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Original article

Identification of yeasts by polymerase-chain-reaction-mediated denaturing gradient gel electrophoresis in *marcha*, an ethnic amylolytic starter of India

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ABSTRACT

Background: *Marcha* is an ethnic amylolytic starter that is used to ferment boiled cereals to produce alcoholic drinks, commonly called *jaanr*, in the Himalayan Regions of Sikkim and Darjeeling of India.
Methods: The aim of this study was to investigate yeast flora of *marcha* collected from Sikkim in India by phenotypic characterization and polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE).
Results: The average load of yeast in *marcha* was 6.0×10^8 colony-forming units/g. The phenotypic characterization of yeast isolates from *marcha* showed the presence of *Candida*, *Pichia*, *Torulospora*, *Schizosaccharomyces*, *Kluveromyces*, *Issatchenki*, and *Saccharomycopsis*. The PCR-DGGE bands showed the dominance of *Wickerhamomyces anomalus* (72%) and *Pichia anomalus* (28%) in *marcha*. *W. anomalus* was reported for the first time from *marcha* using PCR-mediated DGGE.
Conclusion: This is the first report on the yeast community associated with *marcha* analyzed by PCR-mediated DGGE.

1. Introduction

The traditional way of preparation of amylolytic starters is a unique technology of preservation of native microorganisms, consisting of consortia of amylolytic and alcohol-producing yeasts, molds, and some lactic-acid bacteria, with rice or wheat as the base in the form of dry, flattened, or round balls, for alcoholic beverage production in Asia [1]. Amylolytic starters in Asia have different vernacular names such as *marcha* in India, Nepal, and Bhutan, *hamei*, *humao*, and *phab* in India [2–4], *mana* and *manapu* in Nepal [5], *men* in Vietnam [6], *bubod* in the Philippines [7], *chiu/chu* in China and Taiwan [8], *loogpang* in Thailand [9], *ragi* in Indonesia [10], and *nuruk* in Korea [8]. *Marcha* is a nonfood starter culture uses for production of various ethnic alcoholic beverages in the Darjeeling Hills and Sikkim in India, Nepal, and Bhutan [3]. It is a dry, round-to-flattened, creamy to dusty white, solid ball-like starter (Fig. 1). During its preparation, soaked glutinous rice is crushed in a foot-driven heavy wooden mortar, with the addition of the roots of *Plumbago zeylanica* L., leaves of *Buddleja asiatica* Lour, flowers of *Vernonia cinerea* (L.) ginger, red dry chili, and 1% of previously prepared powdered *marcha* for back-sloping fermentation [2]. The mixed dough is kneaded into round or flat cakes of different sizes and shapes that are placed individually on a platform suspended below the bamboo-made ceiling above the earthen kitchen, bedded with fresh fronds of fern *Glaphylopteriolopsis erubescens* (Wall ex Hook.) Ching, and covered with dry fronds of fern and jute bags and are then left to ferment for 1–3 days. Finally, cakes of *marcha* are sun dried for 2–3 days and stored in a dry place at room temperature for > 1 year. Application of polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE), a culture-independent method, is widely applied to study microbial diversity [11–13]. Some species of yeasts such as *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Saccharomycopsis*, *Schizosaccharomyces*, *Torulopsis*, and *Zygosaccharomyces* were previously reported from samples of *marcha* using culture-dependent approaches [7,14,15]. However, a culture-independent method using PCR-DGGE has not

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Fig. 1. *Marcha*, amylolytic starter of Sikkim in India.

been applied yet in profiling of yeast flora in *marcha*. The present study aimed to profile yeast flora directly from *marcha* samples using PCR-mediated DGGE.

2. Materials and methods

2.1. Sample collection

Ten samples of dry *marcha* were collected from the local market or villages of Sikkim in presterile polythene bags, and were stored in a desiccator at room temperature until analysis.

2.2. Culture-dependent analysis

2.2.1. Isolation of microorganisms

Ten grams of powdered *marcha* was mixed in 90 mL physiological saline (0.85%) and homogenized in a Stomacher Lab-Blender 400 (Seward, Worthing, UK) for 1 minute. Serial dilutions were prepared in sterile diluent and mixed with the molten media and poured into plates. Plates of yeast extract–malt extract agar (M424; HiMedia, Mumbai, India) for enumeration of yeasts were incubated at 30°C for 48 hours. Yeast isolates were purified and preserved at −20°C in yeast extract–malt extract broth (M425; HiMedia) mixed with 20% (v/v) glycerol.

2.2.2. Phenotypic characterization

Cell morphology of yeast isolates was determined using a phase contrast microscope (CH3-BH-PC; Olympus, Tokyo, Japan). Yeast cultures have been characterized on the basis of mycelium type, ascospore type, nitrate reduction, growth at 37°C and 45°C, sugar fermentation, and sugar assimilation following the methods of Kurtzman et al [16].

