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Research Article

"Advanced Bimolecular Approaches for Yeast Identification and Molecular Profiling Across Fruit Samples"

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Abstract

Yeasts are an integral part of the microbiome that colonizes fruit surfaces and is involved in spontaneous fermentations and food spoilage, yet there is still little knowledge on their diversity and molecular characteristics in many local fruit systems. We have attempted to isolate, characterise and molecularly identify indigenous yeast strains from grape samples by using a combination of some fundamental microbiological and basic biomolecular techniques. Aseptic collection of grape samples followed by light surface-sterilization, homogenized and plating on Yeast Peptone Dextrose Agar (YPDA). Colony picking, purification, and YPD broth growth: Distinct colonies were selected and cultured on Sabouraud Dextrose Agar by YPD broth (Yeast Peptone Dextrose), followed by microscopy with a stain of Lactophenol Cotton Blue. Genomic DNA was extracted from pure cultures with the Phenol:Chloroform:Isoamyl alcohol (PCI) method and checked on agarose gels. The internal transcribed spacer (ITS) region of rDNA was amplified by PCR as a marker for yeast identification. YPDA supported good and quite selective yeast growth, with morphologically diverse yeast-like colonies. Microscopy confirmed typical unicellular, budding yeast morphology, in line with Saccharomyces and some non-Saccharomyces genera. High-quality genomic DNA was obtained, and PCR produced clear ITS bands of about 500–700 bp, which were suitable for Sanger sequencing. Analysis of the sequencing chromatograms indicated that at least some isolates clustered with Saccharomyces cerevisiae, suggesting that common wine-related strains are present on the grape surface and can be targeted for more detailed future research. Overall, this study sets up a practical workflow for isolating and molecularly characterizing native fruit yeasts and gives a basic platform for later species-level identification, biodiversity studies and possible biotechnological applications of these indigenous strains.

Keywords:
Yeast, Grapes, ITS Region, PCI DNA Extraction, YPDA, Molecular Identification, Fruit Microbiota

1.Introduction

Yeasts are eukaryotic, unicellular fungi that inhabit a broad range of

environments, including soil, water, air, plants and fruit surfaces. Fruits in particular provide a nutrient-rich micro-habitat, supporting yeasts involved in natural spoilage, spontaneous fermentations and complex ecological interactions (Grangeteau et al., 2016). Fruit-associated yeasts thus represent both a potentialresourcefor fermentations and biocontroland a challenge in terms of food quality and safety.

Nutritionally, yeasts are less demanding microorganisms than most bacteria. Ripe fruits provide simple fermentable sugars, amino acids and vitamins and trace minerals that promote their growth [2]. Morphologically, most yeasts are rounded to oval/ellipsoidal in shape and reproduce through budding, though some species reproduce by fission and a few take the pseudohyphal form. The classical identification methods are based on colony morphology, microscopic observation, sugar fermentation and assimilation tests (Walker & Stewart, 2016). These phenotypic approaches, however, are often laborious and may not give species-level resolution. Saccharomyces cerevisiae is the most studied yeast species, commonly employed for wine, beer and bread fermentation as well as a major model organism in molecular biology. The genome of S. cerevisiae was the first completely sequenced eukaryotic genome (Goffeau et al., 1996) and has been found to have high functional conservation with man (Botstein & Fink, 1988; Kataoka et al., 1985). The availability of a rich molecular toolbox for S. cerevisiae has allowed metabolic engineering efforts resulting in biofuels, pharmaceuticals and other value-added products (Da Silva &Srikrishnan 2012).

