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Research Article

# Molecular Characterization of Yeasts and Bacteria Isolated From Handia, an Indian Traditional Rice Fermented Alcoholic Beverage

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**Abstract:** *Handia* is an Indian traditional fermented alcoholic beverage prepared from rice (*Oryza sativa* L.) To date, no information is available about the microorganisms associated with *Handia* fermentation. In this study a total of twelve yeast strains isolated from *Handia* were identified by partial sequencing of the D1/D2 variable domain of 26S of rRNA gene. The isolates were identified and designated as *Saccharomyces cerevisiae* KpY, *S. cerevisiae* 18VSL, *S. cerevisiae* H15, *S. cerevisiae* H17, *Hanseniaspora guilliermondii* G1, *H. guilliermondii* G4, *Pichia kudriavzevii* H21L, *Candida glabrata* H3, C. *glabrata* H8, *C. glabrata* H11, *C. glabrata* H12 and *C. tropicalis* 18VLL. Sequence analysis of 26S rRNA genes revealed 100% identity with the corresponding sequences in the GenBank with the exception of *P. kudriavzevii* H21L which showed 99% identity. The bacterial strains BA, B16 and B4 were identified as *Brevibacillus agri*, *Leuconostoc mesenteroides* and *Kocuria* sp., respectively by 16S rRNA gene sequence analysis. The identification of yeast strains (KpY, 18VSL, H15, H17, G4 and H21L) was further confirmed by 5.8S-ITS-RFLP analysis. M13 PCR-fingerprinting indicated the presence of four different strains of *S. cerevisiae* in *Handia*. The result suggests that *Handia* constitutes a complex microbial consortium comprising a wide diversity of bacterial and yeast strains.

**Keywords**: Handia; PCR-RFLP; ITS; fermentation; M13; diversity

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# Introduction

*Handia* is a traditional spontaneously fermented alcoholic beverage prepared by the tribal people of different states (West Bengal, Orissa, Chhattisgarh and Bihar) of India. From cultural, social and spiritual point of view this beverage plays a vital role in their livelihoods [1]. It is served during social gatherings for happiness, refreshment and cheering. In tribal custom *Handia* is a secret drink offered to God during spiritual festivals. The production of *Handia* from rice using traditional practices involves several steps. The first step in *Handia* making is to cook rice and then spread on a mat over open air for cooling. A powdery mixture of plant ingredients (sun dried rice, roots of *Cissampelos pareira*, *Diospyros melanoxylon*, *Lygodium flexuosum*, *Orthosiphon rubicundus*, *Ruellia tuberosa* and barks of *Terminalia alata* ) known as 'Bakhar' [2] is sprinkled over the cooked rice, mixed well and poured into a clean earthen pot with addition of a proportionate amount of water. The top of the pot is covered with an earthen lid. The mixture is allowed to ferment at room temperature for 4-7days depending on the seasonal temperature. After the fermentation is over an appropriate amount of water is added to it and thoroughly mixed with a stick and then filtered to obtain the final product in the form of liquid which is consumed as *Handia*. The role of the plant ingredients (Bakhar) in *Handia* fermentation is not known. A flow chart of *Handia* preparation is presented in Fig. 1.