2.3. Culture-independent analysis

2.3.1. DNA extraction from sample

Ten grams of powdered *marcha* was homogenized in 90 mL of 0.85% w/v sterile physiological saline, and subsequently filtered. The resulting filtered solutions were centrifuged at 14,000 *g* for 10 minutes at 4°C, and pellets were subjected to DNA extraction using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) after glass bead (0.2–0.5 mm diameter; Sigma-Aldrich, Roth, Germany) beating to rupture the cell walls. The yield and quality of DNA were detected through agarose gel electrophoresis (1.0%), which was stained with ethidium bromide solution.

2.3.2. PCR amplification and DGGE analysis

PCR-DGGE analysis was performed as described previously [17]. Coated PCR with primers sets ITS1-F, ITS4, ITS2, and ITS1F-GC was used to amplify yeast ITS region [18]. A 40-base (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) attached to the 5′ end of the ITS1-F primer was used to stabilize the melting behavior of the DNA fragments during DGGE analysis [19]. The first round of PCRs was carried out in a Mastercycler (Applied Biosystems, Foster City, CA, USA) using 25-μL reaction volumes containing: 1 μL DNA template, 0.25 μL each primer (10μM), 12.5 μL 2× Go Taq Master Mix (Promega), and 11 μL nuclease-free water. PCR cycle was programed as follows: 94°C for 4 minutes followed by 10 cycles of 94°C for 1 minutes, lowering the annealing temperature from 65°C to 55°C in 1°C steps for each cycle for 1 minute, 72°C for 1 minute, and finally 25 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and followed by a final extension at 72°C for 7 minutes. The second PCRs were carried out in a Mastercycler (Applied Biosystems) using 50-μL reaction volumes containing: 1 μL first PCR production, 0.5 μL each primer (10μM), 25 μL 2× Go Taq Master Mix (Promega) and 23 μL nuclease-free water. Cycling parameters were the same as for the first round of PCR. All amplified products were analyzed by electrophoresis in 1.2% (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light. DGGE analysis was carried out using the PCR products in an universal mutation detection system (DGGEK-1001-220; CBS Scientific, Del Mar, CA, USA) following the procedure described by El Sheikha et al [20]. Samples containing approximately equal amounts of PCR amplicons (30 μL) were loaded into 8% (w/v) polyacrylamide gels (acrylamide/N, N0-methylene bisacrylamide, 37.5/1; Promega) in 1× TAE buffer (40mM Tris–HCl pH 7.4, 20mM sodium acetate, 1.0mM Na_2-EDTA). Electrophoresis was performed at 60°C in a denaturing gradient ranging from 40% to 60% [100% corresponded to 7M urea and 40% (v/v) formamide; Promega], at 20 V for 10 minutes and then at 130 V for 4.5 hours, and the gels were stained for 30 minutes with ethidium bromide and then photographed on a gel documentation unit (GelDoc 1000; Bio-Rad, Hercules, CA, USA).

2.3.3. Identification of bands

Individual DGGE bands were excised, resuspended in 20 μL sterile Tris–EDTA buffer, and stored at 4°C overnight. An aliquot of supernatant was used as a DNA template for PCR reamplification as described above, and electrophoresed with DGGE. Band excision, PCR, and DGGE were repeated until a single band was present. PCR products generated from DGGE bands were amplified with primers ITS2 and ITS-1f (without the GC clamp) for sequencing using DNA sequencer (Applied Biosystems). Sequences of major bands obtained from the DGGE gel fragments were compared with the GenBank database using the web-based nucleotide–nucleotide BLAST search engine hosted by the National Center for Biotechnology Information (Bethesda, MD, USA) for identification (http://www.ncbi.nlm.nih.gov) [21].

2.3.4. Phylogenetic analysis

The BLAST program was used for comparing DNA databases for sequence similarities available on the EzTaxon server (http://eztaxon-e.ezbiocloud.net/). Molecular evolutionary genetics analysis software (MEGA version 6, US National Library of Medicine, National Institution of Health) was used for phylogenetic analyses [22].

Table 1
Characterization of yeasts isolates from *marcha*.

