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Full Length Research Paper

Isolation and random amplified polymorphic DNA (RAPD) analysis of wild yeast species from 17 different fruits

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The purpose of this study was to isolate the wild yeast strains present on different fruits and performing random amplified polymorphic DNA (RAPD) analysis to know the genetic inter relationship between different isolated species. Seventeen different fruit namely mango, apple, banana, black grapes, sapotae, orange, plum, jamun, pear, cherry, dates, pomegranate, figs, papaya, pineapple, green grapes and raisins were used as natural sources for yeast isolation. Amplicon fingerprints for the isolated species were obtained by RAPD assay using five different primers. Among them, only three primers allowed discrimination among the 17 isolated species. Jaccard's genetic similarity coefficient varied from 0.00 to 0.98 (as isolate S9 did not show any relatedness with the other clads).

Key words: Random amplified polymorphic DNA, polymerase chain reaction, amplicon, dendrogram, wild yeast strains.

INTRODUCTION

Yeast is a unicellular eukaryotic fungus, very common in the environment and is mostly saprophytic. It has been classified as ascomycetes or basidomycetes under fungi taxonomy Kreger-van et al. (1984) and there are about 1500 species of yeast Kurtzman et al. (2006), Barnett et al. (1990) . The commercial importance of strains of yeast species *Saccharomyces cerevisiae* has made it a model organism of study on both research and industrial importance (Legras et al., 2007) . Fermenting wild yeast species are being isolated from the natural sources for over decades and is being used in various fermentation processes. Yeast has been isolated from variety of natural sources like leaves, flowers, fruits etc (Spencer and Spencer, 1997; Davenport et al., 1980; Tourna, 2005; Li et al., 2008). Being a sugar -loving micro-organism, it is usually isolated from sugar rich materials. Fruits contain high sugar concentration so yeast species are naturally present on them and can be easily isolated from them. Distinct wild yeast species are supposed to be present and associated with different fruits in natural environments (Spencer et al., 1997). As because of yeast fermentative characteristic, there is always a need for yeast strains with better features of fermentation especially high ethanol tolerance for production of ethanol as biofuel on commercial scale (Colin et al., 2006). Moreover, besides *S. cerevisiae*, there is always a search on for new wild/non toxic-fermentative yeast species for their further industrial exploitation in fermentation industry, baking industry, therapeutic production etc (Legras et al., 2007). Traditional methods like morphological, physiological and biochemical studies used for taxonomic identification of yeast isolates (Kurtzman et al., 2006; Barnett et al., 1990; Rosa et al., 2006). But being laborious and time consuming techniques, they are not appropriate for routine identification (Couto et al., 1994). Molecular biology based methods have been developed and can be applied to the field of yeast taxonomy in routine identification works (Quesada and Cenis.1995; Loureiro Malfeito-Ferreira., 2003). One such method is a variant of the polymerase chain reaction (PCR) technique based on the amplification of

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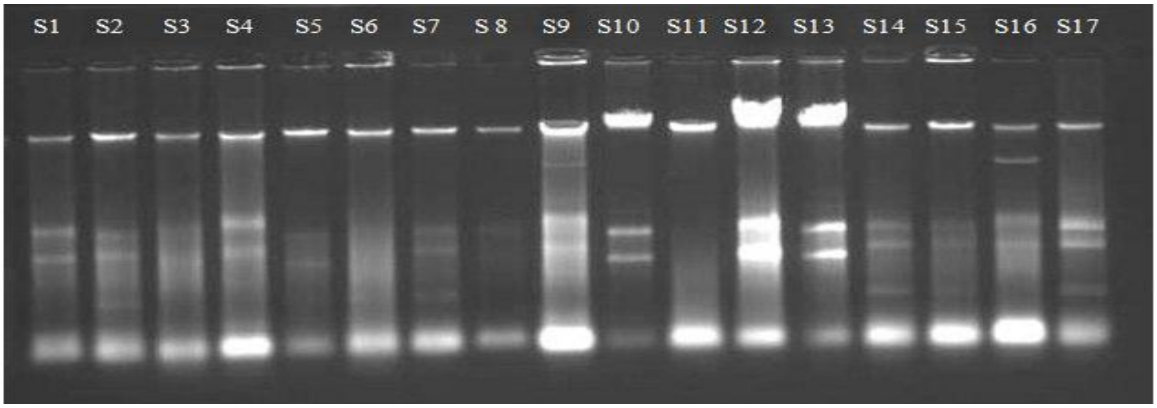

Figure 1. DNAquality testing of different yeast isolates.

random fragments of DNA (RAPD) (Xufre et al., 2000).

