

Isolation, Identification and Molecular Characterization of Different Yeast Sources by using SSR and ADH markers

Ningaraj H. Dalawai*, K.M. Harini Kumar, H.B. Manoj Kumar, K.N. Krupa, S. Raghavendra, V. Hampanna and K.L. Chethan

Department of Plant Biotechnology, UAS, GKVK, Bangalore - 560 065, India.

(Received: 24 September 2015; accepted: 26 October 2015)

Yeasts are unicellular fungi that reproduce by budding (asexual) and also fusion (sexual). *Saccharomyces cerevisiae* is budding yeast mainly used for ethanol production. A well-known property of yeast is that they are responsible for the conversion of fermentable sugars into alcohol and other by-products. Consistent yeast performance during the fermentation process requires both accurate cell counts plus assessment of cellular viability. The budding yeasts (true yeasts) are classified in the order Saccharomycetales. Yeasts are ubiquitous in the environment, but are most frequently isolated from sugar-rich samples of different sources. Some good examples include fruits and berries different ethanol concentrations from 0%-24%. Growth in different ethanol concentrations varied from one strain to another. Yeast strains showed tolerance level from 7-15%. Even though some strains had tolerance at 15 to 16% but the growth was less. Yeast from Sugarcane juice and grapes showed highest tolerance and pineapple showed least tolerance among 10 isolates Ten *Saccharomyces* strains were subjected to SSR and ADH specific primer analysis using eight primers. Cluster diagram was divided into 3 major clusters and 4 sub clusters, two major cluster showing 25% dissimilarity. There was correlation between ethanol tolerance and genetic relatedness shown by SSR and ADH specific primer analysis.

Key words: Yeast, Ethanol, SSR and ADH primers,

Yeast cells (*Saccharomyces cerevisiae*) are facultative anaerobes and under anaerobic conditions can ferment glucose to ethanol. *S. cerevisiae* is ideal for ethanol production due to several properties including fast growth rates, efficient glucose repression, efficient ethanol production and tolerance for environmental stresses, such as high ethanol concentration and low oxygen levels. Glucose is broken down to form pyruvate in most organisms via the glycolytic pathway and this pyruvate can result in the production of ethanol under anaerobic conditions. The energy for growth of cells during ethanol production is provided by the glycolytic and

fermentation pathways. The alcohol produced by yeast is the most valuable product for the biotechnology industry with respect to both value and revenue. Approximately 80% of ethanol is produced by anaerobic fermentation of various sugar sources by *Saccharomyces cerevisiae* (Zuko *et al.*, 2012). *Saccharomyces cerevisiae* has traditionally been used for alcoholic fermentation because of its ability to produce ethanol anaerobically in a low pH and a high osmolality environment with unparalleled productivity and efficient yields. *S. cerevisiae* is also able to ferment xylulose to ethanol since it possesses a xylulose kinase that is expressed in low amounts (Deng and Ho, 1990). Microsatellites or Simple Sequence Repeats (SSR) consist of direct tandem repeats of a short DNA motif, usually less than 10 bp (Charlesworth *et al.*, 1994). These repetitive sequences are a major component of higher

* To whom all correspondence should be addressed.
E-mail: manojmandya.17@gmail.com

organism DNAs. They are hyper variable in length (Tautz, 1989) as a result of DNA-replication errors, such as slipped strand mispairing (Strand *et al.*, 1993). Thus, microsatellites show a substantial level of polymorphism between individuals of the same species and are extensively used for paternity exclusion tests (Helminen *et al.*, 1988), forensic medicine (Hagelberg *et al.*, 1991) and for molecular typing of different organisms including cultivars of *Vitis vinifera* (Bowers *et al.*, 1999) and the pathogen yeast *Candida albicans* (Bretagne *et al.*, 1997). Field and Wills (1998) have conducted computer searches for short tandem repeat patterns on several completely sequenced small genomes, including yeast. They suggest that trinucleotide repeats could be used to genotype yeast strains.

MATERIAL AND METHODS

Isolation of Yeast strains

Preparation of Media

Yeast Extract Peptone Dextrose Agar (YEPDA) medium (Sambrook and Russell, 2001) was used for isolation of yeast strains. Medium for petriplates were prepared in 500ml conical flasks. All components were individually weighed and mixed and the pH was adjusted to 5.4 before addition of agar. Agar was melted prior to autoclaving. Medium was autoclaved together for 15 min. at 121°C and 15 psi.

Isolation of yeast from different sources

Yeasts are naturally associated with sugar rich environments. In the present study sugarcane juice were selected as sources for isolating yeast cells. Samples were collected from ZARS, Jaggeri Park, V. C. Farm, Mandya. Flower nectar were collected from botanical garden UAS GKVK, Bangalore Grape juice, Apple juice, Mosambi, Pomegranate, Pineapple, Watermelon, Muskmelon were also used as sources for isolation of yeast which were procured locally.

The sugarcane juice and other sources were collected in sterilized bottles and kept at room temperature. Fruit samples were washed and rinsed many times in distilled water to remove other contamination. They were then cut, squeezed and the juice was collected in separate sterile flasks. Samples of the juice were serially diluted and 0.1 ml of the diluted samples from 10^{-3} and 10^{-4} were

plated on YEPDA medium. The plates were incubated at 30°C for 48 h.

