Identification and Characterization of Yeast Strains Associated With the Fermented Rice Beverages of Garo Hills, Meghalaya, India

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A B S T R A C T

Fermented Rice Beverage is one of the most popular and ethnic fermented alcoholic beverages of Meghalaya Region located in North-eastern part of India. Locally, rice beverage is known as Chubitchi by Garo, kyiad by Khasi and Sadhiar by Jaintias tribes of Meghalaya, it is prepared by using rice and dried starter culture. Dried starter culture or Wanti is used for traditional rice beverage processing by Garo tribes. The nature of microbes was assessed and their source during fermentation was studied generally to explore the microbial diversity associated with the rice beverage samples. Yeast cultures have health attributes and applied in fermented rice beverages since age old. In this study, six yeast cultures were isolated from twenty indigenous fermented food samples collected from various regions of Meghalaya. Isolation was conducted on specific media: MA, SCA, RBA and YPDA for yeast isolates. Based on the phenotypic characteristics obtained from Gram’s staining, biochemical characterization of selected isolates was accomplished by API 20 C AUX V5.0 kit for yeast followed by 5.8s rRNA amplification for its genotypic identification and the sequences was deposited at Genebank and NCBI bearing their specific accession numbers were obtained. Further, studies on further techno-functional properties of yeast isolates could be analysed.

Keywords: Fermented rice beverage, Yeast, API, Genebank, NCBI, Meghalaya

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Introduction

Meghalaya is one of the seven states of North-east India which is bounded to the south by Bangladesh and to the north and east by India’s State of Assam. In Meghalaya three major tribes prepare and consume indigenous fermented rice beverage which are known as Chubitchi by Garos, kyiad by Khasi and Sadhiar by Jaintias. Fermented rice beverage is a traditional alcoholic beverage which was formerly exclusive to East Asian and Southeast Asian countries and now is popular in countries like China, Korea, Japan, Philippines, Vietnam and some parts of India. About 220 diversified ethnic tribes of North East India consume rice beer on a regular basis in different forms prepared by their own traditional method by using different starter cultures made of locally available rice and
medicinal plants (Mishra et al., 2016). It is seen to have important roles in the socio-cultural life of the tribal people of North-east India as it is found to be associated with many occasions like ritual ceremonies, marry making, festivals, marriages and even death ceremonies (Saikia et al., 2007). It is now a part of Asian cuisine and is believed to possess many therapeutic and medicinal properties (Teron et al., 2006). They are called as apong in Adi, Laopani in Aka, ijasuijiang in Naga, jumai by Bodos, jou by Meches and dimasas, bankchung in Mongpa, Chi in lepcha, morpo by mikris, zu by Tiwas, apong by Mishings, suze by Deoris, laopani and mod by some other tribal communities of North-east India (Deka and Sarma, 2010). These products are similar to shaosingji and laochao of China, sake of Japan, brem bali, tape-ketan and tapuy of Indonesia, khaomak of Thailand, chongju and takju of Korea and tapai pulal of Malaysia (Lee CH, 2009), ruou de or ruou nepin Vietnam, Makgeolli in Korea (Kim, et al., 2013, Dung, 2004) etc. In India, an alcoholic beverage called sura, distilled from rice, was in use between 3000 and 2000 B.C. (Eraly, 2002).

Fermented rice beverage is prepared by brewing sticky rice known as Minal by Garo tribe and Ja-Shulia by Khasi tribe by addition of starter culture known as Wanti by Garo tribe and Thiat (natural yeast) by Jaintia Tribe. Starter cultures cakes are generally made from the ground rice powder mixed with medicinal herbs powders which are made into sticky paste and small round cakes are prepared with standard size of 4.5 cm in diameter and 0.8-1 cm in thickness. These cakes are exposed to sunlight or tied about 1.20-1.50 m above the fireplace/hearth for drying until the cakes get harden and then are used for rice brewing as natural yeast (Samati et al., 2007). These traditional rice beverages have different compositions according to the formulation and processes used, but the principle of their manufacture can be characterized as a biochemical modification that is saccharification of cereal starches brought about by microorganisms in which fungi (yeasts and moulds) play essential roles. Moulds produce the amylases that degrade the starch into dextrins and sugars, and yeasts convert these sugars to alcohol. The preparation and the use of starter cake as a source of inoculums are important in the manufacture of rice alcoholic beverage. Dried starter cakes normally include yeasts, moulds and bacteria and convert starchy materials to fermentable sugars and subsequently to alcohol and organic acids. The use of different starter cultures with varying microbial content, rice variety and medicinal herbs has been associated with the production of wine with different tastes and flavours, the quantity and quality of wine. Glutinous or sticky rice for instance is a rich source of starch, protein and various microelements that are used by microbes during the fermentation process to produce more wine (Palaniveloo et al., 2013). Among yeasts, Saccharomyces cerevisiae is of industrial importance due to its ability to convert sugars (i.e., glucose, maltose) into ethanol and carbon dioxide (baking, brewing, distillery, and liquid fuel industries). S. cerevisiae breaks down glucose through aerobic respiration in presence of oxygen. If oxygen is absent, the yeast will then go through anaerobic fermentation. The net result of this process is two adenosine triphosphate molecules, in addition to two by products; carbon dioxide and ethanol (Mugula et al., 2003).

