Diversity of Filamentous Fungi Isolated From Some Amylase and Alcohol-Producing Starters of India

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Filamentous fungi are important organisms in traditionally prepared amylase and alcohol-producing dry starters in India. We collected 40 diverse types of amylase and alcohol-producing starters from eight states in North East India viz. marcha, thiat, humao, hamei, chowan, phut, dawdim, and khekhrii. The average fungal population was $4.9 \times 10^5$ cfu/g with an average of pH 5.3 and 10.7%, respectively. In the present study, 131 fungal isolates were isolated and characterized based on macroscopic and microscopic characteristics and were grouped into 44 representative fungal strains. Based on results of morphological characteristics and ITS gene sequencing, 44 fungal strains were grouped into three phyla represented by Ascomycota (48%), Mucoromycota (38%), and Basidiomycota (14%). Taxonomical keys to species level was illustrated on the basis of morphological characteristics and ITS gene sequencing, aligned to the fungal database of NCBI GenBank, which showed seven genera with 16 species represented by Mucor circinelloides (20%), Aspergillus sydowii (11%), Penicillium chrysogenum (11%), Bjerkandera adusta (11%), Penicillium citrinum (7%), Rhizopus oryzae (7%), Aspergillus niger (5%), Aspergillus flavus (5%), Mucor indicus (5%), Rhizopus microsporus (5%), Rhizopus delemar (2%), Aspergillus versicolor (2%), Penicillium oxalicum (2%), Penicillium polonicum (2%), Trametes hirsuta (2%), and Cladosporium parahalotolerans (2%). The highest Shannon diversity index $H$ was recorded in marcha of Sikkim ($H: 1.74$) and the lowest in hamei of Manipur ($H: 0.69$). Fungal species present in these amylolytic starters are morphologically, ecologically and phylogenetically diverse and showed high diversity within the community.

Keywords: filamentous molds, amylolytic starter, India, Mucor, Rhizopus, Aspergillus, Penicillium

INTRODUCTION

Drinking alcoholic beverages has a cultural connotation in India from the Indus Valley Civilization dating back to 8,000 years (Sarkar et al., 2016), mostly through fermentation (Singh et al., 2010) and distillation (Achaya, 1991). Traditionally malting, brewing (such as beer), and vinification (fermentation of grapes into wine) processes are unknown in Indian food culture. Instead, traditional alcoholic beverages are prepared either by natural fermentation of plants or cereals, or by using traditionally prepared dry starters in India (Tamang, 2010). Some ethnic people in India traditionally prepare amylase and alcohol-producing starters to ferment alcoholic beverages for
home consumption, which are known by different names in different languages spoken locally in regions such as *marcha* in Sikkim and Darjeeling hills, *thiat* in Meghalaya, *humao* in Assam, *hamei* in Manipur, *chowan* in Tripura, *phut* in Arunachal Pradesh, *dawdim* in Mizoram and *khekhrii* in Nagaland (Anupma et al., 2018), *dhehl, balam, maler, treh*, and *bakhk* of Himachal Pradesh and Uttarakhand (Thakur et al., 2015), and *ranu dabai/goti* of West Bengal, Odisha and Jharkhand (Ghosh et al., 2015). Traditional methods of the preparation of Indian starters are almost the same with some differences in use of starch-rich substrates such as rice or wheat or barley, and wrapping materials either in fern fronds or dry paddy-straw, or in fresh leaves of locally available wild plants (Shrivastava et al., 2012; Tamang et al., 2016). Soaked, dewatered, and ground cereal (rice/wheat/barley) flours are mixed with some wild plants, with a few spices such as sun-dried chilies or garlics and supplemented with 1–2% of previously prepared dry starters in powder forms (“back-slopping method” for sub-culturing the microbiota) to make thick doughs with addition of water. Thoroughly mixed dough mixtures are made into round or flat cakes of varying shapes and sizes, placed on fresh ferns or other plant leaves/dry paddy straws and allowed to ferment under semi-anaerobic conditions for 2–3 days at room temperature inside the room. After desirable fermentation, fermented doughs are then sun dried for 2–3 days to obtain dry starters which are exclusively used to ferment cereals into mild/strong alcoholic beverages (Tamang, 2010; Anupma et al., 2018). However, *khekhrii*, a dry starter from Nagaland in India is prepared by naturally fermenting sprouted-rice grains which are then dried in the sun to obtain dry starter granules to prepare an alcoholic beverage locally called *zutho*. Indian amylase and alcohol-producing starters are similar to starters from Asian countries such as *duaq or chiiu* from China (Zheng et al., 2012), *benh* from Vietnam (Dung et al., 2007), *nuruk* from Korea (Jung et al., 2012), *ragi* from Indonesia (Roslan et al., 2018), *bubod* from the Philippines (Fronteras and Bullo, 2017), *loogpang* from Thailand (Daroonpunt et al., 2016) and *dombea or medombae* from Cambodia (Ly et al., 2018).

Several species of filamentous molds (Hesseltine et al., 1988; Yang et al., 2011; Ly et al., 2012a; Chen et al., 2014; Das et al., 2017); *yeasts* (Hesseltine and Kurtzman, 1990; Tamang and Sarkar, 1995; Tsuyoshi et al., 2005; Jeyaram et al., 2008, 2011; Thanh et al., 2008; Fronteras and Bullo, 2017; Sha et al., 2017, 2018, 2019); and *bacteria* (Hesseltine and Ray, 1988; Tamang et al., 2007; Sha et al., 2017; Roslan et al., 2018) are found to coexist in traditionally prepared dry starters as “micro-resources” which have been sub-cultured to preserve essential microbiota for alcohol production by Asian people for centuries (Tamang et al., 2020). Filamentous fungi present in traditional starters from Asia have several functionalities such as saccharification (Lee and Lee, 2002; Thapa and Tamang, 2004), liquefaction (Suesse et al., 2016), and ethanol production (Dung et al., 2007; Chen et al., 2014) to produce different types of low-alcoholic beverages and high-alcoholic distilled liquor. Filamentous molds are also responsible for the quality of alcoholic beverages including nutritional values and organoleptic properties such as flavor, taste, and color (Zhang et al., 2015; Tamang et al., 2016). Taxonomical identification of filamentous molds isolated from traditionally prepared dry starters from India have not been reported yet except from *marcha* (Tamang et al., 1988; Sha et al., 2017, 2019), *thiat* (Sha et al., 2017, 2019), *amou*, and *perok-kushi* (Das et al., 2017). *Mucor circinelloides, Rhizopus chinensis*, and *Rhizopus stolonifer* were reported earlier from *marcha* samples collected from Nepal, Darjeeling, and Sikkim (Tamang et al., 1988; Tamang and Sarkar, 1995; Thapa and Tamang, 2006; Sha et al., 2017, 2018, 2020). *Amylomyces rouxii* and *Rhizopus oryzae* from samples of *amou*, and *perok-kushi*, traditional starters of Assam (Das et al., 2017). Sha et al. (2017) reported fungal Phylum Ascomycota (98.6%) followed by Mucoromycota (1.4%), while in *marcha* samples only Phylum Ascomycota by high-through sequencing was reported. The present study aimed to identify the filamentous molds isolated from eight different types of traditionally prepared starters from North East India, viz. *marcha, thiat, humao, hamei, chowan, phut, dawdim*, and *khekhrii*, to species level by morphological and molecular identifications, and to profile their diversity within the fungal community.

### MATERIALS AND METHODS

#### Sample Collection

A total of 40 samples of traditionally prepared dry starters viz *marcha* from Sikkim, *thiat* from Meghalaya, *humao* from Assam, *hamei* from Manipur, *chowan* from Tripura, *phut* from Arunachal Pradesh, *dawdim* from Mizoram, and *khekhrii* from Nagaland (*Table 1*) were collected directly from local markets and the homes of local producers in North East India (*Figure 1*) in pre-sterile containers. Dry starter samples were transported to the laboratory and stored in desiccators at room temperature as traditionally prepared dry starters have a shelf life of more than 1 year (Sha et al., 2018).

