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

Birendra Kumar Mishra[1*], Subrota Hati[2], Jonali Brahma[1], Maulik Patel[2] and Sujit Das[1]

[1]*Department of Rural Development and Agricultural Production, North- Eastern Hill University, Tura campus 794 002, Meghalaya, India*
[2]*Department of Dairy Microbiology, S.M.C College of Dairy Science, Anand Agricultural University, Anand-388 110, Gujarat, India*

**Corresponding author*

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ABSTRACT

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.

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, *ijasuijang* 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 *shaosingiji* 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 pulul* of Malaysia (Lee CH, 2009), *ruou de* or *ruou nep*in 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 0.8-1cm 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 32ºC 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 94ºC for 5 minutes followed by cycles of denaturation at 94ºC for 1 min, annealing 57ºC for 1 min and extension was performed at 72ºC 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 muciliginosa.* 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. RNS4C showed positive for D-Glucose, D-Galactose, and Methyl-α D-Glucopyranoside, D-Maltose, D-Saccharose and D-Raffinose respectively. RNL1A 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 beveragein 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 phonotypical 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	
ITS1	5' TCCGTAGGTGAACCTGCGG 3'
ITS4	5' TCCTCCGCTTATTGATATGC 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	-	-	-	-	-	-

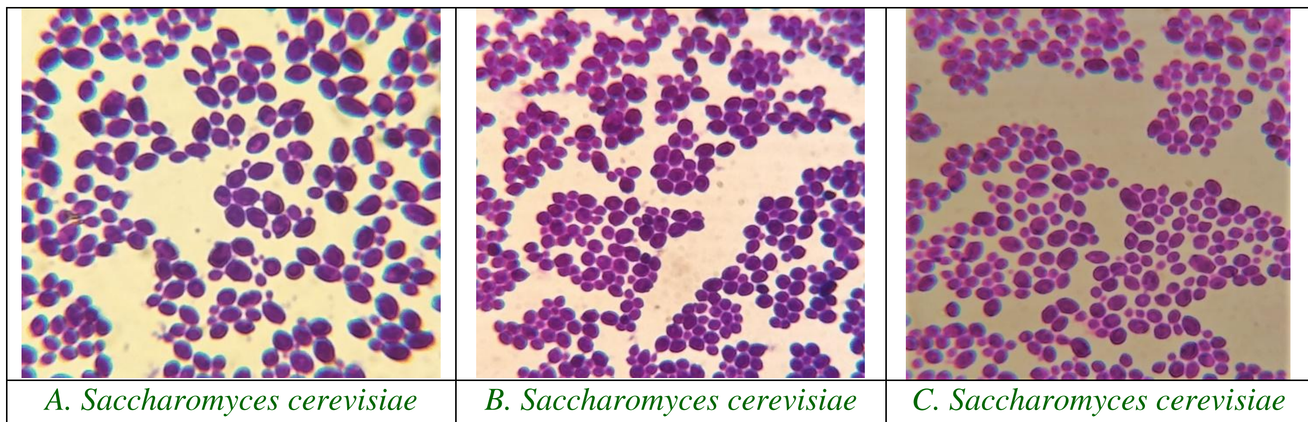
Table.4 Biochemical characterization of selected isolates through API kit (API 20 C AUXV5.0)

