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Microorganisms associated with amylolytic starters and traditional fermented alcoholic beverages of North Western Himalayas in India

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ABSTRACT

Traditional alcoholic brewing is generally a home-based industry mostly practiced by the ethnic people of Himalayas. *Chhang, jau chhang* and *sura* are the three most popular cereal based traditional fermented alcoholic beverages prepared and consumed by tribal and rural folks of Himachal Pradesh (North West Himalayas) using traditional inocula '*phab*' and '*dheli*'. The numbers of lactic acid bacteria (LAB) were in range of 1.7×10^4 CFU/g in *chhang*, 2.9×10^4 CFU/g in *jau chhang*, and 1.9×10^7 CFU/g in *sura*. LAB isolates were strains of *Lactobacillus plantarum, Lactobacillus casei, Enterococcus faecium* and *Pediococcus pentosaceus* based on comparison of the sequence of 16S rRNA genes. Yeasts were identified from all the three beverage samples with cell number ranging from 3.5×10^4 CFU/g for *chhang*, 1×10^5 CFU/g for *jau chhang* and 5.5×10^5 CFU/g for *sura*. Sequencing of D1/D2 26S rDNA regions identified the yeasts as *Saccharomyces cerevisiae, Saccharomyces fibuligera, Pichia kudriavzevii* and *Candida tropicalis*.

1. Introduction

Traditional alcoholic brewing is generally a home-based industry mostly practiced by the tribal and rural folks using their indigenous knowledge in Himalayan belts of India. Especially, the people of the tribal and rural areas of North Western Himalayan regions (Lahaul & Spiti and Kullu districts of Himachal Pradesh) prepare these fermented cereal beverages. *Chhang* is made from cooked rice and *jau chhang* from cooked barley. *Chhang* and *jau chhang* are mild alcoholic sweet beverages prepared using traditional inocula *'phab'* by the tribals of Lahaul. *Sura* is prepared using *'dheli'* as an additive by the highlanders in *Lug* valley of Kullu district of Himachal Pradesh (Thakur, Savitri, & Bhalla, 2004).

Phab is said to be prepared by the tribal people of *Nubra* valley in Ladakh region by using coarse flour of husked roasted barley (*Hordeum vulgare*). Fresh twigs of shrub *Artemisia* sp. locally available in Ladakh is used to incubate the fresh tablet of *phabs* (Angmo & Bhalla, 2014).

Some other ethnic amylolytic starters reported similar to '*phab*' are *Hamei* and *marcha* (Tamang & Kailasapathy, 2010) from North East part of India. The diversity of fungi and bacteria associated with traditional starter *banh men* from Vietnam has also been investigated (Thanh, Mai, & Tuan, 2008).

Previous studies by Sourabh, Kanwar, and Sharma (2011) have also reported the probiotic attributes of the yeasts isolated from indigenous fermented foods. It is thus important to study the microbial population of these fermented cereal based alcoholic beverages. In this study lactic acid bacteria, yeasts and fungi were isolated from all three different cereal based alcoholic beverages and traditional fermentation starters. Identification to species level was done by PCR amplification of 16S rDNA of lactic acid bacteria and D1/D2 domain of 26S rDNA of yeasts using respective universal primers.

2. Materials and methods

2.1. Sample collection and isolation

Traditionally prepared *phab* samples (2) were collected from the tribal village (Tholang) and *dheli* (1 sample) was procured from villagers of *Lug* valley in Kullu district of Himachal Pradesh. *Chhang, jau chhang* and *sura* were prepared using traditional method in laboratory. One gram of each product was serially diluted in physiological saline and spread-plated onto MRS agar (Oxoid, UK) for isolation of lactic acid bacteria (LAB). The plates were incubated aerobically at 30 °C for 24–48 h. In case of yeasts isolation was done on YPD agar (yeast extract 2%, peptone 1%, dextrose 1% and agar 2%). LAB and yeasts were purified by streaking on MRS and YPD agar plates.

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2.2. Identification of lactic acid bacteria

Colonies from MRS agar plates were inoculated into MRS broth and incubated at 30 °C for 24–48 h. Cultures with pH below 4.0 were Gram stained and tested for the presence of catalase and oxidase activity according to methods described by Harrigan and McCance (1976).

LAB colonies were picked on the basis of differences in colony morphology during succession studies in fermentation. Identification to species level was done by PCR amplification of the 16S rRNA gene with lactic acid bacterial universal primers (Thomas, 2004) 27F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). Isolates were grown in MRS broth at 30 °C and DNA was extracted by alkaline lysis method of Sambrook, Fritsch, and Maniatis (1989). PCR reaction was performed in 50 µl of the reaction mixture containing 1 µl of DNA, 5.0 µl of PCR buffer (10 ×) (Promega, A3511, USA), 1.0 µl of 5 mM dNTPs (Promega, U1240, USA), 1.0 µl each of forward and reverse primers 5 mM, 1 µl of Dynazyme (Promega) and 33 µl of autoclaved deionized water (MilliQ, Millipore). The amplification was performed with a total of 34 cycles in a thermal cycler (Eppendorf, USA). The cycling program was started with an initial denaturation at 94 °C for 5 min followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for lactic acid bacteria. The PCR was ended with a final extension at 72 °C for 7 min and then amplified product was cooled at 4 °C. The products of PCR reactions were visualized on 1.0% agarose gel containing ethidium bromide to confirm amplification of fragment. PCR products were purified using the Promega QIA quick PCR purification kit (Qiagen, Germany). Sequencing was conducted at sequencing facility at Viikki Biocentre, University of Helsinki, Finland. The newly acquired sequences were compared to those in the NCBI GenBank by BLAST.