Nevertheless, non-Saccharomyces yeasts are now recognized as important contributors to aroma, flavor and complexity in wine and other fermented foods (Ciani&Maccarelli, 1997; Cordero-Bueso et al., 2013; Canonico et al., 2016). They often dominate early fermentation stages and can produce distinctive volatile profiles. Killer toxins, such as K1, K2 and K28 in S. cerevisiae, further influence yeast–yeast interactions and community structure (Dabhole&Joishy, 2005; Becker & Schmitt, 2017).Molecular methods, particularly PCR-based techniques targeting the internal transcribed spacer (ITS) or D1/D2 domains of the rDNA, have transformed yeast taxonomy and ecology, enabling rapid, accurate identification directly from cultures or environmental samples (Esteve-Zarzoso et al., 2000; Baffi et al., 2011; White et al., 1990). ITS-RFLP, sequencing, qPCR and DGGE have also been used to map yeast biodiversity and population dynamics on grape skins and during spontaneous fermentations in vineyards and wineries worldwide (Sabate et al., 2002; Renouf et al., 2005; González et al., 2007; Granchi et al., 1999; Zott et al., 2010).

Unlike over the world, local yeast biodiversity on fruits and in traditional fermentations is still poorly documented in India but some works have been undertaken continue with rice wine starters (Hamei) from Manipur showed an extraordinary diversity of indigenous yeast communities (Jeyaram et al., 2008). The systematic documentation of this microbial diversity is critically needed by applying similar standardized biomolecular protocols as those used on isolates from Indian fruit systems,

which would also highlight strains with desirable technological or biocontrol traits. This study was therefore aimed at isolation and molecular characterization of indigenous wine yeast from fruit samples – grape in particular. We developed a reliable, multi-step workflow for further species level identification as well as comparative studies based on YPDA as a selective medium, analysis by LPCB microscopy and PCI-based DNA extraction combined with the amplification of the ITS-region.

2. Materials and Methods

2.1 Sample collection and preparation

Aseptic sampling of fresh fruits (mostly grape) was performed from local markets and orchards in Bhopal (Madhya Pradesh, India). Each fruit was carefully removed using sterile nitrile gloves with a sterilised (autoclaved) plastic knife and placed into pre-sterilised (autoclaved) sterile polyethylene bags (Poison Food 1.0 L Seal Zip Bags). Data were collected, including metadata (fruit type, location, date, and code). Samples were held in insulated containers transport to the laboratory and processed within 24 h at ~4°C.

The fruits were first washed with sterile distilled water to eliminate loosely adherent debris in a laminar flow cabinet. Then, we performed surface sterilization of fruits in 70% ethanol for 30 min then rinsed three times with sterile distilled water. The same were crushed with a sterile mortar and pestle (sterilized by wiping with 70% ethanol, air-dried) to form a single diffusion uniform homogenate. Fruit pulp was filtered through sterile Whatman No. 1 paper and the obtained filtrate (the fruit extract) was collected in sterile labelled centrifuge tubes and either placed on ice or at 4 °C until plating (Kallithraka et al., 1995).

2.2 Preparation of YPDA medium and yeast isolation

Yeast Peptone Dextrose Agar (YPDA) was prepared as follows: yeast extract 10 g, peptone 20 g, dextrose 20 g, agar 15–20 g in distilled water to 1 L. The mixture was dissolved, pH was adjusted to 6.5 ± 0.2, and the medium was autoclaved at 121 °C for 15–20 min at 15 psi. After cooling to ~45–50 °C, ~25 mL was poured into sterile Petri plates under aseptic conditions and allowed to solidify.

Serial dilutions (10^{-1}–10^{-5}) of the fruit extract were prepared in sterile distilled water. From the 10^{-5} dilution, 0.1 mL was spread evenly on YPDA plates using a sterile L-shaped spreader. Plates were incubated inverted at 28–30 °C for 48–72 h. Plates with 30–300 colonies were selected for counting and isolation.

Distinct yeast-like colonies (smooth, creamy, opaque, slightly raised) were selected and purified by quadrant streaking on fresh YPDA plates. Plates were incubated at 30 °C for 24–48 h to obtain morphologically uniform colonies regarded as pure cultures.

