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Molecular identification of yeast species associated with 'Hamei' — A traditional starter used for rice wine production in Manipur, India

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ABSTRACT

In Manipur state of North-Eastern India, wine from glutinous rice using traditional solid state starter called 'Hamei' is particularly interesting because of its unique flavour. A total of 163 yeast isolates were obtained from fifty four 'Hamei' samples collected from household rice wine preparations in tribal villages of Manipur. Molecular identification of yeast species was carried out by analysis of the restriction digestion pattern generated from PCR amplified internal transcribed spacer region along with 5.8S rRNA gene (ITS1-5.8S-ITS2). Seventeen different restriction profiles were obtained from the size of PCR products and the restriction analysis with three endonucleases (Hae III, Cfo I and Hinf I). Nine groups were identified as *S. cerevisiae*, *Pichia anomala*, *Trichosporon* sp., *Candida tropicalis*, *Pichia guilliermondi*, *Candida parapsilosis*, *Torulaspora delbrueckii*, *Pichia fabianii* and *Candida montana* by comparing this ITS-RFLP profile with type strains of common wine yeasts, published data and *insilico* analysis of ITS sequence data available in CBS yeast database. ITS-RFLP profile of eight groups was not matching with available database of 288 common wine yeast species. The most frequent yeast species associated with 'Hamei' were *S. cerevisiae* (32.5%), *P. anomala* (41.7%) and *Trichosporon* sp. (8%). The identity of major groups was confirmed by additional restriction digestion of ITS region with Hind III, EcoRI, Dde I and Msp I. The genetic diversity of industrially important *S. cerevisiae* group was investigated using Pulsed Field Gel Electrophoresis (PFGE). Although most of the 53 strains of *S. cerevisiae* examined were exhibited a common species specific pattern, a distinct degree of chromosomal length polymorphism and variable number of chromosomal DNA fragments were observed with in the species. Cluster analysis showed seven major karyotypes (K1–K7) with more than 83% similarity. The karyotype pattern K1 was the most frequent (67.9%) among the strains from different samples. Other karyotypes K2–K7 were very unique with less than 80% similarity. Finally using mitochondrial DNA restriction analysis (mt-DNA RFLP), *S. cerevisiae* strains belonging to the major karyotype K1 were distinctly differentiated with highly polymorphic bands by Hinf I and Hae III endonucleases.

1. Introduction

Rice wine from glutinous rice is a popular traditional alcoholic beverage in North-Eastern states of India. It is manufactured under non-sterile conditions at home scale using traditional solid rice flat cakes as starter. The principle of rice wine manufacturing consist of the saccharification of steamed rice starch by fungal enzymes under aerobic solid state fermentation and the moulded mass is mixed with water and is allowed to undergo submerged alcoholic fermentation by yeasts using traditional starter flat cakes (Blandino et al., 2003; Sujaya et al., 2004; Dung et al., 2007).'Atingba' is an alcoholic beverage traditionally prepared in Manipur state of India from glutinous rice with starter called 'Hamei'. 'Hamei' is a natural starter (flat rice cake), similar to 'Ragi' of Indonesia, 'Budob' of Philippines, 'Chu' of China, 'Naruk' of Korea and 'Marcha' of Sikkim that has been traditionally used for the preparation of rice wine (Tsuyoshi et al., 2005). The 'Hamei' cakes (Fig. 1B) are prepared from crushed raw rice with "Yangli" (*Albizia myriophylla*) bark powder @ 0.25 kg kg^{-1} along with water to form dough like mass with moisture content 65–70%. This is inoculated by dry powdered starter 'Hamei' from previous batches, followed by thorough mixing. The inoculated dough is shaped into form flat cakes approximately 2–7 cm in diameter and 0.6–1.5 cm thickness. The prepared rice cakes are kept over rice husk (Fig. 1A) in the floor/ bamboo basket for 2–3 days at room temperature (20–30 °C). The desired state of fermentation is indicated by the swelling of cakes, alcoholic flavour production and yellowish coloration. These commercial undefined starters have been prepared during summer (May–July) and dried cakes have shelf life up to one year.