This technique utilizes short (5–15 mer) oligonucleotide primers of arbitrary sequence at low annealing temperature that hybridize at the loci distributed at random throughout the genome, allowing the amplification of polymorphic DNA fragments (Quesada et al., 1995).

MATERIALS AND METHODS

All chemicals used in the experiment were of highest purity and were obtained from Sigma Chemical Company (St. Louis, MO), Merck limited (Mumbai, India) and Hi Media (Mumbai, India).

Isolation and medium

Fruit samples were obtained from local sources of Himachal Pradesh and mostly naturally decaying/fermented samples were preferred. 100 g of each fruit sample was taken in a sterile mortar and crushed to a fine paste by mixing with sterile water. Then mixture was kept for overnight at normal room temperature so that natural wild yeast present on fruit samples might grow and develop to enrichment culture. A loopful of liquid portion from each sample was streaked (Quaternary streaking) in plate (with replica) containing MYPG medium (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, glucose 1% and agar 3%), pH 6.4 (phosphate buffer system) and incubated at 26°C for 2 days.

DNA isolation

For isolation of chromosomal DNA from the isolated yeast species, single isolated colony was selected from each plate and grown in test tube with 20 ml of MYPG broth (pH=6.4) at 26°C for 24 h. DNA was isolated from 24 h old broth culture with following materials: Yeast lysis buffer (pH-8.0), TE buffer (pH-8.0), zymolyase enzyme, phenol-chloroform-isopropyl alcohol solution and ethanol (100%). DNA pellet suspended in TE buffer are stored at 4°C for further PCR amplification (Rosa et al., 2006).

PCR amplification and gel run

The PCR reaction mixture (15 l) contains 2 l (~40 to 60 ng) sample DNA, 1X PCR Buffer (10 mM) pH: 8.0, MgCl2 (3mM), dNTP mix (0.2 mM), Primer 1 (0.5 M) and Taq DNA Polymerase (0.5 units, Intron technologie, USA).

Amplification of isolated DNA was done using random primers namely RAPD primer-OPA12, RAPD primer-OPB09, RAPD primer-OPC06, with PCR conditions: Initial denaturation at 94°C for 4 min, 39 cycles of: 45 s at 94°C, 45 s at 35°C, 1 min 30 s at 72°C and followed by final extension at 72°C for 5 min (Bio-Rad-My Cycler Thermal Cycler, USA). Gel electrophoresis (2% agarose) of amplified DNA done under standard electrophoresis procedure.

RAPD analysis

Arbitrary primers were obtained from OPC (Operon Technologies, California, USA). The DNA fragments were visualized with ethidium bromide, photographed and analyzed. A 100-bp DNA ladder (Pharmacia Biotech, Uppsala) used as the size standard. The binary data generated here were used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands. The polymorphism information content (PIC) was calculated by the formula: PIC = 2 Pi (1-Pi) where, Pi is the frequency of occurrence of polymorphic bands in different primers. Pair-wise similarity matrices were generated by Jaccard's coefficient of similarity (Jaccard, 1908) by using the SIMQUAL format of NTSYS-pc (Rohlf, 2002). Jaccard coffiecient was calculated by converity data into binary form. 1 for presence of band and 0 for absence. Matrix was generated for calculating similarity or dissimilarity between yeast strains. A dendrogram constructed by using the unweighted pair group method with arithmetic average (UPGMA) with the SAHN module of NTSYS-pc to show a phenetic representation of genetic relationships as revealed by the similarity coefficient (Sneath and Sokal, 1973).