2.3 Microscopic examination using Lactophenol Cotton Blue

Morphology of the isolates was examined with Lactophenol Cotton Blue (LPCB) staining (Leck, 1999). A small portion of a colony was picked with a sterile loop and mixed in a drop of LPCB on a clean glass slide. A coverslip was gently placed to avoid air bubbles. Slides were observed under a light microscope at 40× and 100× magnification for cell shape, size, budding patterns and

presence/absence of hyphae or pseudohyphae.

2.4 Yeast cultivation in YPD broth

For biomass generation, pure colonies were inoculated into YPD broth (yeast extract 10 g, peptone 20 g, dextrose 20 g in 1 L distilled water). The medium was autoclaved at 121 °C for 15 min. Inoculated flasks were incubated at 28–30 °C with shaking (150–180 rpm) overnight to reach late exponential phase.

2.5 Genomic DNA extraction (PCI method)

Genomic DNA was extracted from yeast cultures using a Phenol:Chloroform:Isoamyl alcohol (PCI) protocol adapted from standard methods (Sambrook& Russell, 2001; Ahmed et al., 2014). Briefly, 1–5 mL of overnight culture was centrifuged at 5000×g for 5 min. The pellet was resuspended in 500 µL lysis buffer (100 mMTris-HCl pH 8.0, 50 mMNaCl, 10 mM EDTA, 1% SDS) and incubated at 65 °C for 30 min with occasional mixing.

An equal volume of PCI (25:24:1) was added, vortexed 30 s and centrifuged at 12 000×g for 10 min. The aqueous phase was transferred to a new tube and optionally re-extracted with chloroform:isoamyl alcohol (24:1). RNA was removed by incubation with RNase A (10 µg/mL, 37 °C, 30 min). DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cold 100% ethanol, incubated at −20 °C for ≥1 h, and pelleted at 12 000×g for 15 min (4 °C). The pellet was washed with 70% ethanol, air-dried and resuspended in TE buffer (10 mMTris-HCl, 1 mM EDTA, pH 8.0).

2.6 Agarose gel electrophoresis

DNA quality was assessed on 1% agarose gels prepared in 1× TAE buffer. After dissolving agarose and cooling to ~60 °C, ethidium bromide (0.5 µg/mL) was added. Gels were cast, and 10 µL of DNA sample mixed with loading dye was loaded alongside a 1 kb DNA ladder. Electrophoresis was performed at 80 V for ~1 h. Bands were visualized under UV illumination and photographed (Voytas, 2000; Straube et al., 2021).

2.7 PCR amplification of ITS region

The internal transcribed spacer (ITS) region of rDNA was amplified as a universal fungal barcode (White et al., 1990; Baffi et al., 2011). Each 20 µL PCR reaction contained: 2 µL 10× buffer, 0.5 µL $MgCl_2$, 0.5 µL dNTP mix, 0.5 µL forward primer, 0.5 µL reverse primer, 0.2 µL Taq polymerase, 1 µL template DNA and 14.8 µL nuclease-free water. Typical cycling conditions were: initial denaturation at 95 °C (3 min); 30–35 cycles of 95 °C (30 s), 50–60 °C (30 s), 72 °C (45–60 s); and final extension at 72 °C (5 min). PCR products were resolved on 1.5% agarose gels with a 1 kb ladder.

3. Results and discussion

After 3–4 days of incubation on YPDA, the plates inoculated with grape dilutions showed heavy microbial growth and many colonies with typical yeast-like appearance. Most of them were smooth, creamy to opaque and slightly raised, with colours ranging from white and cream to a bit pinkish or pale yellow, which suggests the presence of more than one yeast type. Because YPDA is nutritionally rich and only mildly selective, it supported dense yeast growth but still kept bacterial contamination quite low, so it was possible to pick single, well separated colonies. Under the microscope, with lacto phenol cotton blue staining, the selected colonies showed mainly oval

to round cells occurring singly or in small groups. Active budding was clearly visible, and some cells had several bud scars, which points to strong asexual reproduction. No true hyphae and almost no pseudohyphae were seen, so these isolates are probably non filamentous yeasts, for example Saccharomyces or non filamentous Candida like species. Sub culturing of these single colonies onto fresh YPDA plates gave uniform, round, smooth edged colonies within 24–48 hours at 30 °C, and no obvious bacterial or mould contamination was observed, meaning that reasonably pure yeast cultures were obtained for further work.

