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Identification of yeast strains in three different "Arishta" using molecular techniques

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Abstract

In Sri Lanka, "Arishta" is prepared from medicinal plant materials and it is well known as "Herbal wine". Arishta is particularly interesting because of its medicinal value. Dry *Woodfordia fruticosa* flowers are used in order to provide yeast strains needed for fermentation and biotransformation processes in Arishta. However, yeast strains involved in preparation in local arishta have not been identified at the molecular level until this investigation. The *Saccharomyces cerevisiae* is the well known yeast species which is used in many industries. Therefore the main objective of the present study was to determine whether *Saccharomyces cerevisiae* is involved in fermentation in Arishta. Hence the identification of yeast strains present in Arishta was carried out during its fermentation process. In the present study three different Arishta was used i.e. Ashwagandha, Nimba and Dasamula. In total, 33 yeast isolates were isolated from three different Arishta and dry *Woodfordia fruticosa* flowers. The molecular identification of yeast species was carried out by RAPD-M13 PCR profiles and analysis of amplified ITS1-5.8S-ITS2 region of selected yeast lines. *Saccharomyces cerevisiae*, a type strain, was used to determine whether *Saccharomyces cerevisiae* is present in the fermentation process. According to results of ITS region analysis, the yeast strains isolated from Arishta were not *Saccharomyces cerevisiae*. Therefore, the species level identification of this yeast strain in Arishta was carried out by sequence of ITS1-5.8S-ITS2 region and comparison with the sequences in NCBI database. According to the sequence analysis the yeast strains in "Arishta" were identified as *Candida guilliermondi* which is the vegetative stahe of pichia ohmeri.

Keywords: Arishta; Yeast; M13-RAPD PCR; ITS1-5.8S-ITS2 region; ITS1-5.8S-ITS2 region sequence

Introduction

Traditional medicine plays an important role in human health in many countries especially in South East Asia. "Arishta" or "herbal wine" is a very common drug in Ayurveda. It has been used since in ancient time to cure various disorders, as well as enhance physical fitness and appetite as well. They are weak alcoholic products prepared by mixing a decoction of plant materials with sugar. After the addition of dried flowers of *Woodfordia fruticosa* L.kurz (Lythracae) the mixture is fermented in an air tight sealed vessel for nearly one month (Kroes, 1990).

In the preparation of Arishta, yeasts play an important role in both fermentation and biotransformation. Yeast may participate in generation of alcohol during the fermentation process and may have relevance for the biological activity of the fermented product (Kroes, 1990). Besides ethanol, a whole series of other products are released by active yeasts during fermentation. Those products are very important to medicinal value of arishta. Because the original forms of phytochemicals in arishta are transformed into another forms by yeast. Among the yeast, *Saccharomyces cerevisiae* is best documented for biotransformation. *Saccharomyces* spp. are also capable of hydrolyzing carbohydrates by means of extra cellular enzymes including invertase, glucoamylase, melibiase and amylase (Kroes, 1990). Therefore, the investigation of yeast strains in arishta is very important from both of biotransformation and fermentation point of view. On the other hand the fermentation process is important for preservation of arishta for long period.

Traditionally, the classification and identification of yeast species and strains are based on morphological and physiological traits. Molecular techniques utilizing amplification of target DNA provide alternative methods for identification. Ribosomal RNAs (rRNA) provide a powerful taxonomic indicator, because they are highly conserved and are universally found in living cells (Pramateftaki *et al.*, 2000; Lopes *et al.*, 2002). The

genes coding for yeast rRNA occurs as tandem repeated units on chromosome X11, with 18S, 5.8S, and 26S rRNA genes co- transcribe. Sequence comparisons of the genomic regions in the rDNA repeat unit of various organisms have shown a relatively high degree of evolutionary conservation and have been used as basis for inferring phylogenetic relationships. Phylogenetic relationships among the ascomycetes yeasts have been analyzed from 5' end of 26S rDNA D1/D2 region sequence divergence. Between the coding regions are the internal transcribed spacer 1 and 2 regions (ITS 1 and ITS 2 respectively) which evolve more rapidly and therefore, these regions are vary in different species within a genus. The yeast species have been identified by restriction fragment length polymorphism (RFLP) using the polymerase chain reaction (PCR) technique of ITS regions (Alvarez-Marti *et al.*, 2007).

Therefore, the main objective of the present study was to identify yeast strains involved in fermentation and biotransformation of selected Arishta by using molecular tools.

Materials and methods

Sample collection

Three different "Arishta" samples (i.e. Nimba Arishta, Dasamula Arishta, and Ashwagandha Arishta) were aseptically collected during fermentation of the arishta broth (Two weeks old fermented arishta samples) from different Ayurvedic manufactures (Table 1). They were brought into the laboratory and kept at 4 °C.