RESULTS AND DISCUSSION

17 yeast species were isolated, purified and morphological as well as genetic diversity analysis using RAPD method were successfully carried out. DNA quality testing is illustrated in Figures 1 - 5. Yeast specific defined media used was able to inhibit the growth of bacterial population in all the cultures. In some fungal contamination, fungus growth showed after 3 days of incubation of primary cultures and pure cultures were easily made before fungal

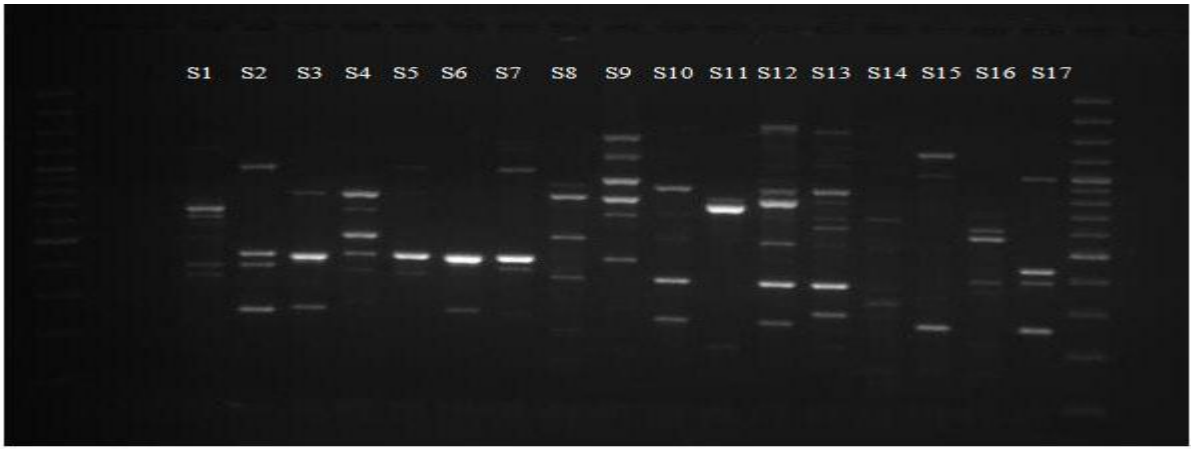

Figure 2. Amplification of genomic DNA of various yeast isolates by PCR using RAPD primer.. OPA12.

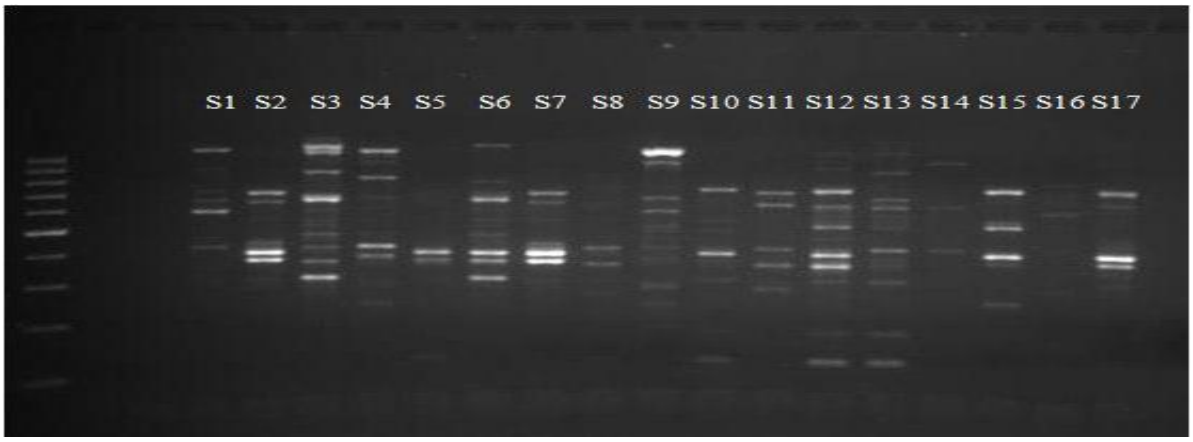

Figure 3. Amplification of genomic DNA of various yeast isolates by PCR using RAPD primer.. OPB02.