The quality and quantity of the genomic DNA extracted from these pure cultures using the PCI method were sufficient for PCR applications. The DNA had negligible smearing which indicates low degradation, and no strong RNA smear suggesting that the RNase treatment was effective as it appeared like clear high-molecular weight bands on agarose gel. All of the 162 isolates tested in this study yielded single, discrete bands (approx. It should also be noted that the gels did not show significant non-specific products or any other strong primer dimers, which suggests both template DNA quality and the PCR conditions are relatively well optimised and these amplicons are ready for sequencing. Collectively, these findings demonstrate that: (i) the pipeline from isolating colonies to establish pure cultures followed by extraction, and amplification of ITS is working robustly with grape associated yeasts.

This small study indicates that the surfaces of cleaved grape berries are a rich and readily available source of indigenous yeasts and that simple culture based methods remain very useful for their isolation and preliminary characterization. Surface rinsing followed by mild sterilization and grinding was likely used to remove some epiphytic contaminants while permitting recovery of a large portion of more firmly adhered or entophytic yeasts, but it may also impose some bias on community composition. Most isolates are unicellular, budding yeasts as confirmed by microscopy but morphology alone is not sufficient to differentiate closely related or cryptic species thus molecular tools such as ITS barcoding were applied. The PCI extraction protocol, although cumbersome and based on toxic reagents still represents a trustworthy and low-cost method to obtain high-quality DNA in areas where commercial kits are unavailable. The ITS sequences of these isolates can then be used for identification and biodiversity study in comparison to other studies on wine and fruit microbiota, thus achieving a higher resolution level. Further, the same methodological framework can be broadened using improved techniques (e.g. ITS-RFLP or high throughput amplicon sequencing) and applied to bigger sample sizes and diverse fruits in order to identify strains of interest for their fermentation quality, stress tolerance or biocontrol potential.