Different studies on traditionally prepared and isolation of potential yeast from fermented rice beverages have been carried out but molecular level study of isolates from fermented rice beverages from Meghalaya has not yet been reported. Keeping in view the potential health benefits and its nutraceutical properties of probiotics, this study is designed
to explore the novel yeast, particularly, *Saccharomyces* spp. from ethnic fermented rice beverages up till molecular level characterization along with phylogenetic studies. In Meghalaya, this study will help to provide valuable functional food with particular health benefits. The aim of this paper was to identify and characterize the predominant species of *Saccharomyces* spp. in naturally fermented rice beverages of Meghalaya, India. These species were characterized using phenotypic and molecular techniques for confirmation of genus and species level of *Saccharomyces* spp. strains, along with DNA sequencing and analysis of phylogenetic studies by utilizing 5.8S rRNA gene.

Materials and Methods

Sample collection and growth enrichment

Indigenous homemade fermented rice beverages and dried starter rice cakes were collected from different parts of Meghalaya for its analysis in the laboratory (Table 1). Rice beverages were collected in sterile sample container and preserved at 4°C for further analysis. Starter rice cakes were grounded to fine powder and kept at 4°C for further analysis. The enrichment process was carried out by inoculating approximately 1 ml/1gm of sample into 50 ml of Yeast peptone dextrose (YPD) broth and incubated at 32°C for (2-5) days. All samples were kept in sterile glass bottles at refrigeration temperature (4-6°C) for further analysis.

Identification of yeast strains

All isolates were serially diluted by adding 1ml of sample into 9 ml of peptone water up to 10^-6 and streaked on Rose Bengal Agar (RBA), Sabouroud Dextrose Agar (SDA) and Malt’s Agar (MA) (Himedia, India). It was incubated at 32°C for 2-5 days. All isolates were tested for catalase activity, Gram’s reaction and cell morphology. The identification of strains was performed according to their morphological, cultural and biochemical properties based on their specific characterization (Salazar et. al., 2016). The strains were analysed for the biochemical sugar fermentation using API 20 AUX 5.0 CH kit (HiMedia, India) according to the manufacturer’s instructions. Results were scored after incubation at 32°C for 24-48-72 hours. These results were put on the apiweb™ identification software with database (V5.1) which uses the phenotypic data to predict a species identity. Interpretations of the fermentations profiles were facilitated by comparing all results obtained for the tested isolates with information from the computer aided database, apiweb™ (https://apiweb.biomerieux.com).

Confirmation of yeast isolates by colony PCR

DNA was isolated using DNA Kit (Himedia, India). The 5.8S-ITS region was amplified by Polymerase Chain Reaction (PCR) using primers (ITS1 and ITS4) (Table 2). Template was prepared by picking freshly grown colony and transferred to Phosphate buffer and was incubated at 32°C for 24 hours. PCR was performed of a reaction mixture containing 50 μl of 1.5 mM MgCl2, 0.2 mM dNTPs, 1 μM of each primer, 0.025 U of *Taq* polymerase and 50 ng of yeast DNA. The PCR mixture was initially heated at 94°C for 5 minutes followed by cycles of denaturation at 94°C for 1 min, annealing 57°C for 1 min and extension was performed at 72°C for 4 minutes (Harju. et. al., 2004). The PCR products and their restriction fragments were subjected to electrophoresis for 1 h at 110 V in 1 % agarose gels, respectively, which were then stained with ethidium bromide (14 mg/ml) for visualization of the DNA bands under UV light. Fragment sizes were estimated by
comparison with DNA size markers (Thermo Fisher Scientific).