Representative strains*	No. of grouped strains	Pellicle formation	Nitrate reduction	Sugar fermentation†									Tentative genera
				Cellobiose	Arabinose	Ribose	Mannose	Raffinose	Aesculin	Galactose	Trehalodse	Xylose	
GM:Y12	2	+	−	−	−	+	+	+	+	+	−	+	*Saccharomyces*
GM:Y21	2	+	−	−	−	+	1/1	+	+	+	−	−	*Saccharomyces*
GM:Y50	3	+	−	2/1	−	+	+	+	+	2/1	+	+	*Saccharomyces*
GM:Y34	3	−	+	+	+	+	+	+	+	+	−	+	*Pichia*
GM:Y43	2	−	+	+	+	+	+	+	+	+	−	+	*Pichia*
GM:Y7	3	−	−	+	−	−	−	−	−	−	−	2/1	*Candida*
GM:Y37	2	−	−	+	−	−	−	−	−	−	−	+	*Candida*
GM:Y4	2	+	−	−	−	+	+	−	+	+	−	−	*Issatchenkia*
GM:Y36	2	+	−	−	−	+	+	−	+	+	−	−	*Issatchenkia*
GM:Y10	2	+	−	−	−	+	+	−	+	+	−	−	*Issatchenkia*
GM:Y29	4	+	−	+	+	3/1	+	+	+	+	+	−	*Kluveromyces*
GM:46	3	+	−	+	+	2/1	+	+	+	+	+	−	*Kluveromyces*
GM:Y5	3	+	−	+	+	+	+	+	+	+	+	−	*Schizosaccharomyces*
GM:49	2	+	−	+	+	+	+	+	+	+	+	−	*Schizosaccharomyces*
GM:Y15	3	+	−	+	+	+	+	+	+	+	+	−	*Schizosaccharomyces*
GM:Y22	2	+	−	+	1/1	+	+	+	+	+	+	+	*Saccharomycopsis*
GM:Y41	2	+	−	+	1/1	+	+	+	+	+	+	+	*Saccharomycopsis*
GM:Y1	4	+	−	−	+	+	3/1	+	+	+	+	+	*Torulospora*
GM:Y18	4	+	−	−	+	+	3/1	+	+	+	+	+	*Torulospora*

+, all strains positive; −, all strains negative; (../..), number of positive/negative strains.
* All yeast cells were oval to circular, colonies of all strains were smooth and creamy white, showed pseudo mycelia. All strains grew well at 37°C, but not at 45°C, except *Kluveromyces* grew at 45°C.
† All strains fermented sucrose, glucose, fructose, galactose, maltose, and no strains fermented lactose, except *Kluveromyces*, and all strains assimilated sugars except lactose.

3. Results

3.1. Phenotypic characterization of yeasts

The average load of yeast in *marcha* was calculated as 6.0×10^8 colony-forming units/g (data not shown). A total of 50 yeasts isolates were isolated from 10 samples of *marcha*. Characterization of yeasts isolated from different *marcha* samples of Sikkim, which were phenotypically identified on the basis of colony morphology, cell morphology sugar fermentation, and sugar assimilation tests (Table 1). Out of 50 isolates, 19 representative isolates were grouped based on colony appearance, cell shape, type of mycelia and ascospores, pellicle formation, nitrate reduction, and growth at 37°C and 45°C (Table 1). All 50 isolates were tested for sugar fermentation and sugar assimilation for identification up to genus level (Table 1). Tentatively the following yeast genera were identified: *Candida* (18%), *Pichia* (14%), *Torulospora* (14%), *Schizosaccharomyces* (16%), *Kluveromyces* (10%), *Issatchenki* (20%), and *Saccharomycopsis* (8%).

3.2. PCR-DGGE analysis

DNA was directly extracted from *marcha* samples. The results of PCR-DGGE analysis showed the diversity of yeast (Fig. 2). Five bands were identified as *Wickerhamomyces anamolus* and two bands as *Pichia anamolus*. Detected in eight of the 10 samples, *W. anamolus* (DGGE bands MY1, MY3, MY4, MY7, and MY8) was found to be the most abundant yeast species. *P. anamolus* (DGGE bands MY5 and MY6) was detected frequently in *marcha* (Fig. 2). Nevertheless, it is worthwhile to note that some yeast species were only detected by DGGE in some samples, for example, *W. anamolus* was found in *marcha* samples with an intense DGGE band. By contrast, *P. anamolus* was detected both by culture independent (PCR-DGGE) as well as culture-dependent techniques (conventional microbiological method). However, we were not able to identify the minor bands (MY9–MY13) since they could not be excised from the gels due to their low intensity.

The selected seven isolates were identified by partial 18S rRNA gene sequencing and were compared to the EzTaxon server database for their phylogenetic relationship using MEGA 6.06 version software (Fig. 3). Five of the isolates were identified as *W. anamolus* (MY1, MY3, MY4, MY7, and MY8) and two as *P. anomalus* (MY5, MY6). Identification based on BLAST comparison in GenBank of the bands obtained by PCR-DGGE gel using universal primers NL1/LS2 is shown in Table 2.