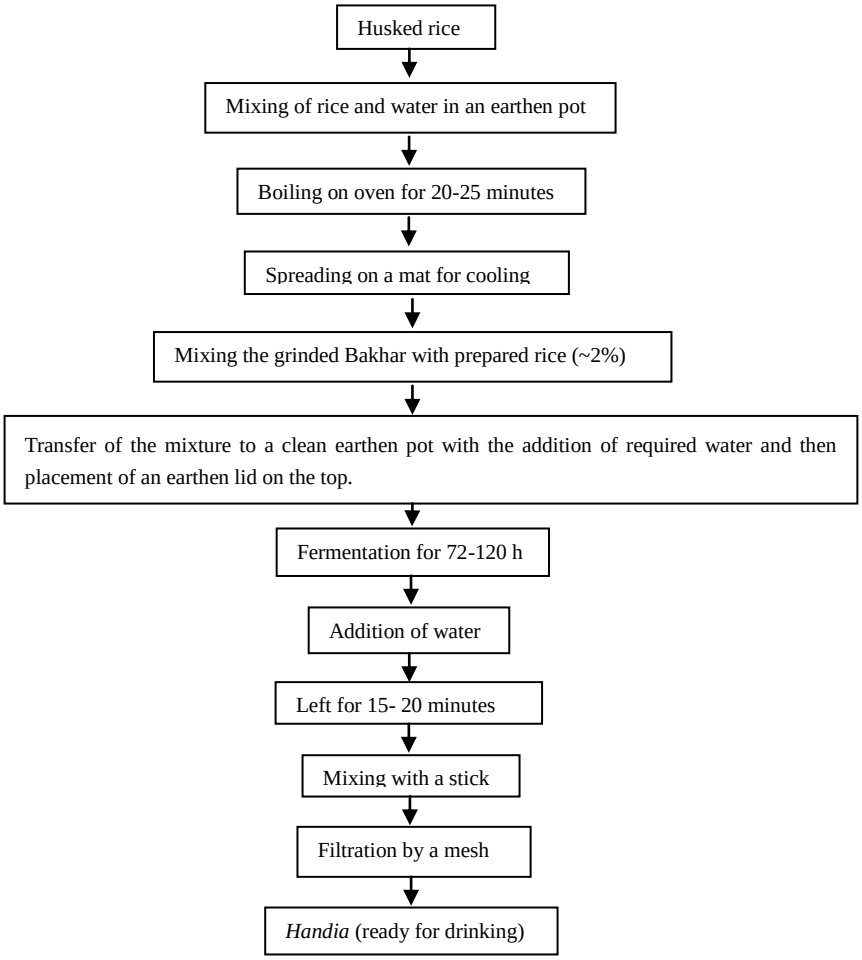


**Fig. 1** Flow chart of traditional *Handia* preparation.

Many studies have been done on the traditional fermented alcoholic beverages like Pito, Sekete and Burukutu in Nigeria [3], Marcha in Sikkim [4], Boza in Turkey, Tapuy and Ruou nep in Philippines [5], Sato and Krachae in Thailand [6] and Tapai and Tuak in East Malaysia [2]. However, no research has been done on microbiological analysis of *Handia*. It has been found that Handia contains antioxidant properties [7]. It is prepared under less hygienic condition which may result in microbial contamination and inconsistency in the quality of the final product. The development of scientific and affordable technology of *Handia* preparation will ensure food safety and quality. As a first step in achieving this goal, the present study attempted to isolate and identify the microorganism from Handia in order to explore the microbial community involved in *Handia* fermentation.

# Materials and Methods

### Microbial growth Kinetics during Handia production

The microbial succession in the fermentation process was determined by growth kinetics. For this purpose Handia was prepared for laboratory use by a local villager residing in Pearson Palli, a place near to this laboratory. Samples were collected on daily basis and immediately processed for microbiological analysis. 1 g of sample was extensively vortexed following addition of 9 ml of saline water and serially diluted upto $10^{-8}$. An aliquot of 100 µl from each dilution was spread in duplicates on TGE (1% Tryptone, 1% Glucose and 1% Yeast extract, w/v, pH 6.5) and YPD (0.5% Yeast extract, 1% Peptone and 2% Dextrose, w/v, pH 6.5) -agar plates. Following incubation for 48 h at 37$^{o}$C, the number of yeasts and bacterial colonies was enumerated as cfu (colony forming units).

### Isolation of microorganisms from Handia

Five samples of 1g spontaneously fermented *Handia* were collected from different tribal villages in West Bengal, India, brought to the laboratory under ice and immediately processed for microbiological analysis. Sampling of the Handia was done at the beginning (0 h fermentation), middle (72 h fermentation) and the end of the fermentation process. One mL of each sample of *Handia* was transferred to 10 mL of YPD or TGE medium. After incubation for 48 h at 25$^{o}$C and 37$^{o}$C, each sample was then serially diluted in saline water up to $10^{-8}$. A 50 µl from appropriate dilutions was spread on YPD and TGE agar plates in duplicates. The plates were incubated for 48 h at 25 $^{o}$C and 37$^{o}$C for the appearance of colonies. A few morphologically distinct colonies were picked up from each plate and repeatedly streaked on the same agar medium to obtain pure culture. Each pure culture was maintained on YPD and TGE agar at 4$^{o}$C.

