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Isolation, Characterization, and Identification Candidate of Probiotic *Rhodotorula mucilaginosa* Isolated from Soaked and unsoaked Rice and Black gram Immersed in sterilized de-ionised Water

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ABSTRACT: In this investigation, soaked and unsoaked rice and black gram were submerged in sterile de-ionized water with the intention of examining the morphology, pigmentation, and identification of any microorganisms that may have grown there. Few studies have been done to determine the presence of particular probiotic bacteria in the soaked rice and black gram water. Over the potato dextrose agar (PDA) media, there were more than 234 yeast and mould colonies discovered. From these colonies, the most unique 4 colonies were chosen based on colour, characteristics, and gram staining, and from those, 2 colonies were used for PCR (Polymerase Chain Reaction) amplification. A single distinct 1050 bp PCR amplicon band was visible on an agarose gel. Through the use of NS1 and NS4 primer, the forward and reverse sequences of the 18S rRNA gene were combined to create the consensus sequence. However, *Rhodotorula mucilaginosa*'s presence was validated by sequences that produced substantial alignments, a distance matrix, and a phylogenetic tree. The results of this investigation point to the confirmed the existence of *Rhodotorula mucilaginosa* in both soaked and unsoaked black gram.

Keywords: PCR amplification, agarose gel, *Rhodotorula mucilaginosa*, 18S rRNA.

INTRODUCTION

Rice is mankind's most significant staple food. South, East, and Southeast Asia account for the growth and consumption of more than 90% of it. However despite this concentration, rice is actually incredibly versatile and adaptable. The quality of the rice grain is determined by a number of elements, including cultivars, production and harvesting circumstances, post-harvest management, milling, and marketing strategies. 80–90% of rice endosperm is made up of starch, with 6-28% of it being amylose and 5-7% of it being protein. Whereas, Pulses, which are renowned as "Poor Man's Meat" and "Rich Man's Vegetable" and are significant sources of proteins, vitamins, and minerals, considerably contribute to the nation's nutritional security (Singh and Singh 2016). India is the world's greatest producer and user of black gram among pulse crops. In many regions of India, black gram (*Vigna mungo* L.) is grown throughout the year (rainy, winter, and summer) (Singh *et al.*, 2022; Singh *et al.*, 2018). Cereals and pulses (edible dry seeds of leguminous plants) are significant dietary components for people in developing countries. A cereal diet is less healthy since it contains complex proteins, insoluble fibres, and antinutritional elements (Garris *et al.*, 2005). Simple domestic fermentations transform these hard to digest cereals and pulses into nutritious food, and this technique is a significant provincial legacy. In general, fermentation in water-soaked rice and black gram batter is mostly caused by the microflora inherent to the black gram, which, in addition to lactic acid bacteria and yeast, are primarily accountable through *Leuconostoc mesenteroides*, *Lactobacillus lactis*, *Lactobacillus delbrueckii*, *Lactobacillus brevis*, *and Lactobacillus*

fermentum, among others. The yeast *Pediococus cerevisiae, Candida glabarata, Aspergillus hansenii, candidum Geotrichum*, Sugar-making yeast, *Torulopsis holmii, Hansenula anomala, Trichosporon pullulans, Trichosporon beigelli, and Candida versatilis, Candida tropicalis, Candida kefyr, Candida fragilola, Candida glabarata*, (Saravanan *et al*., 2015; Regubalan and Ananthanarayan 2018; Singh *et al.*, 2002).

Black gram (*Vigna mungo*) and parboiled rice (*Oryza sativa*) dehulled cotyledons are soaked for a number of reasons, such as to make batter, to consume immediately after soaking, to extract primary and secondary metabolites, and more. There haven't been many studies done to find out if the water from the soaked rice and black gram seeds contains any specific probiotic bacteria. Therefore, there is a critical need to investigate the isolation, characterisation, and identification of bacteria in rice and black gram that have been soaked and unsoaked in sterile deionized water.