Table1. Arishta samples collected from different ayurvedic manufactures

Arishta sample	Manufacture
Nimba Arishta 1	Denagama Ayurvedic dispensary, Matara
Nimba Arishta 2	Withanarachchi Ayurvedic Hospital (Pvt) Ltd.
Dasamula Arishta	Withanarachchi Ayurvedic Hospital (Pvt) Ltd.
Ashwagandha Arishta	Withanarachchi Ayurvedic Hospital (Pvt) Ltd.

Isolation of yeasts

Isolation of yeasts from Arishta

The Arishta samples were centrifuged at 10,000 rpm for 10 min and pellet was washed using 100 mM sorbitol for three times. Then ten fold serial dilutions were prepared and 10 µl aliquots were spread on YPD (Yeast extract 4 g, Peptone 4 g, Glucose 8 g, Agar 8 g per 1 L) medium. The plates were incubated at room temperature for 24 hours. The colonies appeared were counted as colony forming units. The predominant representative colonies were selected based on their colony morphology under microscope. Selected colonies were sub-cultured on new plates and purified by repeated streaking. A total of 33 single yeast isolates were obtained and maintained on YPD plates.

Isolation of Yeasts from Woodfordia fruticosa flowers

Two dry *Woodfordia fruticosa* flowers were put into 100 ml of sterilized 2% glucose solution containing 12 µg ml^{-1} of amoxiline for two hours at 25 °C. Then 10 µl of incubated *Woodfordia fruticosa* flower solution was spread on YPD plate and incubated at room temperature for 48 hours. The predominant representative colonies were selected based on colony morphology under microscope. Selected colonies were sub-cultured on new plates and purified by repeated streaking. Eight single yeast isolates were obtained and maintained on YPD plates at room temperature.

DNA Extraction

Selected yeast isolates were plated on new YPD plates and grown for 24 hours at 25 °C. DNA was extracted from those single yeast isolates as follows. Small amount of glass beads was put into sterilized 1.5 ml vial containing 150 µl of SE buffer (1.2 M Sorbitol and 0.1 M EDTA) and β-mercaptoethanol mixture (2 µl of β-mercaptoethanol in 1 ml SE buffer) and then, lo[illegible]s full of yeast isolates were mixed in the vial. The yeast suspension was vortexed for 40-60 seconds. After that 10 µl of lyticase enzyme was added and incubated for 1 hour at 37 °C. The incubated yeast suspension was centrifuged under 13,000 rpm for 10 min. The supernatant was discarded and pellet was vortexed for 30-50 seconds. Then 200 µl of lysis buffer (0.15 M NaCl, 0.1 M EDTA, 1% SDS and deionized water) and protinase K mixture was added and incubated at 55°C for 30 min. The DNA was extracted according to phenol-chloroform method described by Sambrook *et al.*, (1983). The extracted DNA was precipitated in isopropanol and vial was kept in ice for 10-15 min. Then DNA pellet was dissolved in 30-40 µl of sterilized de-ionized water and stored in 4 °C for 48 hours and then stored in -20 °C.

Randomly Amplified Polymorphic DNA (RAPD) - PCR

The extracted DNA was diluted using sterilized deionized water and adjusted them to a final concentration of 10-20 ng μl^{-1} for PCR analysis. To perform the RAPD-PCR amplification the M13 primer (5' GAGGGTGGCGGTTCT 3') was used (Guerra *et al.*, 2001; Gadanho *et al.*, 2006). The PCR was performed in a total reaction volume of 20 µl consisting of 2 µl of 10X PCR buffer (colour), (Promega, USA), 4.5 mM $MgCl_2$ and 0.15 mM dNTP (Promega, USA), 0.2 pmol μl^{-1} M13 primer and 0.8 unit of Taq DNA polymerase and 12.2 µl of sterilized deionized water and 1.5 µl of diluted DNA. The PCR amplification was performed in programmable thermal cycler. An initial denaturation at 94 °C for 4 min, 37 PCR cycles of amplification were conducted as follows. Denaturation at 94 °C for 30 seconds, annealing at 44 °C for 90 seconds, and extension at 72 °C for 100 seconds. The final extension was at 72 °C for 15 min and amplified product was cooled at 4 °C. The amplified DNA fragment was separated by applying 10 µl of each PCR product to 1.8 % agarose (Promega) gel. The 1 kb DNA ladder (Promega) was included as standard for the calculation of the DNA fragment size. The gel was run in 0.5X TBE buffer for three hours at 50 V. The electrophoresed gel was stained in ethidium bromide for 40 min and photographed using gel documentation system. Band sizes were estimated by comparison against DNA ladder (1 kb).

