases in immune-compromised patients (Dijkshoorn et al. 2007; Peleg et al. 2008). *Stenotrophomonas maltophilia*, a Gram-negative, aerobic, glucose non-fermenting contaminant has also been reported to cause nosocomial infections in immune-compromised patients (Kalidasan et al. 2018). These microbes are often opportunistic contaminants and deteriorate the product quality. The deteriorated product emanates a foul smell and has hazy appearance.

Isolation and screening of useful moulds

Twenty-two morphologically different mould strains were isolated from the starter cultures and segregated based on their performance in starch utilization. Of the 22 strains, 13 were found to produce extracellular amylases. Finally, four strains viz., ABTSJ23, ABTSJ63, ABTSJ72 and ABTSJ82 were selected for further consideration based on the sizes of

the radius of visible clear zone on starch agar medium upon iodine inundation. Based on morphological observation and molecular ITS sequencing, the four selected moulds were identified as *Penicillium* sp. ABTSJ23, *Rhizopus oryzae* ABTSJ63, *Mucor guilliermondii* ABTSJ72 and *Amylomyces rouxii* ABTSJ82 (NCBI accession no. KP790012–KP790015 respectively).

Amylase activity of the selected strains

Alpha-amylase activity of the fungal strains ranged from 5.92 to 23.16 U/ml. It was observed that *Penicillium* sp. ABTSJ23 produced significantly highest α -amylase (23.16 U/ml) and the *Amylomyces rouxii* ABTSJ82 (5.92 U/ml) lowest. *Penicillium* sp. ABTSJ 23 exhibited highest alpha-amylase activity at pH 6.5 and at the temperature 40 °C after an incubation period of 120 h (Fig. 5i–iii).

All the strains showed considerable glucoamylase which is important for breakdown of rice starch into glucose. *Penicillium* sp. ABTSJ23 was observed to have highest glucoamylase activity (15.9 U/ml) followed by *Mucor guilliermondii* ABTSJ72 (8.62 U/ml). Highest glucoamylase activity of fungus *Penicillium* sp. ABTSJ23 was found at 40 °C after 120 h. The strain ABTSJ82 exhibited highest glucoamylase activity of 7.5 U/ml at pH 4.5 after 96 h. Optimum temperature for glucoamylase activity of ABTSJ82 was at 40 °C (Fig. 6i–iii).

Isolation and identification of cultivable yeast

Yeast cultures were identified based on colony characters, microscopic examination and bud formation. Based on the above parameters eight yeast isolates were selected and designated as ABT-Y1S, ABT-Y1J, ADJ1, ADJ2, ADJ4,

Fig. 5 i–iii α -amylase activity of mould strains at different pH, incubation period and temperature; ABTSJ 23: *Penicillium* sp. ABTSJ23; ABTSJ63: *Rhizopus oryzae* ABTSJ63, ABTSJ72: *Mucor guilliermondii* ABTSJ72, ABTSJ82: *Amylomyces rouxii* ABTSJ82

Fig. 6 i–iii Glucoamylase activity of mould strains at different pH, incubation period and temperature; ABTSJ 23: *Penicillium* sp. ABTSJ23; ABTSJ63: *Rhizopus oryzae* ABTSJ63, ABTSJ72: *Mucor guilliermondii* ABTSJ72, ABTSJ82: *Amylomyces rouxii* ABTSJ82

ADJ3, ADJ5 and ADJ6. The API ID32C (bioMérieux, Lyon, France) was used for identification of the yeast strains (Table 2). Based on the results from API ID 32C profiles and molecular ITS sequencing analysis, the yeast strains were designated as *Saccharomyces cerevisiae* ABTY1J, *S. cerevisiae* ABTY1S, *S. cerevisiae* ADJ1, *S. cerevisiae* ADJ5, *Wickerhamomyces anomalus* ADJ2, *Saccharomycopsis malanga* ADJ3, *Saccharomycopsis malanga* ADJ6 and *Saccharomycopsis fibuligera* ADJ4. The assembled and curreted sequences were submitted to GenBank and assigned NCBI accession numbers viz., KF055432 (for ABTY1S), KF055433 (for ABTY1J) and KX904345–KX904350 (for ADJ1–ADJ6).