The "'Hamei'" is used by crushing the flat cake into powder, then mixing with cooked, cooled glutinous rice @ 5 cakes for 10 kg. The mixture is fermented under Solid State Fermentation in mud pots (Fig. 1C) covered with 'Hangla' (*Alocasia* sp.) leaves for 3–4 days during summer and 6–7 days in winter. This is followed by 2–3 days of

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Fig. 1. 'Hamei' — traditional preparation over rice husk layered with gunny bags (A), dried 'Hamei' for storage & marketing (B) and solid state fermentation of glutinous rice inoculated with 'Hamei' powder for rice wine production in traditional earthen pots (C).

submerged fermentation in earthen pots. The filtered fermented beverage is called 'Atingba' and distilled clear liquor (using traditional assembly) is called 'Yu'. The recipes are being kept secret and passed on from generation by generation. This practice produces low yields of wine with variable quality where as wine producers are aware that the choice of starter influences the yield and quality of wine. The limited knowledge about the microbial composition of traditional starters and their effect on rice wine fermentation, obstacles development of defined mixed pure culture for industrialization of rice wine production (Dung et al., 2005).

Traditionally, yeasts have been identified based on morphological, physiological and biochemical characteristics (Jespersen, 2003). These methods are laborious and time consuming. Molecular methods based on the analysis of polymorphism in DNA region that encodes the ribosomal RNA genes (5S, 5.8S, 18S and 26S) (Kurtzman and Robnett, 1998; Couto et al., 2005; Gonzalez et al., 2006) and the non-coding ITS (Internal Transcribed Spacers) (Sabate et al., 2002; Cadez et al., 2002) and IGS (Intergenic Spacer) regions (Diaz and Fell, 2000; Naumova et al., 2003) are being successfully used for the identification of many yeast species. In 1999, Esteve-Zarzoso et al., and Granchi et al., proposed a rapid and easy method for routine identification of yeast associated with fermented foods based on RFLP analysis of 5.8S rRNA gene and the Internal Transcribed Spacers (ITS1 and ITS2). Arias et al. (2002) compared different methodologies for the identification of yeast species and concluded that 5.8S rDNA-ITS-RFLP as the best method for rapid and accurate identification of yeasts species.

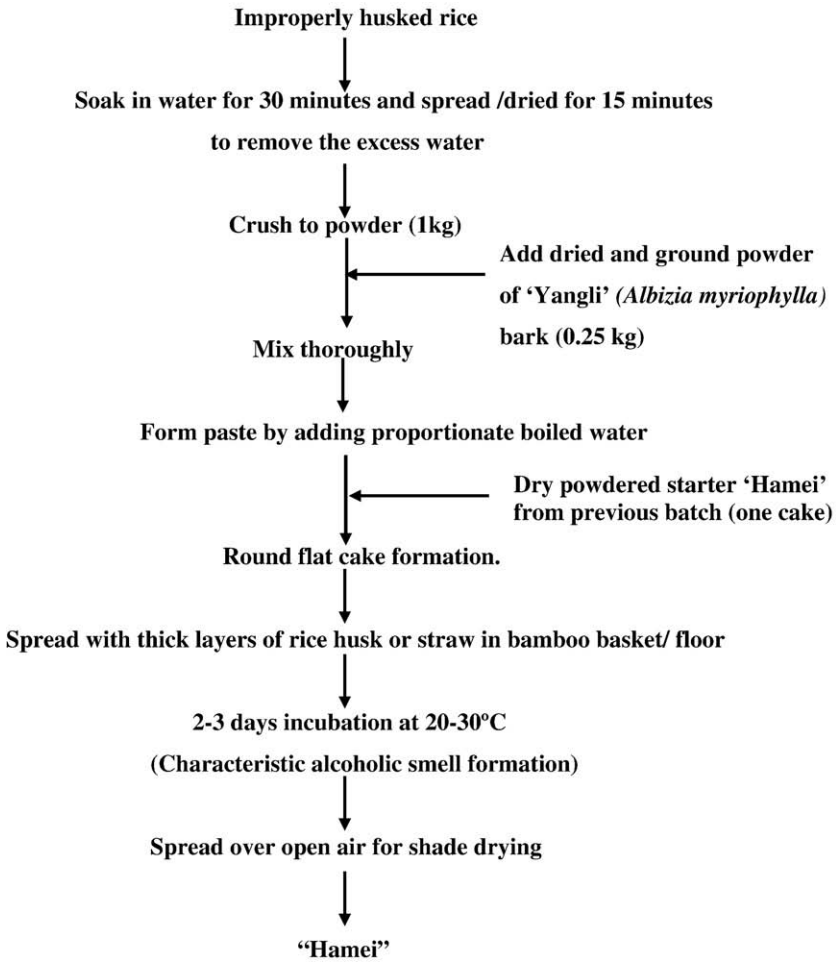

Fig. 2. Flow sheet for traditional 'Hamei' Preparation.