#### Analysis of pH and Moisture Content

The pH of homogenized samples was recorded by digital pH-meter (Orion 910003, Thermo Fisher Scientific, United States). The moisture content of the samples was estimated by a moisture analyzer (OHAUS/MB-45, United States).

#### Microbiological Analysis

Each dry sample starter was taken from the desiccator, then crushed coarsely by sterile spatula and 10 g of the crushed powered sample was homogenized with 90 mL of 0.85% physiological saline in a stomacher lab blender 40 (Seward, United Kingdom) for 2 min to obtain serial dilutions. One milliliter of each diluted sample (10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) was poured onto malt extract agar (M137, HiMedia, Mumbai, India) and potato dextrose agar (M096, HiMedia, Mumbai, India) and potato dextrose agar (M096, HiMedia, Mumbai, India) with an addition of antibiotics (1% streptomycin) to suppress the growth of bacteria, and plates were then incubated under 28°C and observed for the appearance of colonies for up to 1 week. The colonies that appeared on plates were counted as a colony forming unit (cfu/g) on the dry weight of starters. Colonies were selected on the basis of macroscopic and microscopic characteristics. Selected filamentous molds were sub-cultured
on new plates and purified and stored on slants at 4°C for further studies.

Morphological and Physiological Identification
For each isolate, one- or three-point inoculations on petri plates containing 25 mL of media were applied. Fungal morphology was studied macroscopically by observing the colony features (surface color, reverse side color, shape, and diameter), and microscopically by observation of fruiting bodies using a stereomicroscope, and the vegetative and asexual stages were observed by a DE/Axio Imager A1 microscope (Carl Zeiss, Germany) after staining freshly grown mycelia stained with cotton blue in MEA plates (Gaddeyya et al., 2012). Filamentous molds were identified on the basis of morphological features using the taxonomical keys described by Samson et al. (2004) and Pitt and Hocking (2009).

Genomic DNA Extraction
The genomic DNA was extracted from mold cultures following the methods of Umesha et al. (2016). Mycelial mass from the culture plate was scraped out by a sterile surgical blade and ground in a sterile mortar and pestle using 500 µL extraction buffer [100 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB, and 0.2% 2 mercaptoethanol]. The mixture was transferred to a fresh 1.5 mL tube with addition of 4-µL RNase, vortexed and incubated for 60 min at 37°C, and kept in a water bath for 60 min at 55°C. 500 µL phenol: chloroform: isoamyl alcohol (25:24:1) was added to the solution, mixed thoroughly for 5 min, and then centrifuged at 14,000 rpm for 10 min. The aqueous clear phase was recovered and mixed with chloroform: isoamyl alcohol (24:1), centrifuged at 12,000 rpm for 5 min, and the aqueous phase was recovered, adding 0.8 volume of cold 7.5 M ammonium acetate and 0.54 volume of ice-cold isopropanol, and finally mixed well and stored overnight for precipitation of DNA in a deep freezer. The solution was centrifuged at 14,000 rpm for 10 min. The pellet was washed once with 70% ethanol, air-dried, and resuspended in 100 µL of TE [200 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0)] buffer for further use and stored at −20°C. The quality of DNA was checked on agarose gel and the concentration was measured using a Nanodrop spectrometer (ND-1000 spectrometer, NanoDrop Technologies, Wilmington, United States) (Kumbhare et al., 2015).

PCR Amplification
Polymerase chain reactions (PCR) of the internal transcribed spacer (ITS) region of filamentous molds was amplified using the primer ITS1 (5′-TCCGTAGGTAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Adelayo et al., 2017). PCR reactions were performed in 25 µL of PCR pre-master mix solution (Promega, United States). The amplification steps were followed: initial denaturation at 94°C for 5 min followed by 35 cycles consisting of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min, respectively; and a final extension at 72°C for 10 min in a Thermal Cycler (Applied biosystems-2720, United States). The PCR products were verified by electrophoresis on 1.0% agarose gel containing 0.7 mg/mL of

### TABLE 1 | Geographical locations, pH, moisture content, and fungal populations of dry starters from North East India.

| Sample (n⁴) | Region | Collection Site | Altitude (Meter) | Moisture content (%) | pH | cfu/g (x10⁵) |
|------------|--------|----------------|------------------|---------------------|----|-------------|
| Marcha (n = 8) | Sikkim | Gangtok | 1837 | 11.6 (10.1 – 12.1) | 5.2 (4.9 – 5.7) | 5.0 (4.8 – 5.1) |
| | | Basia | 906 | | | |
| | | Pakyong | 1341 | | | |
| | | Recab | 1072 | | | |
| Thiat (n = 4) | Meghalaya | Shillong | 1550 | 9.4 (8.7 – 10.0) | 4.7 (4.5 – 5.0) | 4.8 (4.5 – 5.1) |
| | | Non-grem | 1547 | | | |
| Humao (n = 7) | Assam | Kokrajhar | 49 | 9.7 (8.8 – 10.6) | 4.9 (4.6 – 5.2) | 4.6 (4.3 – 5.3) |
| | | Borhat | 95 | | | |
| | | Sibsagar | 93 | | | |
| | | Moran | 100 | | | |
| Himel (n = 3) | Manipur | Kangchup | 773 | 8.5 (8.0 – 9.6) | 4.6 (4.1 – 5.4) | 2.6 (2.5 – 3.2) |
| | | Kakching | 769 | | | |
| | | Phayeng | 813 | | | |
| Chowan (n = 4) | Tripura | Bangsul | 116 | 9.1 (9.0 – 9.3) | 5.6 (5.4 – 5.9) | 3.1 (3.0 – 3.4) |
| | | Dharmanagar | 98 | | | |
| Phut (n = 6) | Arunachal Pradesh | Doimukh | 152 | 11.2 (11.4 – 11.8) | 5.4 (5.5 – 5.7) | 5.6 (4.9 – 5.9) |
| | | Pasighat | 155 | | | |
| | | Itanagar | 361 | | | |
| | | Banderdewa | 462 | | | |
| | | Nirjuli | 151 | | | |
| Dawdum (n = 3) | Mizoram | Saiful | 438 | 13.7 (13.1 – 13.9) | 6.2 (6.1 – 6.3) | 7.4 (7.1 – 7.9) |
| | | | | | | |
| Khekhri (n = 5) | Nagaland | Kohima | 1092 | 12.8 (12.3 – 13.1) | 5.6 (5.5 – 5.9) | 6.0 (5.7 – 6.8) |

⁴n = number of samples.
ethidium bromide and visualized under UV light (Gel doc 1000, Bio-Rad, 97-0186-02, United States). Approximate size of amplicons was determined using standard molecular markers (Himedia-100 bp DNA ladder, Mumbai, India).

**Purification of the PCR Amplicons**

The amplified PCR products were purified using PEG (polyethylene glycol)-NaCl (sodium chloride) and precipitation solution (20% w/v of PEG, 2.5 M NaCl) with the addition of 0.6 volumes of 20% PEG-NaCl to the final volume of the PCR products (Schmitz and Riesner, 2006). The mixture was centrifuged at 12,000 rpm for 30 min, incubated at 37°C for 30 min, the aqueous solution was discarded, and the pellet was washed twice with 1 mL ice cold 70% freshly prepared ethanol (70%). The collected pellet was then air dried prior to elution in 20 µL of nuclease-free
water, and finally, the purified product was loaded in 1% agarose gel.