### Standard yeast cultures

*Saccharomyces cerevisiae* MTCC 178, *S. cerevisiae* MTCC 180, *S. cerevisiae* MTCC 211 and *Issatchenkia orientalis* MTCC 642 (*Pichia kudriavzevii*) were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The strains were maintained on YPD agar at 4$^{o}$C. All strains were grown on YPD medium.

### Isolation of Genomic DNA

Genomic DNA was isolated from pure cultures of yeast isolates. Each pure culture in 5 mL of YPD medium was grown at 37$^{o}$C for 6 h and the cells were harvested by centrifugation at 5000 × g for 7 minutes. The cell pellet was resuspended in 400 µl of lysis buffer (SDS 1 % and sodium acetate 88 mM) mixture followed by the addition of 400 µl of TE-saturated phenol (pH 8). Samples were

incubated for 10 minutes at 65°C and then centrifuged at 5000 × g for 7 minutes at 4°C. The aqueous supernatant was treated with 20 µl RNase (10 mg/mL) (Genei, India) for 30 minutes at 37°C. After incubation with 10 µl Proteinase K (Genei, India) at 50°C for 1 h an equal volume of phenol/chloroform (1:1) mixture (500 µl) was added to each sample. After centrifugation at 5000 × g for 7 minutes at 4°C, DNA was precipitated with sodium acetate (3M) and isopropanol and finally resuspended in TE or sterile double distilled water. The concentration of DNA was measured spectrophotometrically (UV/Vis spectrophotometer, Beckman Coulter, DU 730, California, USA). Genomic DNA of bacteria was isolated by lysozyme (Amresco)-proteinase K procedure [8].

**PCR amplification of 26S rDNA gene of yeast and 16S rDNA gene of bacteria**

The variable D1 and D2 regions of the 26S rRNA gene (at the 5′ end of the nuclear large subunit of the rRNA gene) were amplified by PCR with the conserved fungal primer pair NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) [10]. The polymerase chain reactions of 26S rDNA was performed in a reaction volume of 50 µl containing NL-1 and NL-4 primers at a concentration of 50 pmol each, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTPs), 1.25 U of *Pfu* DNA polymerase (Fermentas, Hanover, MD, USA), and approximately 50 ng of genomic DNA. The amplification was carried out in 36 cycles with a Thermal Cycler (Applied Biosystem 2720, Foster city, CA) under the following conditions: initial denaturation at 95°C for 5 minutes, followed by 36 cycles at 95°C for 1 minute, 52°C for 1 minute, 72°C for 2 minutes and a final extension at 72°C for 10 minutes. The primers were synthesized commercially from Genei, India.

Bacteria-specific universal primers were used for the amplification of 16S rDNA gene of all bacterial isolates. The forward primer was 27F (5′-AGAGTTTGATCATGGCTC-3′) and the reverse primer was 1327R (5′- CTAGCGATTCCGACTTCA-3′) **[**11**].** The PCR reaction was carried out in a total volume of 50 µl containing 50 ng of the genomic DNA, 1.25 U of *Pfu* DNA polymerase (Fermentas, Hanover, MD, USA), 20 pmol of each primer (forward, reverse), 200 µM each of dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris-HCl and 50 mM KCl. The 16S rDNA gene was amplified in 35 cycles with a Thermal Cycler; initial denaturing at 95°C for 5 minutes, followed by 35 cycles at 95°C for 1 minute, a primer annealing step at 60°C for 1 minute and an extension step at 72°C for 2 minutes and a final extension at 72°C for 5 minutes. The primers were synthesized commercially from Clonitec, Genuine chemical corp., India.

PCR products were checked by electrophoresis on 1% (w/v) agarose gel. A 100-bp DNA ladder was used as the molecular marker (New England BioLabs inc.). PCR products purified by QIAquick PCR purification kit (Qiagen, Hilden, Germany) was sequenced commercially from Chromous Biotech Pvt. Ltd, Bangalore, India using the NL1 and 27f primers for yeast and bacteria, respectively. Sequence comparisons were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) [12].