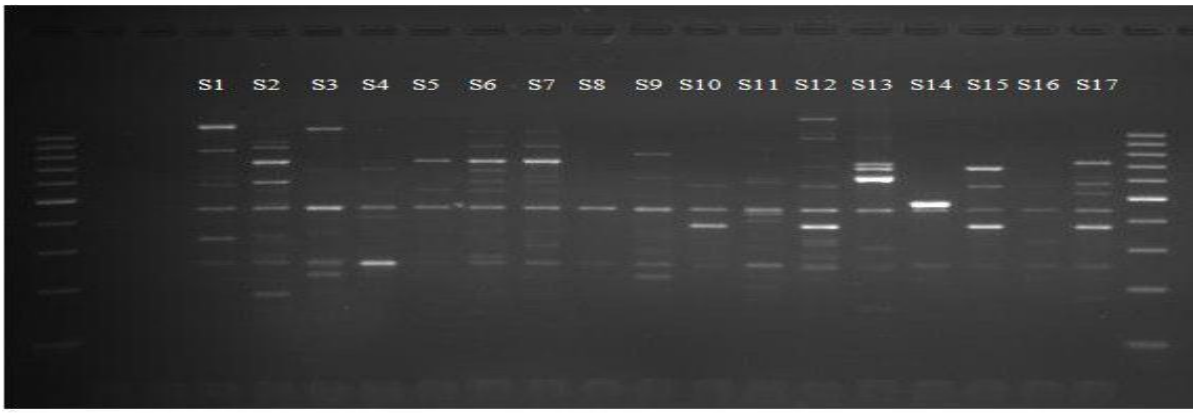

Figure 4. Amplification of genomic DNA of various yeast isolates by PCR using RAPD primer.. OPC06.

Growth became dominant. Studies related to determination of type of fungal contamination were not made as pure cultures were easily made by following the enrichment culturing. Although, in the primary cultures with inoculums from banana, sapota, orange, mango, pineapple and pear fruits more than single type of yeast