### ITS Sequencing

PCR-amplified products had been sequenced in a forward and reverse direction using ITS1 primer (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 primer (5′-TCTTCCGCTTATTGATATGC-3′), respectively, as per the method described by Martin and Rygiewicz (2005). The PCR reaction was carried out in 50 µL reaction volume containing 2.0 mM MgCl2, 0.2 µM each primer, 0.2 mM dNTP, 0.5 mg [mL]−1 bovine serum albumin (BSA) and 0.04 U [µL]−1 tTaq DNA polymerase on a thermal cycler equipped with a heated lid. The thermal program included initial denaturation, enzyme activation at 95°C (6–10 min) followed by 35 cycles to complete the step [95°C (1 min), 40°C (2 min) and 72°C (1 min)] and one cycle at 72°C (10 min). The amplified products were sequenced by an automated DNA Analyzer (ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, United States). These high-quality, double-stranded sequence data were analyzed with the help of the BLASTn program and multiple sequence alignment.

### Bioinformatics

The qualities of the raw sequences were checked by Sequence Scanner version 1.0 (Applied Biosystems, Foster City, CA, United States) and were edited using software ChromasPro version 1.34. Sequences were compared with sequence entries in the GenBank of NCBI (National Center for Biotechnology Information) using the Basic Local Alignment Search Tool (BLASTn) on the NCBI website (Raja et al., 2012). For phylogenetic analysis, the available sequence of similar related organisms was retrieved in FASTA format and aligned using the clustal-W. Sequence alignment and a phylogenetic tree were constructed using MEGA7.0 software by Neighbor-Joining methods using 1000-bootstrap replicates (Lutzoni et al., 2004).

### Statistical Analysis

Percentages of frequency and relative density of fungal species in samples were determined as per the method described by Doi et al. (2018). Frequency (%) was calculated by the equation:

\[
\text{Frequency} (\%) = \frac{\text{Number of quadrats in which the species occurred}}{\text{Total number of quadrats studied}} \times 100
\]

Relative Density (%) was calculated by the equation:

\[
\text{Density} = \frac{\text{Total number of individuals of a species in all quadrats}}{\text{Total number of quadrats studied}} \times 100
\]

Diversity indexes of filamentous molds in samples were calculated by species richness (R), Shannon’s diversity index (H), and species evenness (E) (Panda et al., 2010) using PAST (Paleontological STatistics) software version 3.26 (Hammer et al., 2001).

### Nucleotide Sequence Accession Numbers

The sequences obtained in this study were deposited at the GenBank-NCBI database under accession numbers: MK396469–MK396484, MK396486–MK396500, MK778442–MK778449, and MK796041–MK796045.

### RESULTS

#### Microbial Load, pH, and Moisture

The microbial load of filamentous molds in 40 samples of traditionally prepared dry starters collected from different regions of North East India were 2.5 to 7.9 × 10⁶ cfu/g (Table 1). The pH and moisture contents of all samples analyzed were pH 4.1–6.3 and 8.0–13.9%, respectively (Table 1).

#### Morphological Characterization

We isolated 131 total fungal isolates from 40 different samples of traditionally prepared dry starters (marcha, thiit, humao, hamei, chowan, phut, dawdim, and khekhrii) collected from eight states of North East India (Table 1). Based on the morphological characteristics (such as color, texture, size, and appearance of colony), microscopic characteristics (sporangia, sporangiospores, chlamydospores, conidia, conidiophore, and rhizoid structure), 44 representative fungal isolates were grouped (seven isolates from marcha, five from thiit, six from humao, two from hamei, five from chowan, six from phut, six from dawdim, and seven from khekhrii). Mucor, Rhizopus, Aspergillus, Penicillium, and Cladosporium and a few unidentified basidiomycetes fungi were tentatively identified on the basis of detailed morphological characters using the taxonomical keys described by Samson et al. (2004) and Pitt and Hocking (2009) (Supplementary Table S1).

#### Molecular Identification of Fungal Isolates

Genomic DNA of each isolate of 44 representative fungal strains was extracted and PCR products were prepared for identification by ITS gene sequencing. DNA sequences of fungal isolates were assigned by comparison with those available in the GenBank of NCBI database using the ITS gene sequence (ITS1 and ITS4) based on the Basic Local Alignment Search Tool (BLAST) 2.0 program (Raja et al., 2017). The phylogenetic trees of nucleotide sequences of the 44 fungal isolates from the samples were constructed using the Neighbor-joining method with 1000 replicates bootstrap values (Figure 2). ITS gene sequencing results showed three fungal phyla represented by Ascomycota (48%), Mucoromycota (38%), and Basidiomycota (14%) (Figure 3). Distribution percentage of the phyla in the starter showed the highest percentage of Ascomycota (86%) in...
khekhrii, Mucoromycota (60%) in dawdim, and Basidiomycota (20%) in chowan, dawdim, and thiat, respectively. Phyla Ascomycota and Mucoromycota were present in all starters, whereas Basidiomycota was present only in marcha, thiat, chowan, and dawdim.

Based on results of morphological characteristics and ITS gene sequencing, 44 representative strains of filamentous molds isolated from traditionally prepared dry starters from India were grouped into seven genera with 16 species, which were represented by Mucor circinelloides (20%), Aspergillus sydowii (11%), Penicillium chrysogenum (11%), Bjerkandera adusta (11%), Penicillium citrinum (7%), Rhizopus oryzae (7%), Aspergillus niger (5%), Aspergillus flavus (5%), Mucor indicus (5%), Rhizopus microsorum (5%), Rhizopus delemara (2%), Aspergillus versicolor (2%), Penicillium oxalicum (2%), Penicillium polonicum (2%), Trametes hirsuta (2%), and Cladosporium parahalotolerans (2%) (Table 2 and Figure 4). Interestingly we detected few basidiomycetes fungi represented by Bjerkandera adusta and Trametes hirsuta in marcha, thiat, chowan and dawdim samples. Colony morphology and microscopic images of 16 species of seven genera of filamentous molds isolated from dry starters from India were illustrated for fungal taxonomy (Figure 5).

Frequency and density of fungal species in samples showed that Aspergillus niger was colonized with khekhrii; a species from the Mucor circinelloides complex was observed with a high dominance in samples, whereas Trametes hirsuta was less diversified and observed only in thiat samples (Table 3).

Diversity indexes of filamentous molds of dry starters were characterized by species richness (R), Shannon’s diversity index (H), and species evenness (E) (Table 3). The Shannon diversity index H was recorded as the highest in marcha from Sikkim (H: 1.74) and the lowest in hamei from Manipur (H: 0.69). Species Evenness (E) values were 0.97 in marcha followed by humao from Assam and phut from Arunachal Pradesh. The Species Richness (R), values were recorded highest in marcha and khekhrii samples (Table 3).
DISCUSSION

Drinking of cereal-based mild to strong alcoholic beverages produced by traditionally prepared amylase and alcohol-producing starters has been a traditional food culture of the ethnic people from the North East states of India for centuries. Traditionally prepared dry starters have consortia of co-existed microbiota containing filamentous molds, yeasts, and bacteria and are crudely sub-cultured through a “backslopping” process by traditional starter-makers (Hesseltine et al., 1988; Tamang and Sarkar, 1995; Tamang et al., 2007; Sha et al., 2018, 2019), for alcohol production by the Indian people. The pH of traditionally prepared dry starters from India were slightly acidic in nature, perhaps due to accumulation of metabolic organic acids (Ma et al., 2019). Moreover, low pH is favorable for the growth of mycelial fungi (Abubakar et al., 2013). Low content of moisture in starter cultures is due to the sun-drying process during the traditional method of preparation practiced by the ethnic people of India, which may increase the shelf life of the starter for a year or more at room temperature (Tsuyoshi et al., 2005; Tamang, 2010).

Some traditionally prepared starters from North East India have been microbiologically analyzed in earlier works and several species of yeasts (Tsuyoshi et al., 2005; Jeyaram et al., 2008, 2011; Sha et al., 2017, 2018, 2019) and bacteria (Tamang et al., 2007; Pradhan and Tamang, 2019) were reported. However, detailed taxonomical studies of filamentous molds isolated from traditionally prepared dry starters from North East India have not been reported yet, except for marcha (Tamang et al., 1988; Tamang and Sarkar, 1995; Sha et al., 2017, 2019), thiat (Sha et al., 2017, 2019), amou, perok-kushi (Das et al., 2017).