**PCR-RFLP analysis of 5.8S-ITS of yeast strains**

PCR-RFLP analysis of the ITS1-5.8S rRNA gene-ITS2 region of rDNA gene was performed as described by Esteve-Zarzoso *et al*.[13]. ITS1-5.8S rRNA gene was amplified by PCR with the conserved fungal primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [14]. The primers were synthesized commercially from Clonitec, India. PCR amplification of 5.8S-ITS region was carried out in a total volume of 50 µl containing 50 ng template DNA, 1.5 U of *Pfu* DNA polymerase (Fermentas, Hanover, MD, USA), 50 pmol of each primer, 200 µM of each dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris-HCl and 50 mM KCl. The

PCR amplification was performed with a total of 35 cycles in a Thermal Cycler (Applied Biosystem 2720, Foster city, CA). The cycling program consisted of an initial denaturing at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1 minute. The PCR was ended with a final extension at 72°C for 10 minutes. The amplified DNA was electrophoresed on 1% agarose gel. Gel was stained with ethidium bromide, visualized and photographed. A 100 bp DNA was used as molecular marker. 20 µl of the PCR products (approximately 0.5 µg) were digested without further purification in 25 µl reaction volumes according to manufacturer's instructions with *HaeIII*, *HinfI* and *PstI*. The digestion products were analyzed by electrophoresis on 3% (w/v) agarose gel.

5.8S-ITS PCR product of *P. kudriavzevii* was sequenced commercially from Chromous Biotech Pvt. Ltd., India using ITS1 and ITS4 primers. Homology search was done by BLAST analysis. Multiple sequence alignment of DNA sequences retrieved from GenBank was performed using the online software package (http://www.ebi.ac.uk/Tools/clustalw2/index.html) [15]. Restriction sites within DNA sequence was analyzed with the NEB cutter program (http://tools.neb.com/NEBcutter/index.php3 ).

**RAPD-PCR fingerprinting by M13 primer**

The PCR amplification was carried out in 50 µl of reaction mixture containing approximately 50 ng of genomic DNA, 5 µl of PCR buffer (10X), 5 µl of dNTPs (2.5 mM each), using 4 µl M13 primer (5′-GAGGGTGGCGGTTCT-3′) [16]Sigma- Aldrich, India), 1µl (2.5 U/µl) of *Pfu* DNA polymerase (Fermentas, USA), and 18.5 µl of deionized water. The amplification was performed with a total of 35 Cycles in a Thermal cycler. The cycling program comprised an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation (94°C for 1 minute), annealing (20 s at 40°C), extension (1 minute at 72°C) and a final extension of 72°C for 5 minutes. The amplified DNA product was separated on 1% agarose (Genei, India) gel. A 1 Kb DNA ladder (Genei, India) was used as standard.

# Results

**Growth kinetics of microorganism in Handia**

The growth kinetics of microorganisms associated with *Handia* fermentation was evaluated at daily intervals from 0 to 5 days (Fig. 2).

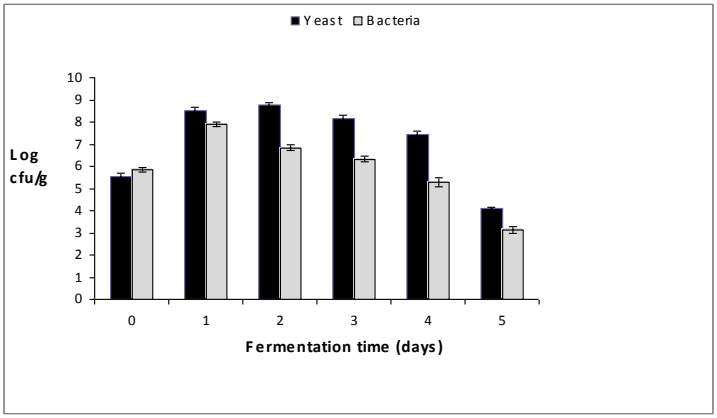


**Fig. 2** Growth kinetics of yeast and bacterial populations during *Handia* fermentation.

The result revealed the presence of both bacterial (5.82 log cfu/g) and yeast species (5.53 log cfu/g) at the beginning (0 h) of fermentation; in the course of fermentation process, population of bacterial species increased to 7.9 log cfu/g at 24 h of fermentation. The yeast population increased during the fermentation process and predominated (8.78 log cfu/g) at 48 h of fermentation, following which both yeast and bacterial population declined.