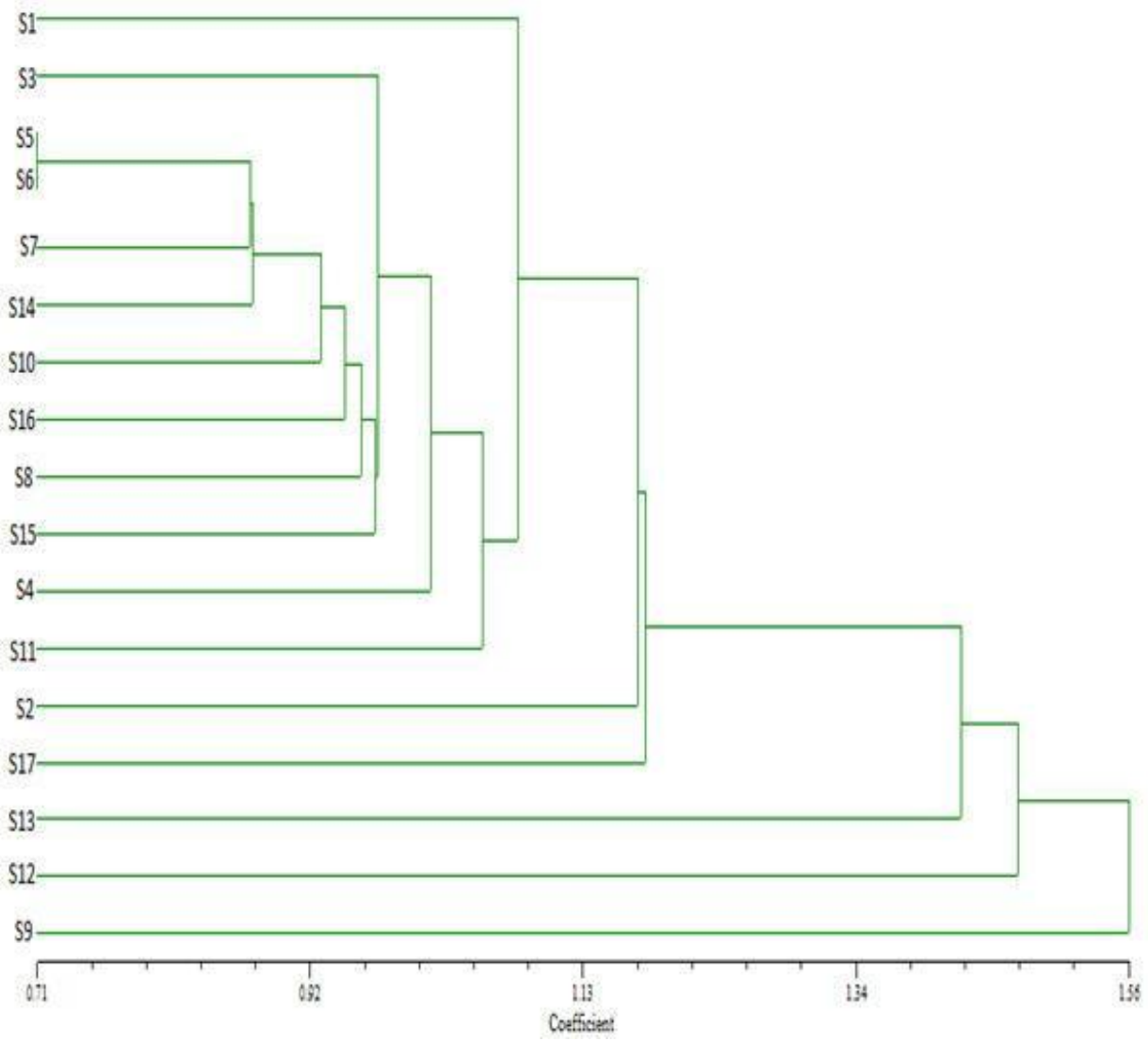

Figure 5. Dendrogram depicting genetic diversity in isolated yeast. The data is on RAPD profiles generated by three primers(OPA-12, OPB-09 and OPC-06).

culture in form of mixed culture existed. From such mixed cultures distinct single isolated colony were picked based on maximum growth among the whole culture and pure cultures of those selected colonies were made and maintained. Morphological data based on different parameters of colony characteristics are tabulated in Table 1. Yeast isolated from fruits namely dates and figs shared some common morphological characteristics. Identical colony characteristics were observed among yeast colonies from green grapes, raisins and black grapes. Yeast isolated from banana, sapota, pear and mango shared some of the common morphological characteristics with maximum growth during incubation period as compared to other isolated species. Few viable isolated colonies were obtained from Papaya under the similar incubation and growth media conditions. Fungal contamination was also observed and mostly in plates with inoculums from banana, mango, jamun and orange.

Jaccard's genetic similarity coefficient varied from 0.00 to the highest genetic similarity coefficient (0.98) observed between S5 and S7 that is yeast isolated from green grapes and black grapes as sources. UPGMA cluster analysis of the Jaccard's similarity coefficient generated a dendrogram that illustrated the overall genetic relationship among the genotypes surveyed. Clusters are formed on the basis of distance. The dendrogram clearly showing the sufficient distance to form apart clusters. Cluster analysis indicated four distinct clusters. Cluster1 includes S1, S3, S5, S6, S7, S14, S10, S16, S8, S15, S4 and S11. Cluster 2 includes S2 and S17, Cluster 3 with S13 and S12, only S9 in Cluster 4. The difference between the 1st and 2nd cluster is lesser than the 3rd and 4th,

Table 1. Yeast Isolate code, corresponding fruit source and colony morphological data of different yeast isolated from 17 fruit sources.