Toxicity screening of moulds and yeasts

None of the mould strains i.e. *Penicillium* sp. ABTSJ23, *Rhizopus oryzae* ABTSJ63, *Mucor guilliermondii* ABTSJ72 and *Amylomyces rouxii* ABTSJ82 produced any aflatoxins (B1, B2, G1, and G2) beyond the test limit qualifying the strains to be atoxigenic. Fungal species *Rhizopus oryzae* is generally recognized as safe by the US Food and Drug Administration (Cantabrana et al. 2015). It has been reported to bioremediate aflatoxins B1, B2 and G1 within 96 h of incubation period by approximately 100% (Hackbart et al. 2014). On the other hand, biogenic amines (BAs) are organic bases frequently found in fermented foods and beverages. The BAs are produced mainly as a consequence of decarboxylation of amino acids by certain microbes (Zaman et al. 2010). High concentration of BAs can cause undesirable physiological effects in sensitive humans, especially when alcohol and acetaldehyde are present (Landete et al. 2005). Therefore, screening of yeast strains having the ability to decarboxylate amines is an important criterion for selection of brewing yeast to reduce the potential health risk for

consumers. The potential of yeast strains to produce biogenic amines through decarboxylation reaction of tyrosine free base, histidine monohydrochloride, tyramine monohydrochloride and lysine monohydrochloride was investigated. Among the yeast strains, *Saccharomyces cerevisiae* ABTY1S and ADJ1 decarboxylated lysine HCl and tyrosine-free base, respectively, indicating possible biogenic amine production. The reference strain ATCC9763 exhibited decarboxylation of tyrosine-free base.

Statistical analysis for the selection of best fit yeast strain

The Pearson correlation analysis was carried out to study the correlation between the important parameters with the test samples including the reference sample. It was found that all the nine samples were significantly positively correlated with temperature at 20 °C, 25 °C, 30 °C and 35 °C and significantly negatively correlated at growth rate after 48 h (Table 3). The alcohol production is positively correlated with temperature at 35 °C while alcohol tolerance showed correlation with temperature at 40 °C, sugar concentration at 10% and 30%. Among the parameters, growth rate at 48 h was found to have maximum significant with 12 other parameters followed by temperature at 25 °C with 9 parameters. This finding will help in improving the alcohol production of the traditional rice wine.

Selection of fungus and yeast as defined mixed starter

Fungal strain *Amylomyces rouxii* ABT82 and the yeast strains *Saccharomycopsis fibuligera* ADJ4 and *Saccharomyces cerevisiae* ABT-Y1J were selected for the development of defined starter culture. *Amylomyces rouxii* ABTSJ82

Table 2 Biochemical, physiological and toxicological characteristics of yeast strains

Characteristics	ABT-Y1S	ABT-Y1J	ADJ1	ABT2	ADJ3	ADJ4	ADJ5	ADJ6
Budding	+	+	+	+	–	+	+	–
True mycelium	–	–	–	–	+	+	–	+
Fragmentation	–	–	–	–	+	+	–	+
Carbohydrate source utilization								
D-Galactose	+	+	+	+	ND	+	ND	+
Cycloheximide	–	–	–	–	–	–	–	–
D-Saccharose (sucrose)	+	+	+	+	+	+	–	+
N-Acetyl-glucosamine	–	–	–	–	–	–	–	–
Lactic acid	+	ND	ND	ND	+	ND	–	–
L-Arabinose	–	–	–	–	–	–	–	–
D-Cellobiose	ND	–	–	–	–	–	–	ND
D-Raffinose	+	+	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+	–	+
D-Trehalose	+	–	–	–	ND	–	–	+
Potassium 2-ketogluconate	–	–	–	–	–	–	–	–
Methyl- D-glucopyranoside	+	+	+	+	+	+	+	+
D-Mannitol	–	–	–	–	–	–	–	–
D-Lactose (bovine origin)	–	–	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	–
D-Sorbitol	–	–	–	–	–	–	–	+
D-Xylose	–	–	–	–	–	–	–	–
D-Ribose	–	–	–	–	–	–	–	–
Glycerol	+	+	+	–	+	+	–	–
L-Rhamnose	–	–	–	–	–	–	–	–
Palatinose	–	–	–	–	–	–	–	–
Erythritol	–	–	–	–	–	–	–	ND
D-Melibiose	–	–	–	–	–	–	–	–
Sodium glucuronate	+	–	–	–	–	–	–	–
D-Melezitose	–	–	–	–	–	–	–	–
Potassium gluconate	ND	+	+	–	–	–	–	–
Levulinic acid	+	+	+	–	+	+	+	–
D-Glucose	–	–	–	+	–	–	–	+
L-Sorbose	–	–	–	–	–	–	–	–
Glucosamine	–	–	–	–	–	–	–	–
Esculin ferric citrate	–	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–	–
Other physiological parameters								
Specific growth rate constant (h^{-1})	0.0637	0.0413	0.0576	0.0408	0.0746	0.0668	0.0529	0.0716
Ethanol production (%v/v)	5.73	9.88	7.59	11.01	6.81	10.11	8.1	7.74
Ethanol tolerance (%v/v)	12.3	12.5	10.1	13	11.1	11.7	11.6	10
Temperature tolerance ($^{\circ}C$)	40	40	40	45	40	40	40	40
Osmotolerance (%v/v glucose)	20	20	15	20	10	20	15	15
Flocculation capacity	+	+	+	–	+	+	+	+
Biogenic amines production	Ly	–	Ty	–	–	–	–	–