Hence, we studied the taxonomy and diversity of filamentous fungi associated with traditionally prepared dry starter cultures from North East India viz., marcha from Sikkim, thiat from Meghalaya, humao from Assam, hamei from Manipur, chowan from Tripura, phut from Arunachal Pradesh, dawdim from Mizoram, and khekhrii from Nagaland based on morphological characters and molecular identifications. The average fungal population in traditionally prepared dry starters from North East India was 10^5 cfu/g, which was in accordance with earlier reports on fungal populations in marcha of Sikkim, and the Darjeeling hills in India (Tamang et al., 1988; Tamang and Sarkar, 1995). No such data on fungal population in other starters of India are available except for marcha. In the present study, we first isolated and characterized 131 fungal isolates from 40 different starters from North East India based on macroscopic and microscopic characteristics and grouped them into 44 representative fungal strains. Morphological examination and identification of fungi are useful for identification up to the family or genus level (Alsohali and Bani-Hasan, 2018). However, morphological-based identification is not adequate to identify the fungi up to species level (Lutzoni et al., 2004). The sequence-based identification tool is widely applied to confirm the exact identity of the fungal species (Romanelli et al., 2010; Xu, 2016).

We applied polymerase chain reactions (PCR) of the internal transcribed spacer (ITS) region of 44 strains of filamentous fungi isolated from starters from North East India using the primers ITS1 and ITS4 and grouped into three phyla represented by Ascomycota (48%), Mucoromycota (38%), and Basidiomycota (14%). A similar type of phylum distribution was also reported earlier in a nuruk dry starter from Korea (Carroll et al., 2017) and daqu from China (Shoubao et al., 2019). Seven genera with 16 species of filamentous fungi, isolated from Indian amylase

FIGURE 3 | Heatmap showing the consensus species diversity resulted by ITS-region gene sequencing of filamentous fungal isolates. We used presence/absence value for fungal species to generate the heatmap, where the yellow color indicates the presence and red indicates absence.
and alcohol-producing starters, were identified based on the morphological and microscopic characteristics, and molecular identification which were represented by Aspergillus flavus, A. niger, A. sydowii, A. versicolor, Bjerkandera adusta, Cladosporium parahalotolerans, Mucor circinelloides, M. indicus, Penicillium chrysogenum, P. citrinum, P. oxalicum, P. polonicum, Rhizopus delemar, R. microsporus, R. oryzae, and Trametes hirsuta. Illustration of taxonomical keys based on morphological and molecular identification is more accurate and reliable in fungal taxonomy (Xing et al., 2018). Our earlier findings of Rhizopus oryzae and species from the Mucor circinelloides complex in traditionally prepared starters of North East India by PCR-DGGE method (Sha et al., 2018) supported the present study. Hesseltine and Kurtzman (1990) reported species from the M. circinelloides complex in bubod from the Philippines. Species from the M. circinelloides complex, M. indicus, Rhizopus oryzae, and R. microsporus were reported in benh men from Vietnam (Dung et al., 2007; Thanh et al., 2008). In marcha and khekhrii

### Table 2

| Product | Isolate code | Identity               | GenBank accession number | Size in base pair (arbitrary primers) |
|---------|--------------|------------------------|--------------------------|--------------------------------------|
| Marcha  | SMM-1        | Aspergillus flavus      | MK396469                 | 519                                  |
|         | SMM-3        | Mucor circinelloides    | MK396489                 | 642                                  |
|         | SMM-4        | Rhizopus microsporus    | MK396495                 | 703                                  |
|         | SMM-10       | Bjerkandera adusta      | MK778445                 | 675                                  |
|         | SMM-16       | Penicillium chrysogenum | MK396477                 | 577                                  |
|         | SMM-22       | Penicillium polonicum   | MK778446                 | 582                                  |
|         | SMM-35       | Penicillium chrysogenum | MK778447                 | 552                                  |
| Thiat   | MTM-1        | Mucor circinelloides    | MK96487                  | 636                                  |
|         | MTM-4        | Rhizopus delemar        | MK96496                  | 788                                  |
|         | MTM-6        | Penicillium chrysogenum | MK96478                  | 583                                  |
|         | MTM-12       | Trametes hirsuta        | MK96492                  | 637                                  |
|         | MTM-16       | Bjerkandera adusta      | MK96500                  | 651                                  |
| Humao   | AEM-1        | Penicillium citrinum    | MK96481                  | 437                                  |
|         | AEM-3        | Rhizopus oryzae         | MK96483                  | 613                                  |
|         | AEM-4        | Mucor circinelloides    | MK96484                  | 648                                  |
|         | AEM-8        | Aspergillus sydowii     | MK96472                  | 467                                  |
|         | AXM-1        | Aspergillus sydowii     | MK96475                  | 546                                  |
|         | AMM-3        | Mucor indicus           | MK778442                 | 565                                  |
| Hamei   | MHM-1        | Mucor circinelloides    | MK96043                  | 601                                  |
|         | MHM-15       | Penicillium citrinum    | MK96042                  | 469                                  |
| Chowan  | TCM-1        | Bjerkandera adusta      | MK96494                  | 520                                  |
|         | TCM-4        | Mucor circinelloides    | MK778449                 | 636                                  |
|         | TCM-7        | Rhizopus oryzae         | MK96491                  | 637                                  |
|         | TCM-9        | Aspergillus sydowii     | MK96041                  | 541                                  |
|         | TCM-12       | Penicillium chrysogenum | MK778448                 | 541                                  |
| Phut    | APM-1        | Aspergillus sydowii     | MK96473                  | 577                                  |
|         | APM-3        | Mucor circinelloides    | MK96482                  | 645                                  |
|         | APM-6        | Aspergillus versicolor  | MK96480                  | 417                                  |
|         | APM-7        | Mucor indicus           | MK96498                  | 627                                  |
|         | APM-12       | Rhizopus oryzae         | MK96490                  | 621                                  |
|         | APM-15       | Aspergillus sydowii     | MK96474                  | 574                                  |
| Dawdim  | MDM-1        | Mucor circinelloides    | MK96497                  | 645                                  |
|         | MDM-10       | Bjerkandera adusta      | MK96493                  | 569                                  |
|         | MDM-11       | Rhizopus microsporus    | MK96488                  | 696                                  |
|         | MDM-14       | Mucor circinelloides    | MK96486                  | 641                                  |
|         | MDM-16       | Bjerkandera adusta      | MK96499                  | 680                                  |
|         | MDM-18       | Penicillium chrysogenum | MK778443                 | 554                                  |
| Khekhrii| NKM-1        | Mucor circinelloides    | MK96045                  | 490                                  |
|         | NKM-6        | Penicillium citrinum    | MK96479                  | 519                                  |
|         | NKM-7        | Aspergillus flavus      | MK96470                  | 519                                  |
|         | NKM-8        | Aspergillus niger       | MK96471                  | 551                                  |
|         | NKM-10       | Penicillium oxalicum    | MK778444                 | 581                                  |
|         | NKM-13       | Aspergillus niger       | MK96476                  | 602                                  |
|         | NKM-15       | Cladosporium parahalotolerans | MK96044                | 546                                  |
Anupma and Tamang

Fungal Diversity in Starters of India

FIGURE 4 | Abundance distribution of the filamentous fungi isolated from dry starters from North East India.

we detected *Aspergillus flavus*, which was also reported in *mana*, an amylolytic starter from Nepal (Nikkuni et al., 1996).