Isolate name	Corresponding fruit source		Colony characteristics					
	Common Indian name	**Scientific/ botanical name**	**Color**	**Shape**	**Surface**	**Elevation**	**Edge**	**Consistency**
S1	Orange	*Citrus sinensis*	Cream	Circular	Dry , Rough	Convex	Serrate	Dull, matte
S2	Date	*Phoenix dactylifera*	Cream	Oval	Glistening	Flat	Curled	Mucoid
S3	Apple	*Malus pumila*	White	Circular	Wrinkled	Bulged	Curled	Stringy
S4	Pear	*Pyrus communis*	Cream	Evenly circular	Shiny	Raised	Undulate	Butyrous
S5	Green Grapes	*Vitis vinifera*	Off-white	Circular	Smooth	Raised	Entire	Butyrous
S6	Raisins	*Vitis vinifera*	Off white	Circular	Smooth, glistening	Raised	Entire	Butyrous
S7	Black grapes	*Vitis vinifera*	Cream	Spheriodal	Smooth glistening	Raised	Entire	Butyrous
S8	Mango	*Mangifera indica*	Chalky white	Irregular	Rough	Convex	Undulate	Brittle
S9	Jamun (Black Plum)	*Eugenia jambolana or Syzygium cumini L*	White	Small circular	Smooth	Raised	Curled	Stringy
S10	Sapota / Zapote (Sapodilla)	*Casimiroa edulis*	Chalky white	Filamentous	Granular	Raised	Serrate	Dry
S11	Pineapple	*Ananas comosus*	Chalky white	Small circular	Mucoid	Raised	Entire	Butyrous
S12	Papaya	*Carica papaya*	Dull White	Spheriodal	Smooth	Umbonate	Entire	Butyrous
S13	Cherry	*Prunus avium*	Off white	Circular	Smooth	Raised	Undulate	Viscous
S14	Plum	*Prunus cultivar*	White	Circular	Smooth	Convex	Curled	Butyrous
S15	Pomegranate	*Punica granatum*	Cream	Evenly circular	Smooth glistening	Raised	Entire	Viscous
S16	Banana	*Musa acuminate*	Chalky white	Irregular	Wrinkled, dry	Umbonate	Undulate	Dry brittle
S17	Figs	*Ficus carica*	Cream	Spheriodal	Shiny	Pulvinate	Entire	Viscous

but its sufficient to form another clad As isolated yeasts were from different sources, high variability both at phenotypic (as seen in colony morphologies) and genotypic (from band pattern of Gel run of randomly amplified DNA generated by 3 primers) was observed. The polymorphism information content (PIC) calculated from the frequency of polymorphic bands in primer OPA12 was 0.63, in primer OPB09 was 0.49 and in primer OPC06 was 0.53. The greater genetic similarity coefficient between yeast isolates from green grapes, raisins and black grapes as well as identical morphological characteristics indicates association of same strains of particular yeast specie with these fruits. Likewise, it is also known that *S. cerevisiae* is naturally presented on grapes Spencer et al. (1995).

The basis of our experiment is the association of different yeast strains with the corresponding fruit and so they have distinct fermentative properties. Further analysis regarding characterization, fermentative properties, ethanol tolerance and subsequent selection of high ethanol yielding species of yeast from different fruits (Colin et al., 2006) were not being evaluated in the study.

Presence of yeast species on fruits can also be dependent on the geographical factors as well as the place from where the fruits are obtained (Rosa et al., 2006) and their further post harvesting treatment. In other manner, analysis variability in yeast species isolated from fruits that are of different geographical origins can provide a procedure to trace the origin in import- export products. Yeast also undergoes a number of changes in accordance with the environment. Isolation of yeast species from different fruit sources and further the RAPD assay is randomized so the present study cannot be compared with any previous work. But it is clearly proved in the dendrogram that yeast isolated from different sources are very much different from each other, being, may be of different species or of different strains.

Conclusion

Presence of genetic diversity using RAPD analysis as well as different observed colony

morphological characteristics in the yeast strains from different fruit sources, it purposes a methodology for easy and quick isolation of yeast strains for both research and industrial analysis.

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