ITS 1-5.8S-ITS 2 region amplification

The amplification of ITS 1-5.8S-ITS 2 region was carried out in 50 µl of reaction mixture containing 5 µl of 10X PCR buffer (Promega), 4.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 pmol μl^{-1} of each forward primer (ITS1 5'-TCCGTAGGTGAACCTGCGG-3) and reverse primer (ITS4 5'-TCCTCCGCTTATTGATATGC-3), 1.6 units of *Taq* DNA polymerase and 27 µl of autoclaved deionized water and 1µl of diluted DNA. The PCR amplification was performed in programmable thermal controller. After an initial denaturation at 94 °C for 4 min, 37 PCR cycles of amplification were conducted as follows. Denaturation at 94 °C for 30 seconds, annealing at 56 °C for 75 seconds, and extension at 72 °C for 120 seconds. The final extension was at 72 °C for 10 min and amplified product was cooled at 4 °C. The amplified DNA fragment was separated by applying 6 µl of each PCR product with 1µl of loading buffer to 2 % agarose (Promega) gel. The 1-kb DNA ladder (Promega) was included as standard for the calculation of the DNA fragment size. The gel was run in 0.5X TBE buffer for two hours at 50 V. The electrophored gel was stained in ethidium bromide for 40 min and photographed using gel documentation system. Band sizes were estimated by comparison against DNA ladder. The amplified products of the unknown strains were compared with the standard strains.

Sequencing of ITS 1-5.8S-ITS 2 region

The ITS 1-5.8S-ITS 2 region of selected yeast isolates were sequenced through Genetic Analyzer in Asiri Centre for Genomic and Regenerative Medicine, Colombo, Sri Lanka using standard protocols. Since the ITS sequences of all known yeast species were available at the GenBank database the sequenced yeast species were identified by direct sequence comparison using the Basic Local Alignment Search Tool (BLAST) available at http://www.ncbi.nlm.nih.

Results and Discussion

Yeast Quantification

Total cell counts were taken for each dilution level of three different arishta using haemocytometer. Five replicates for each dilution level of three different Arishta used for yeast quantification. Considerable variation in the number of colony forming units was observed among the three Arishta. Nimba Arishta showed high colony forming units compared to Ashwagandha and Dashamula Arishta (Table 2).

Table 2: Mean total yeast colony counts from different Arishta

Arishta	Colony forming units (CFU/ml)
Nimba Arishta	7.1 X 104
Ashwagandha Arishta	5.5 X 104
Dasamula Arishta	3.7 X 104

Randomly amplified polymorphic DNA (RAPD) profile

For molecular characterization of yeasts single M13 primer (5' GAGGGTGGCGGTTCT 3') has been used in several studies (Guerra *et al.*, 2001; Gadanho *et al.*, 2006). Therefore, in this study we used the same primer. The randomly amplified DNA products of M13 primer were electrophoresed in a 1.5% agarose gel for three hours under 50 V. The electrophored gel was stained in ethidium bromide solution for 40 min and visualized under UV light (Figure 1). Lane 1 to 16 represents amplified products of DNA from yeast lines isolated from three different Arishta (Table 1).

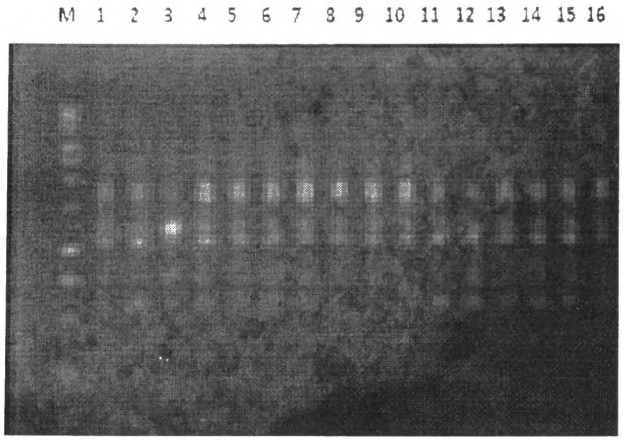

Figure 1. Lane M: 1Kb ladder, Lanes 1, 2, 6, 14, 13, 5: Yeast isolated from Ashwagandha Arishta, Lanes 3, 4, 9, 10, 11: Yeast isolated from Nimba Arishta, Lanes 5, 6, 8, 15, 7: Dasamula Arishta.

The RAPD profile of all yeast isolates except one yeast isolate (lane 3) which are isolated from Nimba Arishta 1 show same RAPD profiles. Therefore, these yeast lines might be the dominant strains in the fermentation process of all types of Arishta included in this study. Thus, in total two RAPD classes of yeast isolates were obtained from Arishta. Most of the yeast lines from Arishta gave ten well separated bands.