**Phylogenetic analysis**

In order to determine the closest known relatives of the partial 16S rDNA sequences obtained, in NCBI GenBank, nucleotide database searches were performed and later those particular sequences were processed by multiple sequence alignment tools using the DNA alignment program MAFFT v6.864 for signifying the evolutionary relatedness (Fig. 3) between the yeast strains by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) (Mishra et al., 2017).

**Results and Discussion**

The study was conducted to isolate yeasts from ethnic fermented rice beverages from various places of Meghalaya (North eastern region of India) and explore their phenotypic and genotypic characteristics for further, development of value added products by identifying productive microbial strains.

**Phenotypic characterization of the isolates**

A total of six yeast strains were isolated from the twenty fermented samples of rice beverages and *Wanti* obtained from Meghalaya, India (Table 1). The Gram’s positive (Fig. 1) and catalase negative isolates from rice beverages and *Wanti* samples were considered as presumptive yeast cultures (Table 1).

Further, biochemical tests (Fig. 2) of all the isolates were carried out by API 20 AUX 5.0 CH kit *(bioMerieux, India)* through sugar fermentation pattern. The result of API test (Table 3 and 4) showed five isolates (NGL3A, NGL4A, NGL1B, RNS4C and RNL1A) as *Saccharomyces cerevisiae* and RNS1C was identified as *Rhodotorula mucilaginosa*. Out of six isolates, NGL3A showed sugar fermentation of D-Glucose, D-Galactose, D-Maltose, D-Saccharose and D-Raffinose. NGL4A and NGL1B also showed positive for Glycerol fermentation along with D-Glucose, D-Galactose, D-Maltose, D-Saccharose and D-Raffinose respectively. RNS4C showed positive for D-Glucose, D-Galactose, Methyl-α D-Glucopyranoside, D-Maltose, D-Saccharose and D-Raffinose. RNS1C showed sugar fermentation in D-Glucose, D-Xylose, D-Galactose, D-Maltose, D-Saccharose and D-Raffinose.

Hence, from the above stated biochemical results, it was assumed that all the six isolates may be of yeast. Earlier, Sefa-Dedeh et al., (2003) and Chiang et al., (2006) also isolated *Saccharomyces cerevisiae*, *Candida krusei*, *C. pelliculosa*, *C. glabrata*, *C. utilis*, *C. sphaerica*, *C. magnoliae*, *Rhodotorula mucilaginosa*, *R. glutinis* and *Cryptococcus laurentii* yeasts from *Tapai*, a fermented rice beverage in Malaysia. Tamang and Sarkar (1996) reported that *Saccharomyces cerevisiae* and *Lactobacillus spp.*, was associated with fermentation of *Kvass*, a rye/wheat-based sour-alcoholic beverage of the Ukraine. Another Indian starter culture used for preparation of for rice wine from Himalayan region (Sikkim and Nepal) called ‘Marcha’ identified yeast species as *Saccharomycopsis capsularis*, *S. bayanus*, *P. anomala*, *C. glabrata*, *Saccharomycopsis fibuligera*, and *Pichia burtonii* (Tsuyoshi et al., 2005). From this, it can be derived that the yeast associated with rice wine starter culture used in Meghalaya differs from the starter culture used in Sikkim and Nepal. In another study it was also found that non-*Saccharomyces* yeast like *P. anomala* is connected with frequent cause of spoilage in fermented food and alcoholic beverages (Caggia et al., 2001).
Table 1: List of selected isolates with their phenotypical characterization