*Aspergillus* belonging to order Eurotiales is a phenotypically polythetic genus and is widely distributed in the environment (Tsang et al., 2018). Samson et al. (2014) proposed phylogenic identification of *Aspergillus* with ITS sequence data, and calmodulin as a secondary identification marker, according to the decision of the International Commission of *Penicillium* and *Aspergillus*. Application of ITS with β-tubulin sequences for identification of *Aspergillus* species has also been reported by Zulkifli and Zakaria (2017). However, in this study we have applied both ITS sequence and morphological characteristics, such as the conidiophore with straight ending in a large vesicle from where primary and secondary sterigmata arise bearing conidia in chains, for identification of species of *Aspergillus*. *Aspergillus niger* and *A. flavus* cannot be distinguished only by their ITS sequences, the morphological characters are also essential in species identification (Zulkifli and Zakaria, 2017). We identified genus *Aspergillus* with four species in dry starter samples from India which included *A. niger*, *A. flavus*, *A. sydowii*, and *A. versicolor*. Among *Aspergillus* *A. flavus*, *A. niger* and *A. sydowii* were most prevalent in food samples due to their sporulating ability in the environment (Adekoya et al., 2017). *Aspergillus* is a dominant fungal genus in *daqu* from China (Ji et al., 2018), and may contribute to the saccharification process (Wang et al., 2019). We detected two strains of *Aspergillus flavus* in a *marcha* sample from Sikkim (*Aspergillus flavus* SMM-1) and in a *khekhrii* sample from Nagaland (*A. flavus* NKM-7). Though the distribution percentage was only 5%, the presence of *A. flavus* in samples of *marcha* and *khekhrii* is alarming. *A. flavus* is a saprotrophic with cosmopolitan distribution (Ramirez-Camejo et al., 2012), which produces aflatoxin (Saori and Keller, 2011; Priyanka et al., 2012; Mudili et al., 2014). Probable sources of *A. flavus* in starters may be from contaminated rice grains (Lai et al., 2015) since rice is the main base substrates for the preparation of starters for the production of alcohol. Moreover starter-makers commonly use low-quality, old-stocked and discarded rice grains for preparation of starters. However due to co-existence of other species of filamentous molds, yeasts and lactic acid bacteria in traditionally prepared starters may antagonize against *A. flavus* in *marcha* and *khekhrii*, which may reduce aflatoxin production in the sample (Karlovsky et al., 2016; Adebo et al., 2019). Lactic acid bacteria isolated from *marcha* showed an antagonistic property (Tamang et al., 2007), similarly, some bacteria have antifungal activity against aflatoxin-producing *A. flavus* (Shakeel et al., 2018). *Rhizopus* spp. from *tempeh*, a fermented soybean food from Indonesia, were reported for detoxification of aflatoxins (Nakazato et al., 1990). *A. sydowii* present in samples *humao*, *phut* and *chowan*, is an industrially important filamentous mold, which produces monosaccharides and indole alkaloids (Zhou et al., 2018). None of the amylolytic starters of North East India showed the presence of *A. versicolor* except in *phut* samples from Arunachal Pradesh. *A. versicolor* is a slow-growing filamentous fungus commonly found in/on damp indoor environments (Samson et al., 2004), foods, and feeds (Jurjevic et al., 2012), and produces toxic metabolites (Piontek et al., 2016). Contamination of *A. versicolor* in *phut* samples might be from the damp room where preparation of *phut* is often practiced by starter-producers in Arunachal Pradesh.
**FIGURE 5** | Images of colony morphology and microscopic features of filamentous molds that grew on MEA media: Aspergillus flavus colonies top (A1), reverse (A2), Conidiophores (A3); Aspergillus niger colonies top (B1), reverse (B2), mature conidia globose conidial head contain conidia (B3); Aspergillus sydowi colonies top (C1), reverse (C2), mature conidiophore with vesicle bearing conidigenous metulae and phialides (biserate) (C3); Aspergillus versicolor colonies top (D1), reverse (D2), mature conidial heads supported vesicles with which are biseriate with metulae about the same size of phialides (D3); Penicillium chrysogenum colonies top (E1), reverse (E2), smooth-walled conidiophores stipes (150–280 µm) and biverticillate (E3); Penicillium citrinum colonies top (F1), reverse (F2), conidiophores stipes (150–280 µm) and biverticillate, phialides ampuliform (flask-shaped) (F3); Penicillium oxalicum colonies top (G1), reverse (G2), mature conidiophores monoverticillate, or biverticillate and asymmetrical, phialides were cylindrical (G3); Penicillium polonicum colonies top (H1), reverse (H2), conidiophore were terverticillate, phialides (H); Mucor circinelloides colonies top (I1), reverse (I2), mature sporangioshores contain sporangiospores (I3); Mucor indicus colonies top (J1), reverse (J2), mature sporangioshores contain sporangiospores (J3); Rhizopus delemar colonies top (K1), reverse (K2), globose sporangia (K3); Rhizopus microsporus colonies top (L1), reverse (L2), sporangia were usually straight, mostly 10–20 µm (L3); Rhizopus oryzae colonies top (M1), reverse (M2), sporangia globose, smooth and released spore (M3); Trametes hirsuta colonies top (N1), reverse (N2), hyphal structure (N3); Bjerkandera adusta colonies top (O1), reverse (O2), dichotomously branched hyphae (O3); Cladosporium parahalotolerans colonies top (P1), reverse (P2), conidiophores and conidial chain (P3).

*Mucor circinelloides* was found to be the most dominant fungus in dry starter cultures from North East India. *M. circinelloides* has a sub-globose sporangiospore with a sympodial branching pattern. Using the ITS sequencing tool, it is difficult to distinguish among the different species of the *Mucor circinelloides* complex (MCC) which include *M. circinelloides*, *M. griseoanus*, *M. janssennii*, *M. lusitanicus*, *M. ramosissimus*, *M. varicolumnellatus*, and *M. velutinosus* (Wagner et al., 2019). We therefore used species from the *Mucor circinelloides* complex. *Mucor circinelloides* contributes in saccharification and liquefaction of cereal during fermentation of *kodo ko jaanr*, an alcoholic product of Sikkim fermented by starter *marcha* (Thapa and Tamang, 2004; Tamang and Thapa, 2006). *M. circinelloides* is an oleaginous fungus (Qiao et al., 2018) which produces lipids (Wei et al., 2013), cellulose degrading enzymes (Huang et al., 2014), and has several functional properties including antioxidants (Hameed et al., 2017). Phylum Mucoromycota does not produce mycotoxins, however, some species that belong to this *M. circinelloides* group has been described to be putatively responsible for human illnesses after consumption of mold-contaminated yogurt (Lee et al., 2014) although its involvement was not clearly proven. *M. circinelloides* was also reported earlier in *marcha* samples (Tamang et al., 1988; Tamang and Sarkar, 1995). *M. indicus*, isolated from *humao* from Assam and *phut* from Arunachal Pradesh, is a dimorphic and ethanolic fungus which is able to produce ethanol from glucose, mannose, fructose and galactose (Karimi and Zamani, 2013) and oil, protein, and glucosamine (Sharifyazd and Karimi, 2017).
### TABLE 3 | Frequency, density, and diversity indices of filamentous molds observed in dry starters from North East India.

