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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; 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 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) was used for phylogenetic analyses [22].

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