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

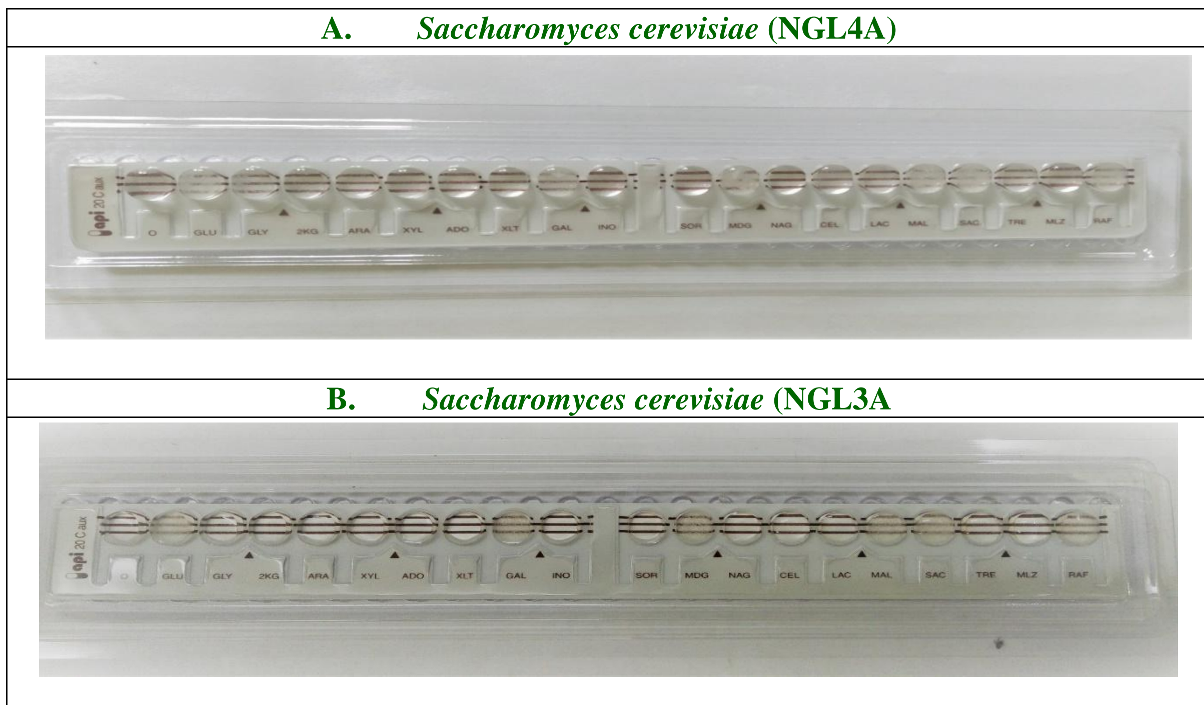
Sl. No	Strains	Partially identified by BLAST	NCBI GeneBank accession no.
1.	NGL3A	*Saccharomyces cerevisiae 1*	MG101823
2.	NGL4A	*Saccharomyces cerevisiae 1*	MG101822
3.	NGL1B	*Saccharomyces cerevisiae 1*	MG183703
4.	RNS4C	*Saccharomyces cerevisiae 1*	MG101827
5.	RNS1C	*Rhodotorula mucilaginosa*	MG101829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

Fig.1 Gram staining of the selected isolates



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

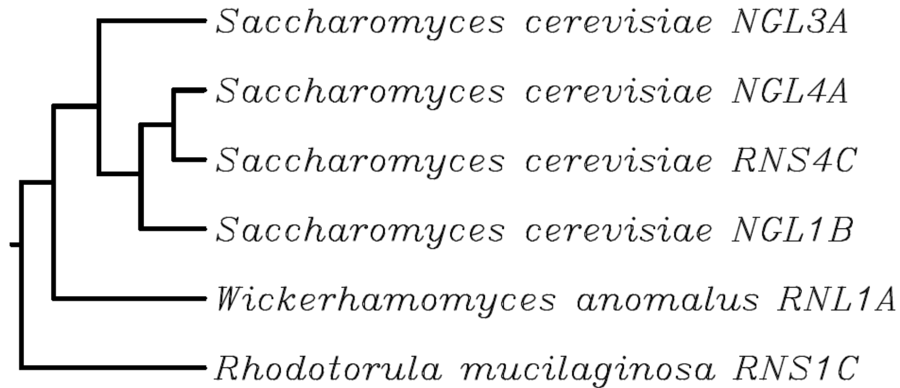
Fig.2 Biochemical analysis of the selected yeast strains



A. *Saccharomyces cerevisiae* (NGL4A)

B. *Saccharomyces cerevisiae* (NGL3A

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 upto 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 RNS4Cconfirmed 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

TTTGGAAATGGATTTTTTTTGGTTTGG
CAAGAGCATGAGAAGCTTTTACTGGGC
AAGAAGACAAGAAGATGAGAGGTCAG
CCGGGCCTGCGGCTTAAGTGCGCGGTC
CTGCTAGGCTTGTAAGTTTCTTTCTTGC
TAATGCAAACGGTGAGAGGTTTCTGTG
CTTTTGTTATTAGACAAATAAAACCGG
TTCAATACAACACACTGTGGAATTTTT
CTATCTTTGCCACTTTTTCTTTGGGCAA
TCGAGCAATCGGGGCCCCGAGGTTACC
AACACCAACAATTTTATTTATTCATTA
AATTTTTGTCAAAAAACAAGAATTTTT
GTAACCGGAAATTTTTAAATTTTAAAA

ACTTTCAACAACCGATTTCTTGGTTCTT
GCCTCGATGAAGGACGCAGCGAAATT
CGATTCGTAAAGTGAATTGCCGAATTC
CCTGAATCCTTGAATCTTTGAACGCCC
CTTGCGCCCCCTGGTTTTTCCGGGGGC
CTGCCTGTTTGAGCGTCCTTTCCTTTTC
CAACCTTTTGTTTGGTAGGGAGTGATT
CTTTTTGGAGTTAACCTGAAATTGCTG
GCCTTTTCCTTGGATGTTTTTTTTTTTC
CAAAGAGAGGTTTCTCTGCGTGCTTGA
GGTATAATGCAAGTACGGTCGTTTTAG
GTTTTACCAACTGCGGCTAATCTTTTTT
ATACTGAGCGTATTGGAACGTTATCGA
TAAGAAGAGAGCGTCTAGGCGAACAA
TGTTCTTAAAGT