| Filamentous molds                  | Marcha | Thiat | Humao | Hamei | Chowan | Phut | Dawadim | Khekhari |
|------------------------------------|--------|-------|-------|-------|--------|------|---------|----------|
|                                    | Fr     | RD    | Fr    | RD    | Fr     | RD   | Fr      | RD       | Fr     | RD    |
| Aspergillus niger                  | 0      | 0     | 0     | 0     | 0      | 0    | 0       | 0        | 0      | 0     |
| Aspergillus flavus                 | 16.6   | 0.16  | 0     | 0     | 0      | 0    | 0       | 0        | 0      | 0     |
| Aspergillus sydowii                | 0      | 0     | 16.6  | 0.16  | 33.3   | 0.33 | 0       | 20       | 0.2    | 33.3  |
| Aspergillus versicolor             | 0      | 0     | 0     | 0     | 0      | 0    | 0       | 0        | 16.6   | 0.16  |
| Penicillium chrysogenum            | 16.6   | 0.16  | 16.6  | 0.16  | 0      | 0    | 0       | 20       | 0.2    | 16.6  |
| Penicillium citrinum               | 0      | 0     | 0     | 0     | 16.6   | 0.16 | 0       | 0        | 0      | 0     |
| Penicillium oxalicum               | 0      | 0     | 0     | 0     | 0      | 0    | 0       | 0        | 0      | 0     |
| Cladosporium parahalotolerans      | 0      | 0     | 0     | 0     | 0      | 0    | 0       | 0        | 0      | 0     |
| Penicillium polonicum              | 16.6   | 0.16  | 16.6  | 0.16  | 16.6   | 0.16 | 50      | 0.5      | 0      | 0     |
| Mucor circinelloides               | 16.6   | 0.16  | 16.6  | 0.16  | 16.6   | 0.16 | 50      | 0.5      | 20     | 0.2   |
| Mucor indicus                      | 0      | 0     | 0     | 0     | 16.6   | 0.16 | 0       | 0        | 0      | 16.6  |
| Rhizopus oryzae                    | 0      | 0     | 0     | 0     | 16.6   | 0.16 | 0       | 20       | 0.2    | 16.6  |
| Rhizopus delemar                   | 0      | 0     | 16.6  | 0.16  | 0      | 0    | 0       | 0        | 0      | 0     |
| Rhizopus microsporus               | 16.6   | 0.1667| 0     | 0     | 0      | 0    | 0       | 0        | 0      | 16.6  |
| Trametes hirsuta                   | 0      | 0     | 16.6  | 0.16  | 0      | 0    | 0       | 0        | 0      | 0     |
| Bjerkandera adusta                 | 16.6   | 0.1667| 16.6  | 0.16  | 0      | 0    | 0       | 20       | 0.2    | 33.3  |

**DIVERSITY INDICES**

| Species richness (R) | 6    | 5    | 5    | 2    | 5    | 5    | 4    | 6    |
|----------------------|------|------|------|------|------|------|------|------|
| Shannon's diversity index (H) | 1.74 | 1.6  | 1.56 | 0.69 | 1.6  | 1.56 | 1.32 | 1.46 |
| Species evenness (E)  | 0.97 | 1    | 0.96 | 1    | 1    | 0.96 | 0.95 | 0.82 |

Fr: Frequency of fungal species; RD, Relative density of fungal species in samples.
Phylogenetic and phyl genomic approaches show that genus Rhizopus has three major clades viz. R. microsorus with its sister taxon R. stolonifer, R. arrhizus, and R. delemar (Gryganskiy et al., 2018). Rhizopus oryzae, commonly inhabits soils, animal excrement, and rotting vegetables (Ghosh and Ray, 2011), and is very similar to Rhizopus stolonifer, except for its smaller sporangia with air-dispersed sporangiospores (Pitt and Hocking, 2009). R. oryzae and R. microsorus are detected in yao qu from China and banh men from Vietnam, which are strong amylase producers (Dung et al., 2007; Thanh et al., 2008; Lv et al., 2012b). R. oryzae is considered as a GRAS filamentous fungus (Londoño-Hernández et al., 2017), which is commonly used for production of some Asian fermented foods (Tamang et al., 2012a). Rhizopus microsorus is the major fungus in tempe, a fermented soybean food from Indonesia (Hartanti et al., 2015), and it probably imparts taste and flavor in kiad, an alcoholic product fermented by the starter thiat. R. delemar has also been reported in xai pitha, starter from Assam in India (Bora et al., 2016). Presence of Rhizopus spp. in starters from North East India may contribute functionalities in end products during acoholic fermentation.

Penicillium chrysogenum was found in only four types of starters viz. marcha (Sikkim), thiat (Meghalaya), chowan (Tripura), and dawdim (Mizoram). The probable entry of P. chrysogenum during traditional preparation may be from damp and moist rooms where preparation for such starters is usually done, since P. chrysogenum is also found in damp buildings (Andersen et al., 2011). Due to the ability of P. chrysogenum to produce antibiotics, mostly penicillin (Bajaj et al., 2014), its presence in starters may have an antagonist property in the end product. P. citrinum was recovered in samples of humao, hamei and khekhrii, probably from indoor environments (Samson et al., 2004). P. oxalicum was found in samples of khekhrii (Nagaland) and P. polonicum in marcha samples. P. oxalicum produces various enzymes and natural products (Li et al., 2016). P. polonicum has also been reported in fermented black table olives (Bavaro et al., 2017).

It is interesting to note that we detected Basidiomycetaceous fungi represented by Bjerkandera adusta in samples of marcha, thiat, dawdim, and chowan, and also Trametes hirsuta in thiat samples. Bjerkandera adusta and Trametes hirsuta are wood decaying white-rot fungi (Rosales et al., 2005; Horisawa et al., 2019). B. adusta grows on a natural cellulosic substrate, imparts a refreshing aroma (Zhang et al., 2015), contributes to saccharification (Quiroz-Castañeda et al., 2009), and produces ethanol (Horisawa et al., 2019). Trametes hirsuta is lignin-degrading fungus due its ability to synthesize laccase (Gilerdzc et al., 2011). Traditional methods of preparation of these amylolytic starter cultures require locally grown wild herbs and spices used as ingredients by local starter-makers (Anupma et al., 2018). We assume that during collection of wild herbs from forest grounds, people might have collected whole wild plants in situ, where wood-rooting fungi have been reported in forests of North East India (Chuzho et al., 2017). There is no practice of filtering and cleaning of collected wild plants during starter preparation, hence chances for contamination of these basidiomycetaceous fungi may be possible during preparation. B. adusta and T. hirsuta were not reported earlier in any starter culture or in any fermented food.

Cladosporium parahalotolerance was found only in samples of khekhrii. C. parahalotolerance mostly occurred in plant debris, foods, and indoors (Bensch et al., 2012). Source of Cladosporium in khekhrii might be from wild herbs used as ingredients during traditional preparation of khekhrii in Nagaland. Species of Bjerkandera, Trametes, and Cladosporium have not been reported in any fermented foods elsewhere.

Diversity indexes determine the phylogenetic relations within different fungal species in a community (Fernandes et al., 2015). We calculated diversity indexes of fungal community present in starters of North East India by Shannon’s diversity index (H), species evenness (E), and species richness (R). Shannon diversity index H for evaluating fungal diversity was recorded highest in marcha samples collected from Sikkim (H: 1.74) and lowest in hamei samples of Manipur (H: 0.69) indicating higher fungal diversity in marcha samples of Sikkim as compared to starters of other states. The diversity index, which considers both the number of species as well as relative abundance of each species for evaluating diversity (Lucas et al., 2017), showed the highest value for marcha of Sikkim. Species evenness refers to how equal the community is numerically, ranging from 0 to 1 (Savary et al., 2018) signifying that the value 1.0 in thiat, hamei, and chowan have a complete evenness in comparison to other starters. Hence diversity index of filamentous fungal community present in dry starters of North East India showed high diversity within the community. It was observed that there was variation in fungal species distribution in each type of amylolytic starters in North East India which determines the quality of the acoholic product, preferred by the local consumers. This might be due to varied geographical regions, environmental conditions, and different plant species used in the preparation methods of amylolytic starters. It therefore shows that fungal diversity, present in amylase and alcohol-producing starters, traditionally prepared by ethnic Indian people using their indigenous knowledge of “back-slopping,” are morphologically, ecologically, and phylogenetically diverse. Our findings on fungal diversity in amylolytic starters from North East India may supplement the microbial diversity in ecosystems of North East India, which is one of the biodiversity hot spots of the world.