| Sl. No. | Fermented Food Sample | Traditional name of collected food samples | Place of procuring fermented food samples | Isolate Code | Morphological characteristics | Gram’s Reaction | Catalase Reaction | Microscopic Examination |
|---------|-----------------------|--------------------------------------------|------------------------------------------|--------------|-----------------------------|----------------|-------------------|------------------------|
| 1       | Rice Beverage         | Chubitchi                                  | Chisin a. Kanang, north garo hills, resubelpara, Meghalaya | NGL3A        | Elevated, circular, entire and shiny | +ve            | -ve               | Round to irregular shaped in clusters |
| 2       | Rice Beverage         | Chubitchi                                  | Chisin a. Kanang, north garo hills, resubelpara, Meghalaya | NGL4A        | Elevated, circular, entire and shiny | +ve            | -ve               | Round cells in single, paired and some in clusters |
| 3       | Rice Beverage         | Chubitchi                                  | North garo hills, Meghalaya               | NGL1B        | Small pale pink circular shiny entire colony | +ve            | -ve               | Oval to circular cells in clusters |
| 4       | Starter Culture       | Wanti                                      | Nongkhrah, nongpoh, dist ri bhoi, Meghalaya | RNS4C        | Creamy, glistening, circular, convex, entire. | +ve            | -ve               | Round small to medium cells |
| 5       | Starter Culture       | Wanti                                      | Umtham, marngar, nongpoh, dist ri-bhoi, Meghalaya | RNS1C        | Large circular dull colony with rough edges | +ve            | -ve               | Oval to elongated cells with bud in single to clusters |
| 6       | Rice Beverage         | Chubitchi                                  | Umtham, marngar, Nongpoh, dist ri-bhoi, Meghalaya | RNL1A        | Small circular smooth edge, shiny and elevated colony | +ve            | -ve               | Round cells in singles and clusters |

Table 2: Oligonucleotide sequences of Primers

| Primers | Primer sequence |
|---------|-----------------|
| ITS1    | 5’ TCCGTAGGTGAACCTGCGG 3’ |
| ITS4    | 5’ TCCTCCGCTTATGATATGC 3’ |
Table 3: Biochemical characterization of selected isolates (on the basis of Morphological and Physiological characteristics) through API kit - API 20 C AUX V5.0

| API 20 C AUX | 1 | 2 | 3 | 4 | 5 | 6 |
|--------------|---|---|---|---|---|---|
| NGL3A | NGL4A | NGL1B | RNS4C | RNS1C | RNL1A |
| 1. Control | - | - | - | - | - | - |
| 2. D-Glucose | + | + | + | + | + | + |
| 3. Glycerol | - | + | + | - | - | - |
| 4. Calcium 2-keto-gluconate | - | - | - | - | - | - |
| 5. L-Arabinose | - | - | - | - | - | - |
| 6. D-Xylose | - | - | - | - | + | - |
| 7. Adonitol | - | - | - | - | - | - |
| 8. Xylitol | - | - | - | - | - | - |
| 9. D-Galactose | + | + | + | + | + | + |
| 10. Inositol | - | - | - | - | - | - |
| 11. D-Sorbitol | - | - | - | - | - | - |
| 12. Methyl-α D-Glucopyranoside | - | + | + | + | - | + |
| 13. N-Acetyl-Glucosamine | - | - | - | - | - | - |
| 14. D-Cellobiose | - | - | - | - | - | - |
| 15. D-Lactose | - | - | - | - | - | - |
| 16. D-Maltose | + | + | + | + | + | + |
| 17. D-Saccharose | + | + | + | + | + | + |
| 18. D-Trehalose | - | - | - | - | - | - |
| 19. D-Melezitose | - | - | - | - | - | - |
| 20. D-Raffinose | + | + | + | + | + | + |
| 21. Hyphae/ pseudo hyphae | - | - | - | - | - | - |

Table 4: Biochemical characterization of selected isolates through API kit (API 20 C AUXV5.0)

| Sl. No | Strains | Identified organism |
|--------|---------|---------------------|
| 1.     | NGL3A   | *Saccharomyces cerevisiae* 1 |
| 2.     | NGL4A   | *Saccharomyces cerevisiae* 1 |
| 3.     | NGL1B   | *Saccharomyces cerevisiae* 1 |
| 4.     | RNS4C   | *Saccharomyces cerevisiae* 1 |
| 5.     | RNS1C   | *Rhodotorula mucilaginosa* |
| 6.     | RNL1A   | *Saccharomyces cerevisiae* 1 |
Table 5 NCBI GeneBank accession no. of the identified yeast isolates

| Sl. No | Strains  | Partially identified by BLAST | NCBI GeneBank accession no. |
|--------|----------|-------------------------------|-----------------------------|
| 1.     | NGL3A    | *Saccharomyces cerevisiae* 1  | MG101823                    |
| 2.     | NGL4A    | *Saccharomyces cerevisiae* 1  | MG101822                    |
| 3.     | NGL1B    | *Saccharomyces cerevisiae* 1  | MG183703                    |
| 4.     | RNS4C    | *Saccharomyces cerevisiae* 1  | MG101827                    |
| 5.     | RNS1C    | *Rhodotorula mucilaginosa*    | MG101829                    |
| 6.     | RNL1A    | *Wickerhamomyces anomalus*    | MG183698                    |