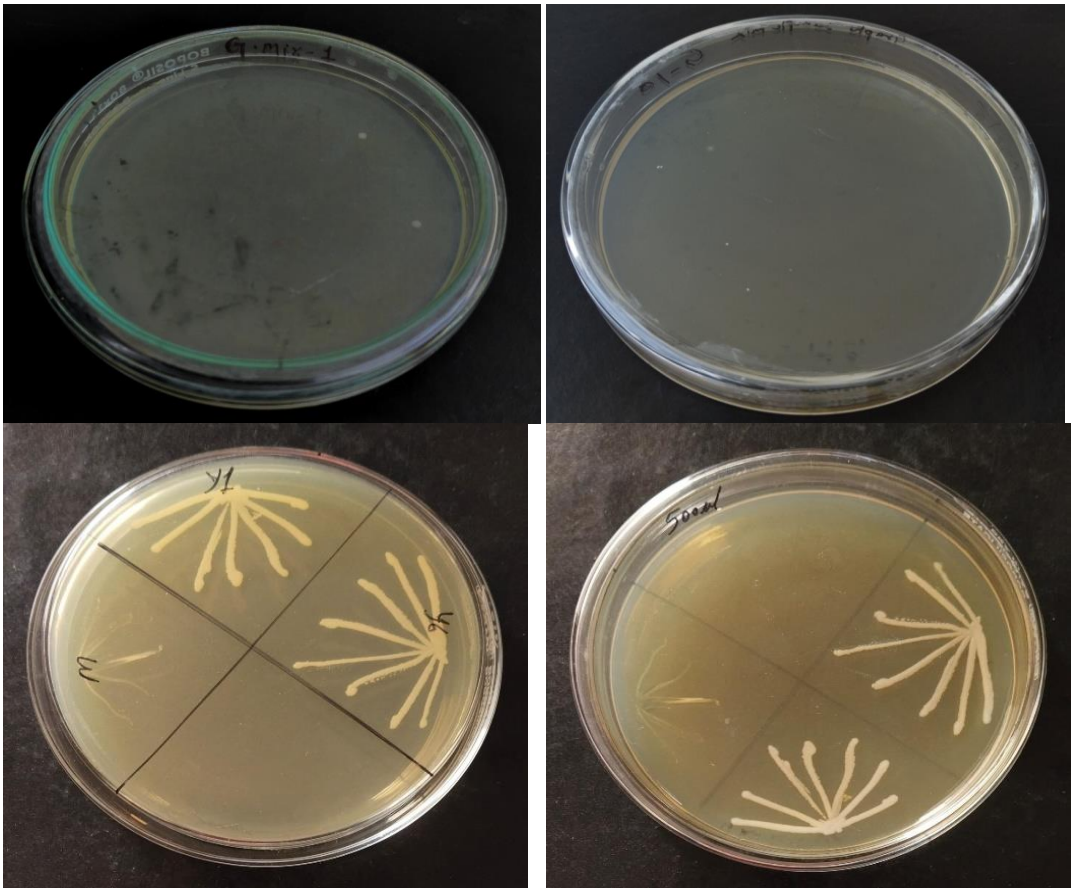

Figure 4.1: Photomicrograph showing the mixed and pure cultures (of yeast species isolated from environmental samples.