ABT-Y1S: *Saccharomyces cerevisiae*; ABT-Y1J: *S. cerevisiae*; ADJ1: *S. cerevisiae*; ADJ2: *Wickerhamomyces anomalus*; ADJ3: *Saccharomycopsis malanga*; ADJ4: *Saccharomycopsis fibuligera*; ADJ5: *S. cerevisiae*; ADJ6: *Saccharomycopsis malanga*; ND: Not determined; Ly & Ty: lysine HCl decarboxylation and TyramineHCl decarboxylation (indicators of biogenic amines production) respectively

Table 3 Correlation matrix of physiological parameters of yeast for wine production

Sample	1	AP	AT	T @ 20 °C	T @ 25 °C	T @ 30 °C	T @ 35 °C	T @ 40 °C	T @ 45 °C	S @ 10%	S @ 15%	S @ 20%	S @ 25%	S @ 30%	S @ 35%	GR1	GR2	GR3
AP	1																	
AT	0.160	1																
T @ 20 °C	0.104	0.614	1															
T @ 25 °C	0.947**	-0.155	-0.200	1														
T @ 30 °C	0.854**	0.153	0.305	0.722*	1													
T @ 35 °C	0.757*	0.236	0.240	0.719*	0.878**	1												
T @ 40 °C	0.692*	0.808**	0.297	0.591	0.918**	0.761*	1											
T @ 45 °C	-0.155	0.226	0.675*	0.036	0.493	0.355	0.524	1										
S @ 10%	0.076	0.257	0.302	-0.220	0.162	0.162	0.091	0.531	1									
S @ 15%	-0.382	0.385	0.705*	0.229	0.706*	0.676*	0.573	0.693*	0.627	1								
S @ 20%	-0.480	0.415	0.569	0.405	0.786*	0.716*	0.843**	0.664	0.405	0.732*	1							
S @ 25%	-0.382	0.508	0.658	.309	0.749*	0.700*	0.835**	0.684*	0.337	0.728*	.980**	1						
S @ 30%	-0.442	0.397	0.638	.375	0.787*	0.740*	0.858**	0.601	0.282	0.746*	0.970**	0.986**	1					
S @ 35%	-0.323	0.518	0.715*	0.182	0.735*	0.730*	0.728*	0.676*	0.471	0.836**	0.896**	0.931**	0.913**	1				
GR1	-0.283	0.650	0.469	0.081	0.528	0.434	0.400	0.819**	0.545	0.639	0.476	0.477	0.387	0.636	1			
GR24	-0.280	0.077	0.472	0.085	0.612	0.486	0.583	0.521	0.743*	0.853**	0.697*	0.658	0.686*	0.731*	0.439	1		
GR48	-0.517	0.102	0.136	0.437	0.710*	0.510	0.852**	0.501	0.248	0.508	0.667*	0.632	0.665	0.476	0.234	0.662	1	
	-0.711*	0.249	0.415	0.552	0.938**	0.712*	0.935**	0.673*	0.299	0.739*	0.841**	0.805**	0.822**	0.746*	0.576	0.720*	0.840**	1

AP Alcohol production, AT alcohol tolerance, T temperature, S sugar concentration, GR1, GR24 7, GR48 growth rate after 1 h, 24 h and 48 h