Fig.1 Gram staining of the selected isolates

![Gram staining](image1.png)

A. *Saccharomyces cerevisiae*  
B. *Saccharomyces cerevisiae*  
C. *Saccharomyces cerevisiae*

Fig.2 Biochemical analysis of the selected yeast strains

A. *Saccharomyces cerevisiae* (NGL4A)

![Biochemical analysis A](image2.png)

B. *Saccharomyces cerevisiae* (NGL3A)

![Biochemical analysis B](image3.png)
Fig.3 Rooted phylogenetic tree (UPGMA) for the strains of yeast from rice beverages and *Wanti*

Molecular confirmation and 5.8S rDNA sequence analysis of yeast strains

Strains were identified to the species level by amplification of the 5.8S rDNA gene and flanking Internal Transcribed Spacer (ITS). In the present study, primer ITS1 and ITS4 were used for amplification conserved regions of 5.8S rDNA, resulted in product of >1.5kb fragments confirming that the isolate was yeast (Table 2). Similarly, Esteve-Zarzoso *et al.*, (1999) and Lentz *et al.*, (2014) also used 5.8S rDNA for the strains identification up to the species level. The tentative phenotypic identification of all six isolates was confirmed by genotypic characterization in which 5.8S rDNA sequence analysis of these isolates as NGL3A, NGL4A, NGL1B and RNS4C confirmed the strains *S. cerevisiae*, whereas rest two isolates were identified as *W. anomalus* (RNL1A) and *R. Mucilaginosa* (RNS4C). The electrophenogram data for 5.8S rDNA sequence was validated using Chromas 2.33 software. Sequences obtained were matched with previously published 5.8S rDNA sequences of yeast strains available in the GenBank database using BLAST. The sequences determined in this study have been deposited in the NCBI GenBank database with their respective accession numbers (Table 5).

The FASTA sequences of the identified strains after 5.8S rDNA sequence analysis are as follows:

16s rRNA sequence of *Saccharomyces cerevisiae* (GeneBank Accession no.MG101823)

>MG101823.1, *Saccharomyces cerevisiae* NGL3A

TTTGGAAATGGATTTTTTTGTTTGG
CAAGAGCATGAGGCTTTTACTGGGG
AAGAAGACAAAGATGAGGTCAG
CCGGGCTGGCCTTTAAGTGGCCCGT
CTGCTAGGCTTGTAAAGTTCTTCTG
TAATGGAAACGTCAGGTTCTCTTCT
CTTTGTTTATTAGACAAATAAAAACCG
TTCAATACAACACACTGGGAAATTTT
CTATCTTTGCCCACCTTTTTGCGAAA
TCGAGCAATCGGGCCCCAGGTAC
AACCAACCAATTTTATTATTACAT
ATTTTTGTCAAAAAAACAAAGATT
GTAACCGGAATTTTTAAATTTAAAA
ACTTTCAACAACCGATTTCTTGTTCTTT
GCCTCGATGAAAGACGACGCGA
CGATTCTGAAAGTAATTGGCGGAAATTC
CCTGAACTCTTTGAACTTGAAGCCC
CTTGCGCCCCCTTGTTTTTCGGGGGC
CTGACCTTGGATGCTTTCTCCTTTT
CAACCTTTTTGTTTGGTAGGGAGTGATT
CTTTTTGGAGTTAACCTGAAATTGCTG
GCGTTTTCTTGGATGTTTTTTTTTTG
CAGGAGAGAGTTTCTTGCTGCTTTGA
GGTTATATGCAAGTACGGTCGTTTTAG
GTTTTACCAACTGCGGCTAATCTTTTTT
ATACTGAGCGTATTGGAACGTTATCGA
TAAGAAGAGAGCTGCTAGGCGAACAA
TGTTCTTTAAAGT