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

>MG101822.1, *Saccharomyces cerevisiae* NGL4A

GGAAGGATCATTAAAGAAATTTAATA
ATTTTGAAAATGGATTTTTTTTGTTTTG
GCAAGAGCATGAGAGCTTTTACTGGGC
AAGAAGACAAGAGATGGAGAGTCCAG
CCGGGCCTGCGCTTAAGTGCGCGGTCT
TGCTAGGCTTGTAAGTTTCTTTCTTGCT
ATTCCAAACGGTGAGAGATTTCTGTGC
TTTTGTTATAGGACAATTAAAACCGTT
TCAATACAACACACTGTGGAGTTTTCA
TATCTTTGCAACTTTTTCTTTGGGCATT
CGAGCAATCGGGGCCCAGAGGTAACA
AACACAAACAATTTTATTTATTCATTA
AATTTTTGTCAAAAAACAAGAATTTTC
GTAACTGGAAATTTTAAAATATTAAAA
ACTTTCAACAACGGATCTCTTGGTTCT
CGCATCGATGAAGAACGCAGCGAAAT
GCGATACGTAATGTGAATTGCAGAATT
CCGTGAATCATCGAATCTTTGAACGCA
CATTGCGCCCCTTGGTATTCCGGGGGG
CATGCCTGTTTGAGCGTCATTTCCTTCT
CAAACATTCTGTTTGGTAGTGAGTGAT
ACTCTTTGGAGTTAACTTGAAATTGCT
GGCCTTTTCATTGGATGTTTTTTTTTTC
CAAAGAGAGGTTTCTCTGCGTGCTTGA
GGTATAATGCAAGTACGGTCGTTTTAG
GTTTTACCAACTGCGGCTAATCTTTTTT
ATACTGAGCGTATTGGAACGTTATCGA
TAAGAAGAGAGCGTCTAGGCGAACAA
TGT

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

> MG183703.1, *Saccharomyces cerevisiae* NGL1B

GTAGCGACGCGATCATGATGGTATACT
ACTGCTTTCATCAGTACGCTGTGTCCG
GAGCAGCGAACGAGTGGATAGTATTA
ATTAGTATATGCGTGCAGTATTTCTGT
GTGTGCACAATATAGTATAATAAATAT
TTGGTGTTTATTCTGCGGGCGCGTGTG
TGTGCCACCACAAGAAAAAGTGGCAC
AAGTATAAAACCCCCCCTGTGTGTTTT
GAAACGTGTTTATATTTCTATAAACAA
AACCGCAGAAACTCCCCCTCCTTGTAA
CCAGAAGAAAGATTTCTACACTCTAGG
AGGCCGGTTTTGGGCGCGCGGGGCGG
CGAGACCTCCCCTCTTGTCTCGCGCCA
GAAAAAGTCTTCTTCTTGGGAAAAAAA
AAAAAAAAAACCCCTTTTACAAAATTA
AAAACTTCAAAATTCTTTCTTTCCGGG
GGGGGACCTGCGGAGGGATCATTAAA
GAAATTTTAATATTTTAGAAAAGGGTT
TTTTTTTTTGTTTCGGCAGGAGCATGA
GAGCTTTTACGGGGCAAGACGACAAG
AGATGGAGACTCCACCCGCGCCTGCGC
TTAAGTCGGCGCTCTTGCTAGGCTAAT
AATTTTCTTTCTTGTTATTCCAAGCGGG
GAGAGATTTGTGTGCTTTTGTTATAGG
ACAAATAAACCCGTTTCAATACCACCC
CCGGGGGAGTTTTCTTTTTTTTGCAACT
TTTTTTTGGGGCATTGGAACAATGGGG
CCCCGGAGGTAACACACACAAACAAT
TTTTTTTCTTCATAAATTTTTTGTAAAA
AACAAGAATTTTTGTAACTGGAAATTT
TAAAATATTAAAAACTTTCAACAACGG
ATCTCTTGGTTCTCGCATCGAAGAAGA

ACGCAGCGAAATGCGATACGTAATGT
GAATTGCAGAATTCCGTGAATCATCGA
ATCTTTGAACGCACATTGCGCCCCGTG
GTATTCCGGGGGGCACGCCTGTTTGAG
CGTCATTTCCTTCTCAAACATTCTGTTT
GGTAGTGAGTGATTCTCTGGGGAGTTA
ACTAGAAATTGCTGGCCTTTTCATGGG
ATGTTTTTTTTTCCAAAGAGAGGTTTCT
CTGCGTGCTTGAGGTATAATGCAAGTA
CGGTCGTTTTAGGTTTTACCAACTGCG
GCTAATCTTTTTTATACTGAGCGTATTG
GAACGTTATCGATAAGAAGAGAGCGT
CTAGGCGAACAATGTTCTAAAGTTGAC
CTCAATCAGTACGATATCCGTTCCCCT