**Identification of isolates from Handia**

***16S rRNA gene sequence analysis of bacteria isolates***

Three strains of bacteria were isolated from *Handia*, which were identified and designated as *Brevibacillus agri* BA, *Leuconostoc mesenteroides* B16 and *Kocuria* sp. B4. The size of the 16S rRNA from all the bacterial strains was 1300 bp. The partial nucleotide sequence of 16S rRNA gene from the bacterial isolates showed 100% identity with the sequences in GenBank (Table 1).

**Table 1 Comparison of 26S and 16S rRNA-gene sequence of yeast and bacterial isolates to the closely related species.**

| Isolates | Identity (%) | Number of bases of 26S rRNA gene sequenced | Number of bases of 16S rRNA gene sequenced | Closest relative | Accession number |
|---|---|---|---|---|---|
| KpY | 100 | 265 | | *Saccharomyces cerevisiae* | HQ641267 |
| 18VSL | 100 | 366 | | *S. cerevisiae* | JF757233 |
| H15 | 100 | 573 | | *S. cerevisiae* | HQ711330 |
| H17 | 100 | 244 | | *S. cerevisiae* | EU386759 |
| G1 | 100 | 539 | | *Hanseniaspora guilliermondii* | AB618029 |
| G4 | 100 | 556 | | *H. guilliermondii* | AB618029 |
| H21L | 99 | 565 | | *Pichia kudriavzevii* | FJ919397 |
| H3 | 100 | 446 | | *Candida glabrata* | HQ641276 |
| H8 | 100 | 561 | | *C. glabrata* | HM591730 |
| H11 | 100 | 357 | | *C. glabrata* | HM591715 |
| H12 | 100 | 336 | | *C. glabrata* | HQ641276 |
| 18 VLL | 100 | 297 | | *Candida tropicalis* | EF644470 |
| BA | 100 | | 601 | *Brevibacillus agri* | HM629394 |
| B4 | 100 | | 527 | *Kocuria* sp. | JN390957 |
| B16 | 100 | | 461 | *Leuconostoc mesenteroides* | JN990379 |

### *26S rRNA gene sequence analysis of yeast isolates*

A total of twelve yeast strains representing four genera were isolated from *Handia* samples and identified by molecular techniques based on sequence analysis of 26S rRNA gene. The PCR amplification of 26S rRNA in all yeast strains yielded amplicon of the expected 600 bp. These strains were identified and designated as *Saccharomyces cerevisiae* KpY, *S. cerevisiae* 18VSL, *S. cerevisiae* H15, *S. cerevisiae* H17, *Hanseniaspora guilliermondii* G1, *H. guilliermondii* G4, *Pichia kudriavzevii* H21L, *Candida glabrata* H3, *C. glabrata* H8, *C. glabrata* H11, *C. glabrata* H12 and *C. tropicalis* 18VLL. With the exception of *P. kudriavzevii* H21L, all the yeast strains, *S. cerevisiae* KpY, *S. cerevisiae* 18VSL, *S. cerevisiae* H15, *S. cerevisiae* H17, *H. guilliermondii* G1, *H. guilliermondii* G4, *C. glabrata* H3, *C. glabrata* H8, *C. glabrata* H11, *C. glabrata* H12 and *C. tropicalis* 18VLL had sequence identity of 100% with their corresponding species in the GenBank. *P. kudriavzevii* H21L (accession no. JN108878) showed 99% identity with the corresponding species in the database (accession no. FJ919397).