16s rRNA sequence of Saccharomyces cerevisiae (GeneBank Accession no. MG101822)

>MG101822.1, Saccharomyces cerevisiae

GGAAGGATCATTAAGAAAGAAATTAATA
ATTTTGAAATGGAATTTTTTTTTTTTT
GCAAGAGCCTGAGAAGCTTTTACTGGGC
AAGAAGACAGAGATGGAGAGTCCAG
CCGGGCTCGGCCTTAAAGTGCGCGGTCT
TGCTAGGCTTAAAGT

16s rRNA sequence of Saccharomyces cerevisiae (GeneBank Accession no. MG183703)

> MG183703.1, Saccharomyces cerevisiae

GCGAGCGACGCGATCATGATGGTATACT
ACTGCTTTTCATCACTGCGTCTGCGG
GAGCAGCGACAAGATTGAATGATAT
ATTTAGTATATGCGGCTGATTTCTGTG
GTGTCACAATATAGTAAATATATAT
TTGTTGTTTTATCTGCAGCCGCGTGTTG
TGGTCACCAGAAAAGTGGGCAC
AAGTTAATACCCCTCTTCTATAC
GAAACGGTTTATATTTTTTCCACG
AACCGCAGAAACTCCCCCTCTCTTGAA

GAAATTTTAATTTTAGAAAAGGGTT
TTTTTTTTTGTTTCGGCAGGAGCATAG
GAGCTTTTACGGGCGAGGATCATAAA
AGATGGAGACTCCACCCGCGCTGCGC
TTAAGTGCGCGCTTGGCTGACTGAT

GCACTTGGAAAATTTTATTTAAAA
AACTTTTCTTTTGTTATCTGCAAGC
GAGAGATTGTTGTGCTTTTGTTATAGG
GAGAAGATTTTGTGCTTTTTGGTAAGG

ACATCGCAGAAGCAGCAGGAAAT
GCCGATCGTAATGGAATGCGAATT
CCGTAATCAGAATCTTGGAAACGCA
CATGCGCCCTGTTGGATTTGGGGGGG
CATGCTGTGGATGCGTCATTTCCTCT
CAACCATCTGTTTTGGTAGGAGTGAT
ACTCTTTGGAGTTAACCTGAAATTGCT
GGCTTTTTACCTGGAGATTTTTTTTTTT
ACGCAGCGAATGCGATACGTAATGT
GAATTGCAAGAATCCGTGAATCATCGA
ATCTTGGACGCACTTGCAGCCCCGTG
GTATTTCCGGGGGCACCGCCTTGGTAG
CGTCTATTCTTCCTCACAACATCTCTTT
GTAGTGTAGTGTCTTCTGGGAGGGTA
ACTAGAAATTGTCTGGCGCTTTTTCATGG
ATGTGTTTTTTTTCAAGAGAAGGTTTCT
CTCAGGTGCTTTGAGTAATGCAAGAAGTA
CGGTCGTTTCTAGTTTACAAACTGCG
GCTAATCTTTTTATACGGATATCTGA
GAAACGTTCGATAAGAAGAGCCGAT
CTAGGCCGAACTGTCTTCTAAAAGTGAC
CTCAATCAGTACGATATCCGTTCCCT

16s rRNA sequence of *Saccharomyces cerevisiae* (GeneBank Accession no. MG101827)

>`MG101827.1, Saccharomyces cerevisiae RNS4C`

GGATTTTTTGTGGTGGCAAGAGCAT
GAGAGCTTTTCTGCGCAGAAGACA
AGAGATGGAAGACCGACCCGGCCCTG
CGCTTAAATGCGGCTCTTCTGAGGCT
TGAGTAAAGTTCTCTGCTATTCACAC
GGGACATTTAAAACGGTTTCAATCA
CACACTGTGGAGTTTTCTATTTGTC
AATCCTTTTTCTGCGGATTCCTGAAGA
CCGGGCCAGAGTTAAACACCAAAC
CAATTTTTTTTTATCTTAAATTTTTG
CAAAAAAACAGAATTTGCTAATCGG
AAATTTTTAAATATTTAAAACTTCAA
CAACGGGATCTCTTTGTGCATCATC
TGAGGAAGCCAGCAGAATTCGTAAC
TAATGTAATTGCAAGAATTCGGTGAAT
CATCGGAATTTGCGAAGCATTGCCGC
CCCTTGATTTCCGGGGGCATGCTGG
TTTGGAGCGTCTTCCCTTCCTCACAACATT
CTGTGTTGGTATGATGATCTTCTTTG
GAGTTAATCTGAAATTTCTGTGGCCTTTT
CAATGGATGTGGGGTCTTTCCTCACAAGAG
AGGTTTTCTCTCGGTGGGCTAGGTATAAA
TGCAAGTACCGTGTTTTAGGTTCCTAC