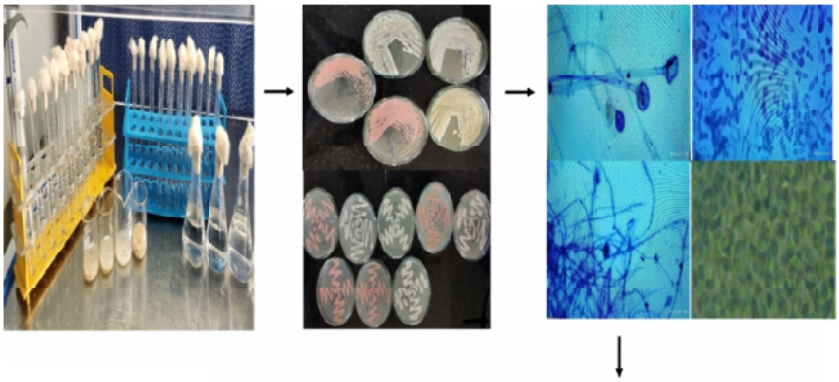
MATERIAL AND METHOD

Materials. Parboiled rice variety IR20, Aduthurai 3 (ADT3) decorticated black gram (*Phaseolus mungo* L.) acquired from a local grocery, and the agar obtained from Himedia, Tiruchirapalli, India. Other chemical compounds used in this investigation, such as staining, were all analytical-reagent grade (Merck, Chennai, Tamil Nadu, India).

Sampling of Rice and Black gram. Rice and black gram were immersed in sterile de-ionised water separately to obtain unsoaked and soaked (kept for 4 hrs) water, at ambient temperature 28 ± 2 ºC (Fig. 1.).

Isolation of Yeasts and Molds. For each of the four water samples, the total yeast and mould colony was identified using potato dextrose agar (PDA) media. Samples were serially diluted (upto a dilution of 10^{-8}), plated on PDA media, which contains Potatoes infusion 20%, dextrose (Glucose) 2%, and agar 1.5%) and then incubated for 4 to 5 days at 25 ºC under aerobic conditions. Specific colonies were chosen based on their morphology and colour. To ensure pure culture, the colonies were restreaked on PDA media, cultured for 4 days at 25ºC, and then stored at 4ºC until processing (Jasmine *et al.*, 2022; Kurtzman and Fell 1998; Banjara *et al.*, 2015). Its morphology and pigmentation were used as the main criteria for identification. For microscopic examination, only colonies with distinctive morphologies (pink, white, and yellow colors) were chosen. An analytical microscope's Gram staining was used for additional confirmation. To confirm the genus and species, 18srRNA sequencing was used to characterize the isolates on a molecular level.

Microbial Identification using 18S rRNA gene based molecular method. DNA was isolated from the culture prepared from soaked and unsoaked black gram de-ionised water. To evaluate its quality, a single band of high-molecular weight DNA was seen on a 1.0% agarose gel. A portion of the 18S rRNA gene was amplified using the NS1 (5'-GTAGTCATA TGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') primers. A single distinct 1050 basepair (bp) PCR (Polymerase Chain Reaction) amplicon band was seen on an agarose gel. The PCR amplicon was purified to get rid of contaminants. A forward and reverse DNA sequencing reaction of the PCR amplicon was then performed using the BDT v3.1 Cycle sequencing kit and an ABI 3730xl Genetic Analyzer. The 18S rRNA gene consensus sequence was created from forward and reverse sequence data using aligner software. Subsequently used the 18S rRNA gene sequence to perform BLAST searches against the "nr" database of the NCBI GenBank database. The first 10 sequences were selected and aligned using the multiple alignment programme Clustal W based on the maximum identity score. MEGA 10 was used to generate the distance matrix and phylogenetic tree (Kimura 1980; Kumar *et al.*, 2018).



Microbial identification using 18S rRNA gene based molecular method

Fig. 1. Systematic process design of soaked and unsoaked rice and black gram.

RESULT AND DISCUSSION

Identification of microbes in rice and black gram soaked water. The rice and black gram soaked and unsoaked 4 water samples produced more than 234 yeast and mould colonies. From these colonies, the most unique 4 colonies were chosen and obtained in pure culture from numerous subcultures. From those 2 isolated pure colonies, DNA was extracted and submitted for sequencing, which was provided and conducted by Genurem lab, Tiruchirapalli. A single distinct 1050 bp PCR (Polymerase Chain Reaction) amplicon band was seen as shown in Fig. 2 when the pure culture made from sample of unsoaked and soaked rice and black gram de-ionized water was run on an agarose gel. By using the 18S rRNA technique and the Mi Seq amplicon sequence of yeast strains, the soaked and unsoaked water of rice and black gram were thoroughly investigated. The gene sequence was utilised to perform BLAST searches against the NCBI GenBank database's "nr" database. The first 10 sequences were chosen and aligned using the multiple alignment application Clustal W based on the maximum identity score. Using MEGA, a distance matrix and phylogenetic tree were created.