2.3. Identification of yeasts

Colonies of yeast were picked from YPD agar (HiMedia, Mumbai, India) plates and cell morphology was observed under light microscope. Isolates were cultured in YPD broth for 18 h at 30 °C and genomic DNA was extracted using the Bust and Grab method (Harju, Fedosyuk, & Peterson, 2004). In the case of yeast, the divergent D1/D2 domain of the 26S rRNA gene was amplified using the universal primers UniF63(5'-GCATATCAATAAGCGGAGGAAAAG-3')and UniLR3 (5'-GGTCCGTGTTTCAAGACGG-3') (Minegishi, Miura, Yoshida, Usami, & Abe, 2006).PCR reaction was performed in 50 µl of the reaction mixture containing 1 µl of DNA, 5.0 µl of PCR buffer (10 ×) (Promega, A3511, USA), 1.0 µl of 5 mM dNTPs (Promega, U1240, USA), 1.0 µl each of forward and reverse primers 5 mM, 1 µl of Dynazyme (Promega) and 33 µl of autoclaved deionized water (MilliQ, Millipore). The amplification was carried out with a total of 34 cycles in a thermal cycler. The cycling program was started with an initial denaturation at 94 °C for 5 min followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for yeasts. The PCR was ended with a final extension at 72 °C for 7 min and then amplified product was cooled at 4 °C. The products of PCR reactions were purified, sequenced and analyzed similarly as for the amplified fragments of the LAB.

2.4. Taxonomic studies and phylogenetic tree construction

Taxonomic strain identification was done by comparing the sequences of each isolate with 16S rRNA and 26S rRNA gene sequences available in the Genebank databases by Basic BLASTn search. Partial sequences were aligned using multiple sequence alignment software ClustalW2 (Thompson, Higgins, & Gibson, 1994). A distance matrix and phylogenetic tree was generated for all the representative isolates of LAB and yeasts using the neighbor-joining method in MEGA 5 software (Tamura, Dudley, Nei, & Kumar, 2007). The robustness of phylogeny was tested by bootstrap analysis using 1000 replications. The sequences in this study were deposited in GenBank and are available under accession numbers as shown in Table 2.

Table 1
Identification of isolates according to 16S rDNA (LAB) and D1/D2 26S rDNA (yeasts) sequencing.

Isolate no.	Isolated from	% homology with the gene bank sequence	Gene bank accession number with corresponding sequence	Identification
SAA 595	*Jau Chhang*	96	EU559598.1	*Lactobacillus. plantarum*
SAA 596	*Jau Chhang*	99	JX141320.1	*Lactobacillus sp.*
SAA 597	*Jau Chhang*	97	AB713901.1	*Lactobacillus plantarum*
SAA 599	*Chhang*	99	AB681648.1	*Pediococcus pentosaceus*
SAA 600	*Jau Chhang*	97	JX141317.1	*Pediococcus pentosaceus*
SAA 601	*Jau Chhang*	99	KC514128.1	*Serratia proteamaculans*
SAA 602	*Chhang*	98	FJ538538.1	*Lactobacillus sp.*
SAA 603	*Chhang*	97	NR075052.1	*Pediococcus pentosaceus*
SAA 604	*Sura*	89	HQ609504.1	*Lactobacillus sp.*
SAA 605	*Sura*	98	FJ514026.1	*Enterococcus sp.*
SAA 606	*Sura*	96	EU600909.1	*Lactobacillus sp.*
SAA 608	*Sura*	97	NR075052.1	*Pediococcus pentosaceus*
SAA 609	*Sura*	98	JX141323.1	*Enterococcus faecium*
SAA 610	*Chhang*	96	GQ280044.1	*Bacillus sp.*
SAA 612	*Chhang*	99	JQ512832.1	*Saccharomyces cerevisiae*
SAA 613	*Jau Chhang*	99	KC94703.1	*Candida tropicalis*
SAA 614	*Sura*	99	JX848640.1	*Pichia kudriavzevii*
SAA 615	*Sura*	99	HM123747.1	*Pichia kudriavzevii*
SAA 616	*Sura*	99	JX141335.1	*Saccharomyces cerevisiae*
SAA 617	*Chhang*	99	HM101473.1	*Saccharomyces cerevisiae*
SAA 618	*Chhang*	99	AB550115.1	*Saccharomyces fibuligera*
SAA 620	*Jau Chhang*	100	HM191663.1	*Saccharomyces cerevisiae*
Isolates from traditional inocula				
SAA 607	*Phab*	92	AB494717.1	*Lactobacillus plantarum*
SAA 598	*Phab*	99	CP000422.1	*Pediococcus pentosaceus*
SAA 611	*Dheli*	99	JX141327.1	*Enterococcus faecium*