CAACTGCGGGCTAATCCTTTTTTATACGT
AGCGTATTGGAAAAGTTATCGAATAAGAA
GAGAGCGTCTCTAG

16s rRNA sequence of *Rhodotorula mucilaginosa* (GeneBank Accession no. MG101829)

>`MG101829.1, Rhodotorula mucilaginosa RNS1C`

CTAATGATCCTTCCGTAGTTGAACCTTG
CGTAGAGATCATTAGTGAATATACGA
CGTCCAACCTAACCTGGATCGAATCCTT
GGATATGTAACCTTCGCAAGAGCGAG
AATCTCTATCTACTATTTAAACACAAACAG
TCTATGAGATTAAATTTCTATTTTATAT
AAATAAACTTTACCAACGGATCTCTCT
CTGCTTCTCGCATCGATAGCTGTTAGTG
CGTAATGACATTGCCATCGCAATCCGA
CTTCCGGATAGCTTTGCGAATAGACT
ATTCCGTAAGATTCTAGTCTCGGAG
CTAGAGCCGGTTGTTGTTAAAGGAAAG
CTTCTAACTGTAATGCTACAT

16s rRNA sequence of *Wickerhamomyces anomalus* (GeneBank Accession no. MG183698)

>`MG183698.1, Wickerhamomyces anomalus RNL1A`

GGCAATAGAAATCTATAATGATCCTTC
CTGTGTTGGTATGATGATCTTCTTTG
GAGTTAATCTGAAATTTCTGTGGCCTTTT
CAATGGATGTGGGGTCTTTCCTCACAAGAG
AGGTTTTCTCTCGGTGGGCTAGGTATAAA
TGCAAGTACCGTGTTTTAGGTTCCTAC

GGCAATAGAAATCTATAATGATCCTTC
CTGTGTTGGTATGATGATCTTCTTTG
GAGTTAATCTGAAATTTCTGTGGCCTTTT
CAATGGATGTGGGGTCTTTCCTCACAAGAG
AGGTTTTCTCTCGGTGGGCTAGGTATAAA
TGCAAGTACCGTGTTTTAGGTTCCTAC

3088
Phylogenetic analysis

To determine the closest known relatives of the partial 5.8S rDNA sequences obtained, nucleotide database searches were performed in NCBI GenBank and later the sequences were analysed by multiple sequence alignment tools using the DNA alignment program MAFFT v6.864 to signify the evolutionary relatedness between the strains by UPGMA (Unweighted Pair Group Method with Arithmetic Mean). From the phylogram as depicted in Figure 3, it can be depicted that the two isolates, S. cerevisiae (NGL4A and RNS4C) are closely related due to the sequence similarity match as well the nodal distance which in turn is significantly related to S. cerevisiae (NGL1B) distantly connected to S. cerevisiae (NGL3A). This branch is again distantly related to W. anomalous (RNL1A) followed by R. mucilaginosa (RNS4C) as represented by the branch length. Each node with descendants represents the inferred most recent common ancestor of the descendants which in this case is Saccharomyces spp.

The present study concluded that S. cerevisiae was predominant yeast in microflora of rice beverages and starter culture. The tentative phenotypic identification of these six isolates were confirmed by the phenotypic as well as genotypic (5.8S rDNA sequence analysis) identification which derived that four isolates viz. NGL3A, NGL4A, NGL1B and RNS4C belonged to Saccharomyces cerevisiae, RNL1A as Wickerhamomyces anomalous and RNS4C as Rhodotorula mucilaginosa. Later, phylogenetic tree of the most closely related yeast isolates have been constructed by using MAFFT sequence alignment tool. Further, the isolated strains could be checked for their specific probiotic attributes and be exploited for the development of value added fermented foods which will be appropriate for glutinous rice fermentation and can improve the quality of traditional rice wine production.

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