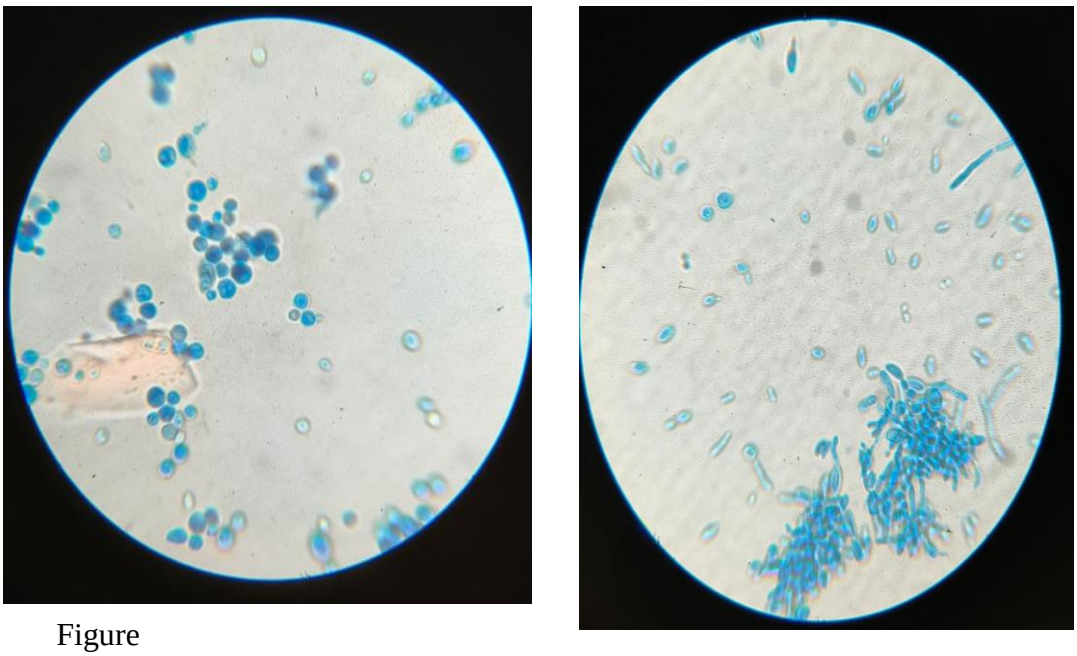

Figure 4.2:Lactophenol Cotton Blue Staining of Yeast Species

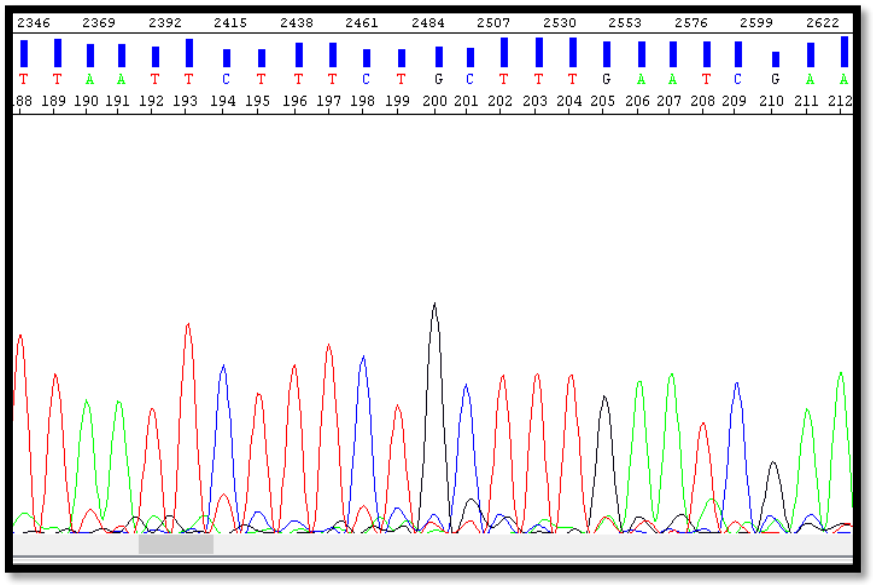

Figure 4.3 :
Present Figure Showing the Electropherogram of Saccharomyces cerevisiae

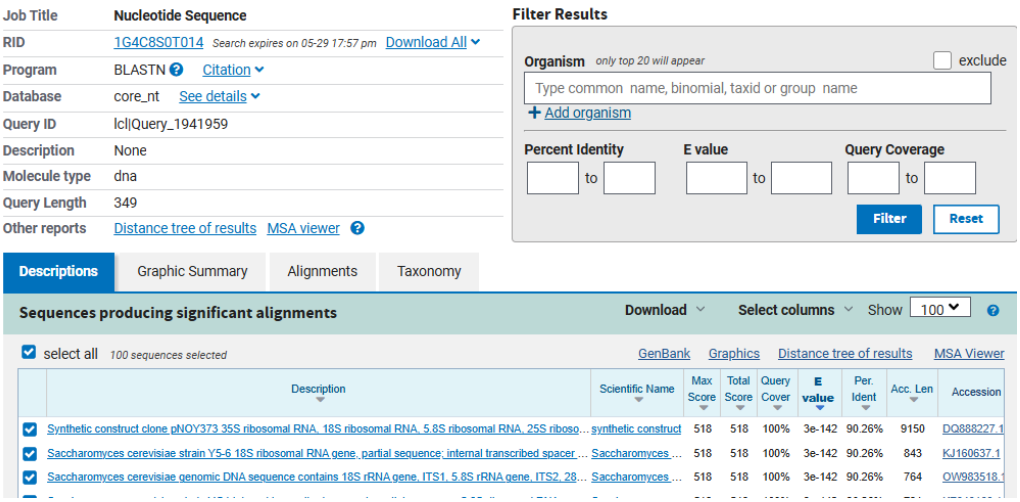

Figure 4.4: Present Figure Showing Saccharomyces cerevisiae species after the NCBI blast.

5.Conclusion: this study demonstrates that simple culture based and molecular methods can successfully recover and preliminarily identify indigenous yeasts from grape surfaces. The established workflow, from YPDA isolation through PCI DNA extraction and ITS based PCR/Sanger sequencing, yielded high quality data and revealed the presence of Saccharomyces cerevisiae among the isolates. This pipeline now provides a solid foundation for expanded species level identification, biodiversity assessment and screening of strains for future biotechnological use

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