4. Discussion

The diversity of yeasts associated with amylolytic starters may be closely related to the raw material used and the regional climate

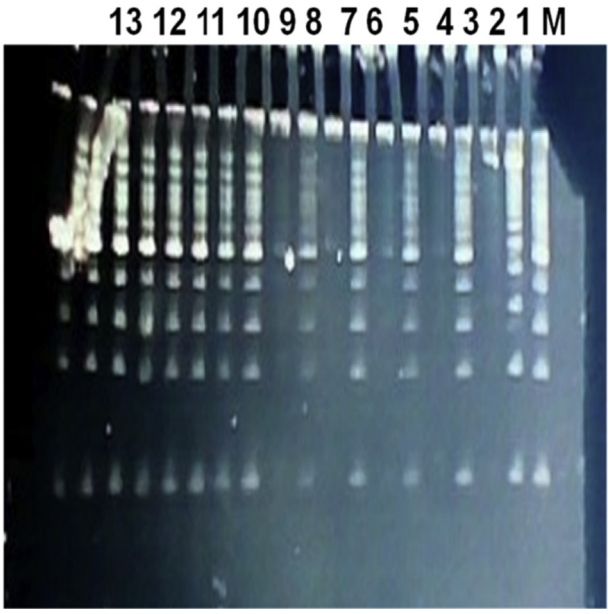

Fig. 2. Denaturing gradient gel electrophoresis profile of bands. 1, *Wickerhamomyces anomalus*; 2, 9, 10, and 11–13 unidentified; 3, *W. anomalus*; 4, *W. anomalus*; 5, *Pichia anomalus*; 6, *P. anomalus*; 7, *W. anomalus*; 8, *W. anomalus*.

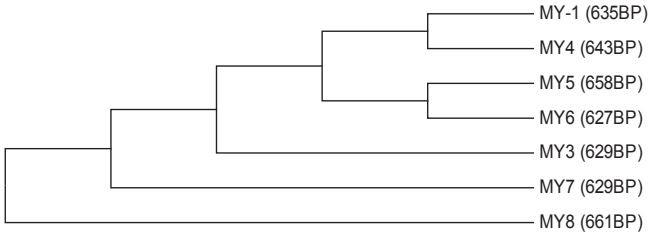

Fig. 3. Phylogenetic tree of yeasts retrieved from bands (MY1–MY8) in denaturing gradient gel electrophoresis profile by neighbor-joining distance tree constructed by MEGA 6 for the seven yeast isolates with the phylogenetic neighbors obtained from EzTaxon server.

Table 2
Identification based on BLAST comparison in GenBank of the bands obtained by polymerase chain reaction–denaturing gradient gel electrophoresis using universal primers NL1/LS2.

Band	Closest relative species	Accession No.	% identity
MY1	*Wickerhamomyces anomalus*	KT175181.1	99
MY3	*W. anomalus*	KT175201.1	99
MY4	*W. anomalus*	G0280811.1	99
MY5	*Pichia anomalus*	E0798697.1	98
MY6	*P. anomalus*	AY349435.1	99
MY7	*W. anomalus*	KT175181.1	99
MY8	*W. anomalus*	KT175181.1	99

where they are produced [23]. *Saccharomycopsis fibuligera*, *Saccharomyces cerevisiae*, *W. anomala*, *Pichia guilliermondii*, and *Candida* sp. are the most common yeasts present in rice-based starters of Asia [24–26]. It is interesting to find that yeast species (*W. anomalus*) could not be detected by conventional media but has been detected by PCR-DGGE analysis. *W. anomalus* has been reported in *hong-qu* and *yao-qu*, traditional amylolytic starters of China [27,28] and *banh men*, a traditional Vietnamese starter [29]. However, we noticed that *P. anomala*, *Issatchenkia*, *S. cerevisiae*, *Torulospora*, *Kluveromyces*, *Candida musae*, *S. fibuligera*, and *Saccharomycopsis* spp., which were detected in *marcha* through culture-dependent methods, were not detected in *marcha* in PCR-mediated DGGE assay. This discrepancy may have been due to the selective amplification, migration of PCR products from different species, and efficiencies of genomic DNA extraction kits for different species [30,31]. Such discrepancies between culturing and DGGE results have also been reported earlier [11,32]. Therefore, it is suggested that the DGGE technique be supplemented with culture-dependent methods, and their combination seems the best strategy to have a complete overview of yeast ecology of traditional alcoholic starters. *W. anomalus*, probably nonculturable yeast, was reported for the first time from *marcha* using PCR-mediated DGGE technique.

This is believed to be the first report on the yeast community associated with *marcha* of India analyzed by PCR-mediated DGGE. The results may enrich our knowledge of nonculturable native microorganisms that may be present in the traditionally prepared starters of Asia.

Conflict of interest

There is no conflict of interest.

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