Amplified ITS1-5.8S-ITS2 Region profile

In order to identify yeast isolates at species level it has been used ITS1-5.8S-ITS2 region in number of studies (Pramateftaki *et al.*, 2000; Lopes *et al.*, 2002). The amplified ITS products with ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers were analyzed on 1.8% agarose gel and visualized under UV light (Figure 2). For this analysis yeast lines representing two RAPD classes (different RAPD profiles) which were identified by M13 primer (Figure 1) were used. According to the amplified products in Figure 2, about 450 bp fragment resulted from arishta (lanes 1-9) and about 800 bp product resulted from the type strain *S. cerevisiae* (lane 10) used in this study as the positive control. Therefore, it can be concluded that both in arishta have yeast strains different from S. cerevisiae. It has been reported that the ITS product of primers used in this study gives 400-550 bp fragment sizes specific for Candida spp. (Jeyaram et al., 2008). Therefore, in order confirm this yeast lines sequencing of the amplified products were performed.

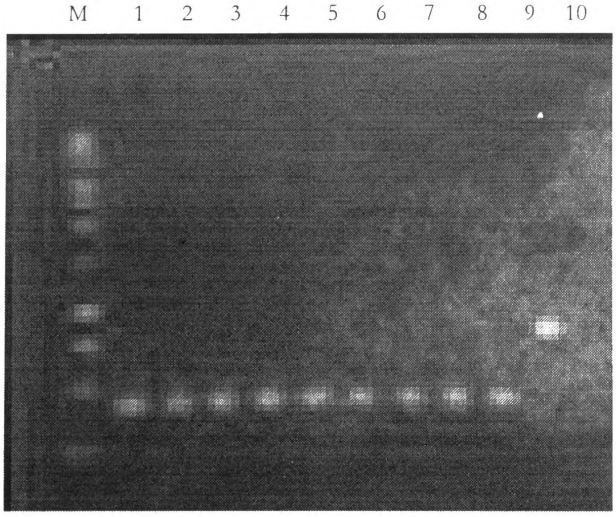

Figure 2. Lane M: 1Kb ladder, Lanes 1, 6, 7: Yeast isolated from Ashwagandha Arishta, Lanes 2, 4, 5: Yeast isolated from Nimba Arishta, Lanes 3, 8, 9: Yeast isolated from Dasamula Arishta, Lane 10: *Saccharomyces cerevisiae* type strain.

Query 24CACTGttttttACAACAAAACAAATCTATCTAAAAACAATTCTTTACAAGAAATTCT
TA 83 |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct558CACTGTTTTTTTTACAACAAAACAAA TCTATCTAAAAACAATTCTTTACAAGAAAT
TCTTA499 Query
84AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGC
GAT143 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct498AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAA
ATGCGAT439
Query144ACGT AATACGAATCGCAGCTCTCGGAATCATCGAATCTTTGAACGCACATTGC
ACCATTG203 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct438ACGTAATACGAATCGCAGCTCTCGGAATCATCGAATCTTTGAACGCACATTGC
ACCATTG379
Query204GGTATTCCCAATGGTATGCTTGTTTGAGCGAATACTTCCCTAATCCTCACGGA
TTGTATT263 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct378GGTATTCCCAATGGTATGCTTGTTTGAGCGAATACTTCCCTAATCCTCACGGAT
TGTATT319
Query264GTGTTTGCACGAAAATAATGACGACAGTACTCTACAAAACGGTACCGTCAGT
ACACTCAt323 |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct318GTGTTTGCACGAAAATAATGACGACAGTACTCTACAAAACGGTACCGTCAGTA
CACTCAT259
Query324ttttttttCCTCAAATCAAGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCG
GAG-382 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct258TTTTTTTTCCTCAAATCAAGTAGGAC TACCCGCTGAACTTAAGCATATCAATAAG
CGGAGG199 Query383AAAAAA388 |||||| Sbjct198AAAAAA193

Figure 3. Yeast species in Arishta was identified as *Candida guilliermondii* which is the vegetative stage of *Pichia ohmeri*

Sequencing analysis of ITS 1-5.8 -ITS 2 region

Two yeast lines representing Arishta were used for sequencing. Since the ITS sequences of all known yeast species were available at the GenBank database the sequenced yeast species were identified by direct sequence comparison using the Basic Local Alignment Search Tool (BLAST) available at http://www.ncbi.nlm.nih. According to those results the yeast species in Arishta was identified as *Candida guilliermondii* which is the vegetative stage of Pichia ohmeri and the similarity of identity of both yeast strains gave 99% similarity (Figure 3). Therefore it can be concluded that S. cerevisiae do not exist in Arishta as a dominant yeast strain.

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