**CONCLUSION**

Traditionally prepared amylolytic starters are consortia of filamentous fungi, yeasts, and bacteria which are traditionally sub-cultured and preserved using traditional methods of
“back-slopping” by the ethnic people of North East India for production of alcoholic beverages. Yeasts and bacteria present in these starters have already been reported in earlier studies. However, no information on fungal communities and their diversity in Indian amylolytic starters is available. We therefore identified the filamentous molds isolated from Marcha, thiat, humao, hamei, chowan, phut, dawdim, and khekhiir based on morphological and sequence-based identifications. We identified seven genera with 16 species represented by Aspergillus flavus, Aspergillus niger, Aspergillus sydowi, Aspergillus versicolor, Bjerkandera adusta, Cladosporium parahalotolerans, Mucor circinelloides, Mucor indicus, Penicillium chrysogenum, Penicillium citrinum, Penicillium oxalicum, Penicillium polonicum, Rhizopus delemar, Rhizopus microsporus, Rhizopus oryzae, and Trametes hirsuta. Fungal species present in these traditionally prepared dry starters are morphologically, ecologically, and phylogenetically diverse and showed high diversity within the community.

DATA AVAILABILITY STATEMENT

The sequences of the internal transcribed spacers (ITS) region obtained in this study were deposited at the GenBank-NCBI database 6S rRNA sequencing were deposited at GenBank-NCBI numbers: MK396469-MK396484, MK396486-MK396500, MK778442-MK778449, MK796041-MK796045.

AUTHOR CONTRIBUTIONS

AA performed the experiments. JT supervised the experiments and finalized the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00905/full#supplementary-material

REFERENCES

Abubakar, A., Suberi, H. A., Bello, I. M., Abdullahi, R., Daudu, O. A., and Lateef, A. A. (2013). Effect of pH on mycelial growth and sporulation of Aspergillus parasiticus. J. Plant Sci. 1, 64–67. doi: 10.11648/j.js.20130104.13

Achaya, K. T. (1991). Alcoholic fermentation and its products in ancient India. Indian J. History Sci. 26, 123–129.

Adebo, O. A., Kayitesi, E., and Njobeh, P. B. (2019). Reduction of mycotoxins during fermentation of whole grain sorghum to whole grain ting (a Southern African Food). Toxins 11:180. doi: 10.3390/toxins11030180

Adekoja, I., Obadina, A., Phoku, J., Nwinyi, O., and Njobeh, P. (2017). Contamination of fermented foods in Nigeria with fungi. LWT-Food Sci. Technol. 86, 76–84.

Alsohaili, S. A., and Bani-Hasan, B. A. (2018). Morphological and molecular identification of fungi isolated from different environmental sources in the Northern Eastern desert of Jordan. Jordan J. Biol. Sci. 11, 329–337.

Andersen, B., Frisvad, J. C., Sendergaard, I., Rasmussen, I. S., and Larsen, L. S. (2011). Associations between fungal species and water-damaged building materials. Appl. Environ. Microbiol. 77, 4180–4188. doi: 10.1128/AEM.0213-10

Anupma, A., Pradhan, P., Sha, S. P., and Tamang, J. P. (2018). Traditional skill of ethnic people of the Eastern Himalayas and North East India in preserving microflora as dry amylolytic starters. Indian J. Trad. Knowl. 17, 184–190.

Baij, I., Veiga, T., van Dissel, D., Pronk, J. T., and Daran, J. M. (2014). Functional characterization of a Penicillium chrysogenum mutantase gene induced upon co-cultivation with Bacillus subtilis. BMC Microbiol. 14:114. doi: 10.1186/1471-2180-14-114

Bavaro, S. L., Susca, A., Frisvad, J. C., Tufariello, M., Chytiri, A., Perrone, G., et al. (2017). Isolation, characterization, and selection of molds associated to fermented black table olives. Front. Microbiol. 8:1356. doi: 10.3389/fmicb.2017.01356

Bensch, K., Braun, U., Groenewald, A., and Crous, P. W. (2012). The genus Cladosporium. Stud. Mycol. 72, 1–401. doi: 10.3114/sim0003

Bora, S. S., Keot, J., Das, S., Sarma, K., and Barooah, M. (2016). Metagenomics analysis of microbial communities associated with a traditional rice wine starter culture (Xaj-pitha) of Assam. India. 3 Biotech. 6:153. doi: 10.1007/s13205-016-0471-1

Carroll, E., Trinh, T. N., Son, H., Lee, Y. W., and Seo, J. A. (2017). Comprehensive analysis of fungal diversity and enzyme activity in nuruk, a Korean fermenting starter, for acquiring useful fungi. J. Microbiol. 55:357. doi: 10.1007/s12275-017-7114-z

Chen, B., Wu, Q., and Xu, Y. (2014). Filamentous fungal diversity and community structure associated with the solid-state fermentation of Chinese Maotai-flavor liquor. Int. J. Food Microbiol. 179, 80–84. doi: 10.1016/j.ijfoodmicro.2014.03.011

Chuzho, K., Dkhar, M. S., and Lyngdoh, A. (2017). Wood-rotting fungi in two forest stands of Kohima, North-East India: a preliminary report. Cur. Res. Environ. Appl. Mycol. 7, 1–7.

Cildrezic, J., Stajic, M., Vukojevic, J., Dulitic-Lausevic, S., and Knezecic, A. (2011). Potential of Trametes hirsuta to produce ligninolytic enzymes during degradation of agricultural residues. BioresResearch 6, 2885–2895.

Daroonpunt, R., Tanasupawat, S., and Keeratipibul, S. (2016). Characterization and amylolytic activity of yeast and mold strains from Thai sweet rice. Malaysian J. Microbiol. 12, 121–131.

Das, A. J., Miyaji, T., and Deka, S. C. (2017). Amylolytic fungi in starter cakes for rice beer production. J. Gen. Appl. Microbiol. 63, 236–245. doi: 10.2323/jgam.2016.11.004

Doi, S. A., Pinto, A. B., Canali, M. C., Polezzi, D. R., Chinellato, R. A. M., de Oliveira, A. J. F. C. (2018). Diversity and density of filamentous fungi in the water and sediment of Araçá bay in São Sebastião, São Paulo, Brazil. Biota Neotrop. Campinas 18:e20170416. doi: 10.1590/1674-0611-bn-2017-0416

Dung, N. T. P., Rombouts, F. M., and Nout, M. J. R. (2007). Characteristics of some traditional Vietnamese starch-based rice wine fermentation starters (men). LWT-Food Sci. Technol. 40, 130–135.

Fernandes, E. G., Pereira, L. O., da Silva, C. C., Bento, C. B. P., and de Queiroz, M. V. (2015). Diversity of endophytic fungi in Glycine max. Microbiol. Res. 181, 84–92. doi: 10.1016/j.micrez.2015.05.010

Frontiers, J. P., and Bullo, L. L. R. (2017). Raw starch-digesting amylase from Saccharomyces fibuligeru 2074 isolated from bubod starter. Philippine J. Sci. 146, 27–35.

Gaddeyya, G., Niharika, P. S., Bharathi, P., and Kumar, P. R. (2012). Isolation and identification of soil mycoflora in different crop fields at Salur Mandal. Adv. Appl. Sci. 3, 2020–2026.