Based on nucleotide homology and phylogenetic analysis, it was determined that both Samples of black gram which was labelled as sample B and C were *Rhodotorula mucilaginosa*. These samples shared a high degree of similarity as shown in Table 1 and 2. Similarly, Banjara et al. (2015) pure cultures the yeast *Debaryomyces hansenii* were obtained and identified by DNA sequence of the ITS region, as well as growth characteristics and colony morphology.

Maximum Likelihood analysis of evolutionary data. Using the Tamura-Nei model and the Maximum Likelihood method, the evolutionary history was inferred. The tree with the highest log probability for samples B and C (soaked and unsoaked black gram) is shown as -2761.99 and -1577.20, respectively (Fig. 3). The initial trees for the heuristic search were produced automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances measured using the Maximum Composite Likelihood (MCL) method, and then selecting the topology with the best log likelihood value. The branch lengths are shown as substitutions per site, and the tree is shown to scale. In this experiment, 11 nucleotide sequences were used. 1st+2nd+3rd+Noncoding codon locations were included. The final dataset contained for sample B and C are 1188 and 1127 locations altogether respectively. MEGA X was used to conduct evolutionary analysis (Kimura, 1980; Kumar *et al*., 2018).

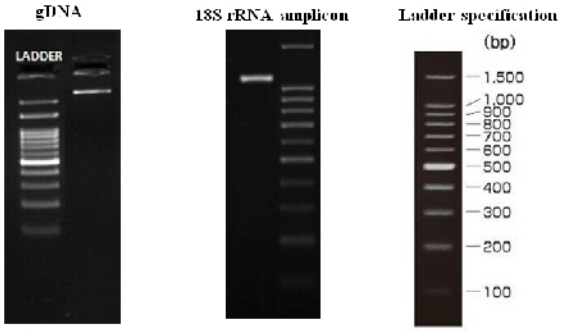


Fig. 2. gDNA and 18S Amplicon QC data of the both sample soaked (B) and unsoaked (C) black gram on an agarose gel.

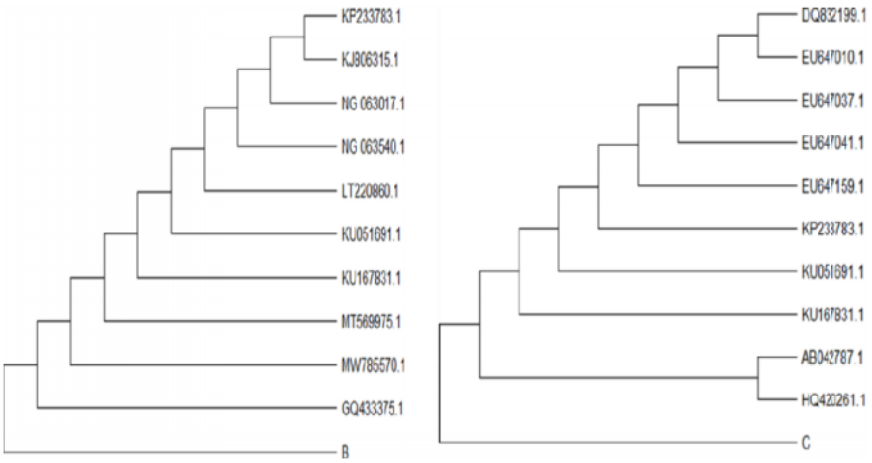


Fig. 3. Phylogenetic Tree: Evolutionary analysis by Maximum Likelihood method of sample B and C.

Sequences producing significant alignments:

Table 1: Sequences that produce significant alignments.