**Table 2** Molecular typing of strains isolated from *Handia*.

| Strains | PCR of ITS (bp) | PCR/RFLP of ITS with *Hae III* (bp) | PCR/RFLP of ITS with *Hinf I* (bp) | PCR/RFLP of ITS with *pst I* (bp) |
|---|---|---|---|---|
| *S. cerevisiae* KpY | 880 | 320 – 240 – 180 – 140 | 370 – 370- 130 | *880* |
| *S. cerevisiae* 18VSL | 880 | 320 – 240 – 180 – 140 | 370 – 370- 130 | 880 |
| *S. cerevisiae* H15 | 880 | 320 – 240 – 180 – 140 | 370 – 370- 130 | 880 |
| *S. cerevisiae* H17 | 880 | 320 – 240 – 180 – 140 | 370 – 370- 130 | 880 |
| *S. cerevisiae* MTCC 178 | 880 | 320 – 240 – 180 – 140 | 370 – 370- 130 | 880 |
| *S. cerevisiae* MTCC 180 | 880 | 320 – 240 – 180 – 140 | 370 – 370- 130 | 880 |
| *S. cerevisiae* MTCC 211 | 880 | 320 – 240 – 180 – 140 | 370 – 370- 130 | 880 |
| *P. kudriavzevii* H21L | 500 | 380 – 100 | 250 – 150 | 500 |
| *P. kudriavzevii* MTCC 642 | 500 | 320 – 100 | 280 – 130 | 500 |
| *H. guilliermondii* G4 | 775 | 775 | 385 – 200 – 160 - 100 | 775 |

### *RFLP analysis of the 5.8S- ITS rDNA region of yeast strains*

In order to further confirm species identification intraspecific variation of yeasts isolated from *Handia* was studied by restriction digestion of their 5.8S ITS region between 18S rRNA and 26S rRNA genes amplified by PCR with ITS 1 and ITS 4 primers. The PCR amplified product for all four strains of *S. cerevisiae* was 880 bp which is in agreement with those previously reported for yeast strains [17, 18]. Restriction analysis of the PCR amplified product of the 5.8S-ITS region of all four *S. cerevisiae* with *HaeIII*, *HinfI* and *Pst*I generated a restriction fragment pattern consisting of four fragments with *HaeIII* (320, 240, 180 and 140 bp) and three fragments with *HinfI* (370, 370 and 130 bp) (Table 2). The PCR product 5.8S-ITS region of *S. cerevisiae* remained uncut after *PstI* digestion (Table 2).

Similar banding patterns with *HaeIII* and *HinfI* were also observed for the reference strains of *S. cerevisiae* MTCC 180, *S. cerevisiae* MTCC 178 and *S. cerevisiae* MTCC 211 and the reported strains [13,19]. Although this technique could not differentiate the four isolates of *S. cerevisiae* at strain level, their identity to species level was confirmed.

The PCR amplification of 5.8S ITS region of the genomic DNA of *H. guilliermondii* G4 yielded 775 bp fragment (Table 2), similar to the results reported previously [20]. The restriction digestion of the 5.8S-ITS region of the PCR amplified product of *H. guilliermondii* G4 with the PCR amplification of 5.8S ITS region of the genomic DNA of both *P. kudriavzevii* H21L and *P. kudriavzevii* MTCC642 yielded 500 bp fragment (Table 2), similar to the results reported previously [21]. Digestion of this product from *P. kudriavzevii* H21L with *HaeIII* yielded two fragments of approximately 380 and 100 bp whereas *HinfI* produced fragments of 250 and 150 bp (Table 2). The strain *P. kudriavzevii* MTCC 642 displayed a restriction pattern consisting of approximately 320 and 100 bp with *HaeIII* and 280 and 130 bp fragments with *HinfI* (Table 2). The PCR products of both the *P. kudriavzevii* remained uncut after digestion with *PstI* (Table 2). Although the 5.8S-ITS pattern of *P. kudriavzevii* H21L generated with *HaeIII* was found to be similar to published strains [22] it differed from that of *P. kudriavzevii* MTCC 642.

***RAPD-PCR analysis of yeast strains***

The four isolates of *S. cerevisiae* were further characterized in order to determine their geneological relationship at strain level by RAPD-PCR using M13 primer [19]. In RAPD-PCR analysis the banding pattern was found to be unique for each isolate of *S. cerevisiae* (Fig. 3, Lane 7-10). The result suggests that all four isolates of *S. cerevisiae* are different at strain level. RAPD-PCR of each strain of *S. cerevisiae* also revealed different genotypic patterns from the standard strains, *S. cerevisiae* MTCC 211, *S. cerevisiae* MTCC 180 and *S. cerevisiae* MTCC 178 (Fig. 3, Lane 4- 6). *HinfI* produced approximately 385, 200, 160 and 100 bp (Table 2) fragments similar to results reported previously **[**20**]** The PCR product remained uncut after digestion with *HaeIII* and *PstI* (Table 2). The previous work also demonstrated that the PCR product of ITS-5.8S rDNA of reported strain of *H. guilliermondii* remained uncut with *HaeIII* [20]. The results confirm the identification of G4 as *H. guilliermondii.* In previous studies, the organism has been reported for 2-phenylethyl acetate production that contributes fruity and flowery flavors in wine [23, *24*, 25]. Hence, *H. guilliermondii* creates further interest to evaluate its sensorial properties in Handia production.