Ghosh, B., and Ray, R. R. (2011). Current commercial perspective of Rhizopus oryzae: a review. J. Appl. Sci. 11, 2470–2486.
producing *Aspergillus* species from major food crops grown in India. *Adv. Microbiol.* 2, 577–586. doi: 10.4236/aim.2012.24075

Qiao, W., Tao, J., Luo, Y., Tang, T., Miao, J., and Yang, Q. (2018). Microbial oil production from solid-state fermentation by a newly isolated oleaginous fungus, *Mucor circinelloides* Q531 from mulberry branches. *R. Soc. Open Sci.* 5:180551. doi: 10.1098/rsos.180551

Quiroz-Castañeda, R. E., Balcázar-López, E., Dantán-González, E., Martínez, A., Folch-Mallol, J., and Martínez Anaya, C. (2009). Characterization of cellulolytic activities of *Bykerianda adusta* and *Pycnoporus sanguineus* on solid wheat straw medium. *Electr. J. Biotechnol.* 12, 5–6. doi: 10.2225/vol12-issue6-fulltext-3

Raja, H. A., Miller, A. N., Pearce, C. J., and Oberlies, N. H. (2017). *Fungal Qiao, W., Tao, J., Luo, Y., Tang, T., Miao, J., and Yang, Q. (2018). Microbial Microbiol.* 2, 577–586. doi: 10.1007/s13205-016-0436-4

Ramírez-Camejo, L. A., Zuluaga-Montero, A., Lázaro-Escudero, M. A., Hernández-Kendall, V. N., and Bayman, P. (2012). Phylogeny of the cosmopolitan fungus *Aspergillus flavus*: is everything everywhere? *Fungal Biol.* 116, 452–463. doi: 10.1016/j.funbio.2012.01.006

Romanelli, A. M., Sutton, D. A., Thompson, E. H., Rinaldi, M. G., and Wickes, B. L. (2010). Sequence-based identification of filamentous basidiomycetous fungi from clinical specimens: a cautionary note. *J. Clin. Microbiol.* 48, 741–752. doi: 10.1128/JCM.01948-09

Rosales, E., Couto, S. R., and Sanromán, M. A. (2005). Reutilisation of food processing wastes for production of relevant metabolites: application to laccase producing *Mucor indicus* *J. Inst. Brew.* 125, 443–452. doi: 10.1016/j.jib.2007.0024-8

Shrivastava, K., Greesha, A. M., and Srivastava, B. (2012). Biotechnology in tradition – a process technology of alcoholic beverages practiced by different tribes of Arunachal Pradesh. North East India. *Indian J. Trad. Knowl.* 11, 81–89.

Singh, N. L., Ramprasad Mishra, P. K., Shukla, S. K., Kumar, J., and Singh, R. (2010). Alcoholic fermentation techniques in early Indian tradition. *Indian J. Sci. Hist. Sci.* 45, 163–173.

Suesse, A. R., Norton, G. A., and van Leeuwen, J. (2016). Pilot-scale continuous-flow hydrothermal liquefaction of filamentous fungi. *Energy Fuels* 30, 7379–7386. doi: 10.1021/acs.energyfuels.6b01229

Tamang, J. P. (2017). *Himalayan Fermented Foods: Microbiology, Nutrition, and Ethnic Values*. New York, NY: CRC Press.

Tamang, J. P., Cotter, P., Endo, A., Han, N. S., Kort, R., Liu, S. Q., et al. (2020). Fermented foods in a global age: east meets west. *Comprehen. Rev. Food Sci. Food Saf.* 19, 184–217. doi: 10.1111/1541-4337.12520

Tamang, J. P., Dewan, S., Tamang, B., Rai, A., Schilling, U., and Holzapfel, W. H. (2007). Lactic acid bacteria in hameci and marchi of North East India. *Indian J. Microbiol.* 47, 119–125. doi: 10.1007/s12088-007-0024-8

Tamang, J. P., and Sarkar, P. K. (1995). Microflora of murcha: an amylolytic fermentation starter. *Microbols.* 81, 115–122.

Tamang, J. P., Sarkar, P. K., and Hesseltine, C. W. (1988). Traditional fermented foods and beverages of Darjeeling and Sikkim - a review. *J. Sci. Food Agri.* 44, 375–385.

Tamang, J. P., and Thapa, S. (2006). Fermentation dynamics during production of bhaati jaanr, a traditional fermented rice beverage of the Eastern Himalayas. *Food Biotechnol.* 20, 251–261.

Tamang, J. P., Watanabe, K., and Holzapfel, W. H. (2016). Diversity of microorganisms in global fermented foods and beverages. *Front. Microbiol.* 7:377. doi: 10.3389/fmicb.2016.00377

Thakur, N., Saris, P. E., and Bhalla, T. C. (2015). Microorganisms associated with amylolytic starters and traditional fermented alcoholic beverages of North Western Himalayas in India. *Food Biosci.* 11, 92–96.

Thanh, V. N., Mai, L. T., and Tuan, D. A. (2008). Microbial diversity of traditional Vietnamese alcoholic fermentation starters (banh men) as determined by PCR-mediated DGGE. *Int. J. Food Microbiol.* 128, 268–273. doi: 10.1016/j.ifl.2008.08.020

Thapa, S., and Tamang, J. P. (2014). Product characterization of kodo ko jaanr: fermented finger millet beverage of the Himalayas. *Food Microbiol.* 21, 617–622.

Thapa, S., and Tamang, J. P. (2006). Microbiological and physio-chemical changes during fermentation of kodo ko jaanr, a traditional alcoholic beverage of the Darjeeling hills and Sikkim. *Indian J. Microbiol.* 46, 333–341.

Tsang, C. C., Tang, Y. J., Lau, S. K., and Woo, P. C. (2018). Taxonomy and evolution of *Aspergillus, Penicillium* and *Talaromyces* in the omics era–Past, present and future. *Computer Struct. Biotech.* 16, 197–210. doi: 10.1016/j.csbt.2018.05.003

Umesha, S., Manukumar, H. M., and Raghava, S. (2016). A new species concept for the clinically relevant *Mucor circinelloides* complex. *Persiona* 44, 67–97.

Wang, J., Chio, C., Chen, X., Su, E., Cao, F., Jin, Y., et al. (2019). Efficient saccharification of agave biomass using *Aspergillus niger* produced low-cost enzyme cocktail with hyperactive pectinase activity. *Biore. Technol.* 272, 26–33. doi: 10.1016/j.biotech.2018.09.069

Wei, H., Wang, W., Yarbrough, J. M., Baker, J. O., Laurens, L., and Van Wychen, S. (2013). Genomic, proteomic, and biochemical analyses of oleaginous *Mucor circinelloides* evaluating its capability in utilizing cellulolytic substrates for lipid production. *PLoS One* 8:e72068. doi: 10.1371/journal.pone.0072068

Xing, J. H., Sun, Y. F., Han, Y. L., Cui, B. K., and Dai, Y. C. (2018). Morphological and molecular identification of two new Ganoderma species on *Casuarina*
*Equisetum* from China. *Mycol. Keys* 34, 93–108. doi: 10.3897/mycokeys.34.22593

Xu, J. (2016). Fungal DNA barcoding. *Genome* 59, 913–932.

Yang, S., Lee, J., Kwak, J., Kim, K., Seo, M., and Lee, Y. W. (2011). Fungi associated with the traditional starter cultures used for rice wine in Korea. *J. Korean Soc. Appl. Biol. Chem.* 54, 933–943.

Zhang, Y., Fraatz, M. A., Müller, J., Schmitz, H. J., Birk, F., Schrenk, D., et al. (2015). Aroma characterization and safety assessment of a beverage fermented by Trametes versicolor. *J. Agric. Food Chem.* 63, 6915–6921. doi: 10.1021/acs.jafc.5b02167

Zheng, X. W., Yan, Z., Han, B. Z., Zwietering, M. H., Samson, R. A., Boekhout, T., et al. (2012). Complex microbiota of a Chinese "Fen" liquor fermentation starter (Fen-Daqu), revealed by culture-dependent and culture-independent methods. *Food Microbiol.* 31, 293–300. doi: 10.1016/j.fm.2012.03.008

Zhou, B., Ma, C., Wang, H., and Xia, T. (2018). Biodegradation of caffeine by whole cells of tea-derived fungi *Aspergillus sydowi*, *Aspergillus niger* and optimization for caffeine degradation. *BMC Microbiol.* 18:53. doi: 10.1186/s12866-018-1194-8

Zulkifli, N. A., and Zakaria, L. (2017). Morphological and molecular diversity of *Aspergillus* from corn grain used as livestock feed. *HAYATI J. Biosci.* 24, 26–34.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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