Description	Max Point	Total Point	Query Covered	E value	Per. Ident	Accession
Rhodotorula mucilaginosa strain LBMH1012	1884	1884	100%	0	98.87%	MW785570.1
Rhodotorula sp. strain EXF-10854, JG1b	1882	1882	99%	0	98.87%	MT569975.1
Rhodotorula mucilaginosa isolate B3	1882	1882	99%	0	98.87%	KU167831.1
Rhodotorula mucilaginosa strain JGTA-R1	1882	1882	99%	0	98.87%	KU051691.1
Rhodotorula mucilaginosa	1882	1882	99%	0	98.87%	LT220860.1
Rhodotorula alborubescens JCM 5352	1882	1882	99%	0	98.87%	NG_063540.1
Rhodotorula evergladensis CBS 10880	1882	1882	99%	0	98.87%	NG_063017.1
Rhodotorula mucilaginosa	1882	1882	99%	0	98.87%	KP233783.1
Rhodotorula mucilaginosa isolate H-4	1882	1882	99%	0	98.87%	KJ806315.1

Table 2: Sequences that produce significant alignments.

Description	Max point	Total point	Query Covered	E value	Per. Ident	Accession
Rhodotorula mucilaginosa isolate B3	2047	2047	99%	0	99.64%	KU167831.1
Rhodotorula mucilaginosa strain JGTA-R1	2047	2047	99%	0	99.64%	KU051691.1
Rhodotorula mucilaginosa	2047	2047	99%	0	99.64%	KP233783.1
Uncultured *Pucciniomycotina* clone D0810_53_M	2047	2047	99%	0	99.64%	EU647159.1
Uncultured *Pucciniomycotina* clone D0735_39_M	2047	2047	99%	0	99.64%	EU647041.1
Uncultured *Pucciniomycotina* clone D0735_35_M	2047	2047	99%	0	99.64%	EU647037.1
Rhodotorula mucilaginosa AFTOL-ID 1548	2047	2047	99%	0	99.64%	DQ832199.1
Uncultured *Pucciniomycotina* clone D0735_02_M	2045	2045	99%	0	99.64%	EU647010.1
Rhodotorula mucilaginosa gene	2045	2045	99%	0	99.64%	AB042787.1

Distance matrix. It shows the number of base substitutions per site from various sequences. The standard error estimate(s) shown above the diagonal were generated using the bootstrap method (500 repetitions). Analysis was conducted using the Maximum Composite Likelihood model (Kimura, 1980). In this experiment, 11 nucleotide sequences were used. Codon positions 1st+2nd+3rd+Noncoding were included. For each sequence pair, all unclear places were eliminated (pairwise deletion option) (Kumar *et al.*, 2018).

Table 3: Distance Matrix: Estimation of Evolutionary Divergence between Sequences.

C		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002
KU167831.1	0.001		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
KU051691.1	0.001	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
KP233783.1	0.001	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.001
EU647159.1	0.001	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.001
EU647041.1	0.001	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.001
EU647037.1	0.001	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.001
DQ832199.1	0.001	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.001
EU647010.1	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.001
AB042787.1	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.001
HQ420261.1	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

CONCLUSION

Findings of this study show that soaked and unsoaked rice and black gram submerged in sterile de-ionized water on the potato dextrose agar (PDA) media; there were more than 234 yeast and mould different colonies shown. From these colonies, the most identicle 4 colonies and from those 2 colonies were used in PCR amplification. A single species 1050 bp PCR amplicon band was visible on an agarose gel. The 18S rRNA gene's forward and reverse sequences were synthesized using the NS1 and NS4 primers to constructed the consensus sequence. However, *Rhodotorula mucilaginosa*'s presence was validated by sequences that produced substantial alignments, a distance matrix, and a phylogenetic tree. Moreover, morphological and pigment also confirmed the availability of the

Rhodotorula mucilaginosa in different water of black gram. Therefore, all the results of colour, characteristics, gram staining, and PCR amplification confirmed the existence of *Rhodotorula mucilaginosa* in both soaked and unsoaked black gram.

FUTURE SCOPE

Probiotic microorganisms have a high likelihood of becoming available through identification, isolation, and characterization in the soaked and unsoaked water of rice and black gram. *Rhodotorula mucilaginosa* and other probiotic microbes may be present in deionized water of soaked and unsoaked rice and black gram. As a result, the use of PCR for the isolation, characterization, and identification of microorganisms has increased potential.

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