RAPD-PCR differentiated the strain *P. kudriavzevii* H21L from *Handia* from the standard strain *P. kudriavzevii* MTCC 642. *P. kudriavzevii* H21L displayed nine distinct bands (Fig. 3, Lane 3) whereas it was eight for *P. kudriavzevii* MTCC 642. (Fig. 3, Lane 2). Both the strains had only one band in common. The result suggests that these two strains of *P. kudriavzevii* were different at strain level. Since the two strains of *P. kudriavzevii* (*P. kudriavzevii* H21L and *P. kudriavzevii* MTCC 642) showed different banding patterns with identical restriction enzymes, the strain *P. kudriavzevii* H21L was further characterized genetically by sequencing of its 5.8S-ITS region. In BLAST analysis, partial nucleotide sequence (456 bp) of *P. kudriavzevii* H21L (accession no. JN164664) showed 98% identity with the sequence of *P. kudriavzevii* in GenBank (accession no. FJ697171). The result suggests that strain H21L is closely related to *P. kudriavzevii.* The nucleotide change at position 111 (T in place of C) created two new restriction sites *ApoI* (AAATTT) and *MluCI* (AATT) in ITS1 region of *P. kudriavzevii* H21L (JN164664) whereas three restriction sites (*SalI, TaqI* and *AccI*) were lost due to the nucleotide change (T in place of C ) at position 98. Although *Issatchenkia* sp. YF04A (DQ667976) possessed the *ApoI* and *MluCI* sites at 111 positions, it differed from H21L in having the *SalI*, *TaqI* and *AccI* sites at position 98 (Fig. 4).

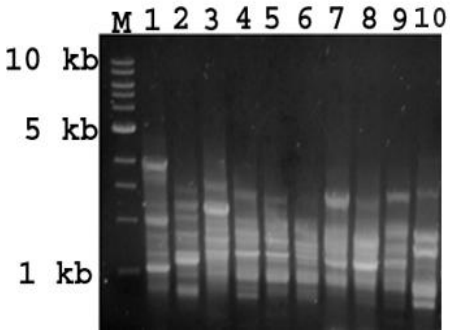


**Fig. 3** RAPD-PCR patterns generated by M13 primer. Lane M, 1 Kb DNA ladder; Lane 1, *H. guilliermondii* G4; Lane 2, *P. kudriavzevii* MTCC 642; Lane 3, *P. kudriavzevii* H21L; Lane 4, *S. cerevisiae* MTCC 211; Lane 5, *S. cerevisiae* MTCC 180; Lane 6, *S. cerevisiae* MTCC 178; Lane 7, *S. cerevisiae* H17; Lane 8, *S. cerevisiae* H15; Lane 9, *S. cerevisiae* 18 VSL and Lane 10, *S. cerevisiae* KpY.

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FJ515204    GGTGAACCTGCGGAAGGATCATTACTGTGATTTAGTACTACACTGCGTGAGCGGAACGAA 60
DQ667972    GGTGAACCTGCGGAAGGATCATTACTGTGATTTAGTACTACACTGCGTGAGCGGAACGAA 60
FM178339    GGTGAACCTGCGGAAGGATCATTACTGTGATTTAGTACTACACTGCGTGAGCGGAACGAA 60
HM053475    GGTGAACCTGCGGAAGGATCATTACTGTGATTTAGTACTACACTGCGTGAGCGGAACGAA 60
FJ697171    GGTGA-CCTGCGGAAGGATCATTACTGTGATTTAGTACTACACTGCGTGAGCGGAACGAA 59
DQ667976    GGTGAACCTGCGGAAGGATCATTACTGTGATTTAGTACTACACTGCGTGAGCGGAACGAA 60
JN164664    GGGGGACCTGCGGAAGGATCATTACTGTGATTTAGTACTACCCTGCGTGAGCGGAACGAA 60
            ** *  ********************************** ******************