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Fig. 7 Effects of fungal and yeast cultures on saccharification and gelatinization of glutinous rice starch. The samples were analysed after 5 days of incubation at 30 °C. Y1: *S. cerevisiae*; Y2- *S. fibuligera*; M: *A. rouxii*; MY1: *A. rouxii* + *S. cerevisiae*; MY1Y2: *A. rouxii* + *S. cerevisiae* + *S. fibuligera*

could efficiently saccharify rice starch through the production of alpha-amylase and glucoamylase enzymes (5.92 U/ml and 7.50 U/ml, respectively). It was found atoxigenic for aflatoxins (B1, B2, G1, and G2). This fungus has also been reported to be involved in most of the amylolytic fermentation starters of Asian countries (Saono et al. 1996; Dung et al. 2005). The major selection criteria for industrial yeasts are ethanol production and tolerance as well as intracellular ethanol accumulation which affect yeast survivability and performance (Peres and Lalue 1998; Wang et al. 2013). *Saccharomyces cerevisiae* ABTY1J could tolerate the maximum ethanol concentration (12.5% v/v) and produced 9.88% ethanol (v/v) without the formation of any biogenic amines.

These selected yeast and moulds were grown together on the same plate to check their compatibility. The strains did not display any inhibition as visible from the plate. Different combinations of fungus, *Amylomyces rouxii* and yeasts, *Saccharomyces cerevisiae* and *Saccharomycopsis fibuligera* were grown in 30 °C for 5 days under solid state fermentation and the amounts of free glucose, ethanol and liquid were quantified (Fig. 7). Compatible mix cultures of MY1Y2 (*A. rouxii* + *S. cerevisiae* + *S. fibuligera*) were able to produce higher amount of glucose, ethanol and liquid (glucose 10.91% w/v; ethanol 7.5% w/v; liquid 51.0% w/v) as compared to MY1 (*A. rouxii* + *S. cerevisiae*) or MY2 (*A. rouxii* + *S. fibuligera*).

Our findings are in congruence with the study by Dung et al. (2005, 2006) that used a defined mixed starter culture to obtain Vietnamese rice wine with favorable flavor and

overall acceptability. The compatible mixed culture comprising of *Saccharomyces cerevisiae* and *Amylomyces rouxii* was found to produce 8.6% (w/v) ethanol, 19.2% (w/v) glucose and 48% (w/v) liquid from purple glutinous rice after 3 days of fermentation in 30 °C. From this present study, it was evident that a defined mixed starter culture can be used to minimize contamination and health risks associated with traditional rice wine starter cultures. This defined mixed starter culture also provides interesting opportunity for the improvement and modification of *Xaj* through careful manipulation of microorganisms. In this line, yield and sensory attributes (clarity, aroma, flavor and overall acceptability) can be evaluated for *Xaj* production towards commercial scale production.

Conclusion

Our culture-independent analysis revealed that ethnic *Xaj-pitha* samples were significantly contaminated with plant pathogens, environmental contaminants and several opportunistic human skin commensals. Presence of *Acinetobacter guillouiae* and *Stenotrophomonas maltophilia* reinforces the necessity for the development of defined mixed starter cultures towards controlled and safe rice wine fermentation. Our culture-dependent approaches in this line enabled the isolation and characterization of four high amylase producing moulds, viz. *Penicillium* sp., *Rhizopus oryzae*, *Mucor guilliermondii* and *Amylomyces rouxii* and eight various *Saccharomyces* and non-*Saccharomyces* yeast strains. All these fungal strains were found atoxicogenic when tested for their potential to produce aflatoxins; whereas, *Saccharomyces cerevisiae* ABTY1S and ADJ1 indicated capability of biogenic amine production. Based on the initial characterization and other literature-based toxicological studies, our study concluded that yeast strains *Saccharomycopsis fibuligera* and *S. cerevisiae* and a mould partner *Amylomyces rouxii* have the potential to be used in defined mixed starter culture preparation for rice-based alcoholic beverage production optimization.

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Compliance with ethical standards

Conflict of interest All authors declare no conflicts of interest.

Metagenome data accession number The metagenome is hosted at the MG-RAST server (accession number: mgm4556318.3) and Sequence Read Archive (SRA) database (accession number: PRJNA597963).

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