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

> MG101827.1, *Saccharomyces cerevisiae* RNS4C

GGATTTTTTTGTTTTGGCAAGAGCAT
GAGAGCTTTTACTGGGCAAGAAGACA
AGAGATGGAGAGTCCAGCCGGGCCTG
CGCTTAAGTGCGCGGTCTTGCTAGGCT
TGTAAGTTTCTTTCTTGCTATTCCAAAC
GGTGAGAGATTTCTGTGCTTTTGTTAT
AGGACAATTAAAACCGTTTCAATACAA
CACACTGTGGAGTTTTCATATCTTTGC
AACTTTTTCTTTGGGCATTCGAGCAAT
CGGGGCCCAGAGGTAACAAACACAAA
CAATTTTATTTATTCATTAAATTTTTGT
CAAAAAACAAGAATTTTCGTAACTGG
AAATTTTAAAATATTAAAAACTTTCAA
CAACGGATCTCTTGGTTCTCGCATCGA
TGAAGAACGCAGCGAAATGCGATACG
TAATGTGAATTGCAGAATTCCGTGAAT
CATCGAATCTTTGAACGCACATTGCGC
CCCTTGGTATTCCGGGGGGCATGCCTG
TTTGAGCGTCATTTCCTTCTCAAACATT
CTGTTTGGTAGTGAGTGATACTCTTTG
GAGTTAACTTGAAATTGCTGGCCTTTT
CATTGGATGTTTTTTTTTTCCAAAGAG
AGGTTTCTCTGCGTGCTTGAGGTATAA
TGCAAGTACGGTCGTTTTAGGTTTTAC
CAACTGCGGCTAATCTTTTTTATACTG
AGCGTATTGGAACGTTATCGATAAGAA
GAGAGCGTCTAG

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

> MG101829.1, *Rhodotorula mucilaginosa* RNS1C

CTAATGATCCTTCCGTAGGTGAACCTG
CGGAAGGATCATTAGTGAATATAGGA
CGTCCAACTTAACTTGGAGTCCGAACT
CTCACTTTCTAACCCTGTGCACTTGTTT
GGGATAGTAACTCTCGCAAGAGAGCG
AACTCCTATTCACTTATAAACACAAAG
TCTATGAATGTATTAAATTTTATAACA
AAATAAAACTTTCAACAACGGATCTCT
TGGCTCTCGCATCGATGAAGAACGCAG
CGAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTG
AACGCACCTTGCGCTCCATGGTATTCC
GTGGAGCATGCCTGTTTGAGTGTCATG
AATACTTCAACCCTCCTCTTTCTTAATG
ATTGAAGAGGTGTTTGGTTTCTGAGCG
CTGCTGGCCTTTACGGTCTAGCTCGTT
CGTAATGCATTAGCATCCGCAATCGAA
CTTCGGATTGACTTGGCGTAATAGACT
ATTCGCTGAGGAATTCTAGTCTTCGGA
CTAGAGCCGGGTTGGGTTAAAGGAAG
CTTCTAATCAGAATGTCTACAT

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

> MG183698.1, *Wickerhamomyces anomalus* RNL1A

GGCAATAGAATACTATAATGATCCTTC
CGTAGGTGAACCTGCGGAAGGATCATT
ATAGTATTCTATTGCCAGCGCTTAATT
GCGCGGCGATAAACCTTACACACATTG
TCTAGTTTTTTTGAACTTTGCTTTGGGT
AGGCTTTTATGGCTTGCCCAGAGGACA

ACTAAACACATTTTTTACAAATGTTTT
AAACCTTTAACCAATAGTCATGAAAAT
TTTTAACAAAATTAAAATCTTCAAAAC
TTTCAACAACGGATCTCTTGGTTCTCG
CAACGATGAAGAACGCAGCGAAATGC
GATACGTATTGTGAATTGCAGATTTTC
GTGAATCATCGAATCTTTGAACGCACA
TTGCACCCTCTGGTATTCCAGAGGGTA
TGCCTGTTTGAGCGTCATTTCTCTCTCA
AACCTTTGGGTTTGGTATTGAGTGATA
CTCTGTTAAATAGGGTTAACTTGAAAT
AATGTCTTAGCAAGAGTGTACTAATTT
ATACGTCTTTCTGAAATAATGTATTAG
GTTCTTCCAACTCGTTATATCAGCTAG
GCAGATGAATAGTATTTTAGGCTCGGC
TTAACAATTAAACTAAAAGT

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. anomalus* (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 anomalus* 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 can 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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