FJ515204    AACAAAAACACCTAAAATGTGGAATATAGCATATAGTCGACAAGAGAAATCTACGAAAAA 120
DQ667972    AACAAAAACACCTAAAATGTGGAATATAGCATATAGTCGACAAGAGAAATCTACGAAAAA 120
FM178339    AACAAAAACACCTAAAATGTGGAATATAGCATATAGTCGACAAGAGAAATCTACGAAAAA 120
HM053475    AACAAAAACACCTAAAATGTGGAATATAGCATATAGTCGACAAGAGAAATCTACGAAAAA 120
FJ697171    AACAAAAACACCTAAAATGTGGAATATAGCATATAGTCGACAAGAGAAATCTACGAAAAA 119
DQ667976    AACAACAACACCTAAAATGTGGAATATAGCATATAGTCGACAAGAGAAATTTACGAAAAA 120
JN164664    AACAAAAACACCTAAAATGTGGAATATAGCATATAGTTGACAAGAGAAATTTACGAAAAA 120
            ***** ****************************** ************ *********

FJ515204    CAAACAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAA 180
DQ667972    CAAACAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAA 180
FM178339    CAAACAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAA 180
HM053475    CAAACAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAA 180
FJ697171    CAAACAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAA 179
DQ667976    CAAACAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAA 180
JN164664    CAAACAAAACTTTCAACAACGGATTTTTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAA 180
            ********************** * ********************************

FJ515204    TGCGATACCTAGTGTGAATTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCACATT 238
DQ667972    TGCGATACCTAGTGTGAATTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCACATT 238
FM178339    TGCGATACCTAGTGTGAATTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCACATT 238
HM053475    TGCGATACCTAGTGTGAATTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCACATT 238
FJ697171    TGCGATACCTAGTGTGAATTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCACATT 237
DQ667976    TGCGATACCTAGTGTGAATTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCACATT 238
JN164664    TGCGATACCTAGTGTGAATTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCCCCTT 238
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**Fig. 4** Multiple sequence alignment of 5.8S ITS region of *Pichia kudriavzevii* H21L with the homologous sequences in the GenBank. The shaded nucleotides represent changes in restriction sites. The numbering is according to the 5′ end of the submitted sequence (JN164664) of 5.8S- ITS of *P. kudriavzevii* H21L.

# Discussion

In microbiological analysis it was found that *Handia* fermentation is a result of a microbial consortium composed of a variety of yeast strains (*Saccharomyces* and non-*Saccharomyces*) and bacteria. *S. cerevisiae* is considered to be generally regarded as safe (GRAS) organism because of a long history of safe use in food industry. Besides *S. cerevisiae*, *H. guilliermondii* and *P. kudriavzevii* have been previously reported as starter cultures for wine production [26, 27]. The isolated *Saccharomyces*, non-*Saccharomyces* strains thus may be regarded as having a positive influence in *Handia* fermentation. It has been previously demonstrated that the specific characteristics and quality of foods and beverages is strain-specific [28]. Each strain of *S. cerevisiae* isolated from *Handia* is not only different among themselves but also show differences from the standard strains. The identification of four different strains of *S. cerevisiae* from *Handia* is thus important in this regard. It is interesting to note that *Handia* contained a new strain, *P. kudriavzevii* H21L as determined by the sequence analysis of 5.8S ITS region. A single nucleotide change in this region at 111 created two restriction sites, which will serve as genetic marker to distinguish this organism from other strains of *P. kudriavzevii* in the database.

Although *Handia* is a popular drink, the presence of *C. glabrata* and *C. tropicalis* in *Handia* raises a concern to the safety of this drink because of their implications in human health and disease [29]. Microbial composition of *Handia* as explored in the study is first of its kind and necessitates further microbiological investigation in the context of tracking the sources of the contaminants, the improvement of quality, acceptability and safety of the product. Such a drink of improved quality is expected to ensure global commercial success. The study further offers opportunity to exploit the biotechnological potential of these microorganisms in the production of beer, wine and other alcoholic beverages with unique sensory properties.

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