Identification of the yeast isolates

Yeast is a unicellular fungus. The precise classification can be made by using morphological characteristics of the cell, ascospore and colony. The physiological characteristics are also used to identify the species. One of the most well-known characteristics is the ability to ferment sugars for the production of ethanol. Budding yeasts are true fungi of the phylum Ascomycota, class Saccharomycetes (also called Hemiascomycetes). The true yeasts are separated into one main order Saccharomycetales. The isolated cultures were identified up to generic level through colony characters and cell morphological characters (mainly budding character).

Colony morphology

The colony type was used as a tool for preliminary identification. Each isolate was streaked aseptically on Petri plates containing YEPDA medium. Yeast isolates were tentatively identified as *Saccharomyces spp.*

Microscopic observation

The isolated yeast cultures from different samples were studied for their growth characteristics on YEPDA and cell morphology. Later simple staining technique was followed using crystal violet stain for microscopic observation using 24 h old culture. The stained cells were observed under microscope with under oil immersion. Yeast isolates showing oblong cell shape with budding character under microscopic field were purified and maintained on YEPDA slants. Microphotograph was used to study the cell characters and budding characters of yeast isolates and microphotographs were recorded. The isolates were given with specific names for further experimentation and easy recognition.

Maintenance of yeast isolates

The yeast isolates were maintained using glycerol stocks and the isolates were preserved at -20°C in the Department of Plant Biotechnology, UAS, Bengaluru, for further studies. A working stock of culture was prepared on the YEPDA slant for further analysis.

Procedure

1. Yeasts were inoculated into 10ml tubes containing 5ml portion of YEPD broth.
2. Incubated for 24 h at 30°C.

3. 10 µl portions were then inoculated into 10ml tubes containing 4ml portion of YEPD supplemented with 0, 5, 7, 9, 10, 12.5, 15, 16, 17 and 18% ethanol and incubated for 24 h at 30° C.
4. Growth expressed as generation time, was determined by measuring the optical density of cultures at 595 nm.
5. The initial optical density of each tube was read off on a spectrophotometer at 595 nm against the medium as blank.
6. The inoculated tubes were transferred to a shaker set at 30°C for 48 hours.
7. The increase in optical density in a tube was recorded as evidence for growth of the yeast.
8. The concentration of alcohol at which the yeast just inhibited was assessed for ethanol tolerance.

Molecular Characterization of Yeast strains using SSR and ADH specific primer

Totally ten *Saccharomyces spp.* isolated from different samples were used for SSR and ADH specific-PCR characterization.

Isolation of DNA

Yeast DNA was isolated by using protocol provided in Sambrook and Russell, (2001).

Procedure

Yeast cells, freshly grown overnight cultures on YEPD medium were used.

1. The yeast cells for lysis.
- a. 1.5 ml of overnight culture of yeast cells was transferred to a microfuge tube.
- b. Cells were pelleted by centrifuging at maximum speed for 1 minute at room temperature in a microfuge.
- c. Culture medium was removed by aspiration and the pellet was resuspended in 200µl of lysis buffer
2. Pellet was incubated in dry ice ethanol bath till it is frozen.
3. The frozen sample was kept in 95°C water bath for 1minute and repeated the process once.
4. The tubes were vortexed for 30 seconds and 200 µl of phenol: chloroform (1:1) was added.
5. The solution was mixed by vortexing for 2 minutes and then centrifuged at 12,000 rpm for 3 minutes.
6. The aqueous layer was transferred to a fresh microfuge tube containing 400 µl of ice cold absolute ethanol.
7. The microfuge tube was incubated at room temperature for 5 minutes and centrifuged at 12,000rpm for 5 minutes. The supernatant was vacuum aspirated using a micropipette.
8. DNA pellet was washed with 50 µl of 70%

ethanol and vacuum aspirated and air dried for 5 minutes at 60°C.

9. DNA was resuspended in 40 µl of TE Buffer.

Quantification of DNA

The specific amount of DNA was quantified by taking the spectrophotometer readings at a wavelength of 260 nm, which allows the calculation of nucleic acids in the sample. Double stranded DNA at 50 µg/ml in aqueous solution has an absorbance (OD) of 1.0 (Sambrook *et al.*, 2001). The procedure used for quantification of DNA is as follows.

1. Five µl of DNA sample was added to 995 µl of Millipore distilled water, mixed thoroughly and the absorbance (OD) at 260 nm and 280 nm was read using the spectrophotometer.

2. The concentration of DNA in the solution was calculated according to the following formula.

$$\text{DNA concentration. (}\mu\text{g/ml)} = [\text{OD}_{260} \times 50 \mu\text{g/ml}] / 5$$

The ratio between the absorbance readings at 260 and 280 nm ($\text{OD}_{260} / \text{OD}_{280}$) was used as an estimate of the purity of the DNA samples. Pure preparation of DNA has $\text{OD}_{260} / \text{OD}_{280}$ values ranging between 1.7 and 1.8 (Sambrook *et al.*, 2001). Computed OD values were used to dilute the DNA samples to working concentration.

The DNA degradation and contamination with other substance were checked by electrophoresis of an aliquot of sample in the agarose gel of 0.7%.

SSR and ADH-specific PCR reaction Reagents used in the PCR Mix (standard) 10 X Taq assay buffer A: 50mM KCl, 1.5 mM MgCl₂, 10 mM Tris.HCl pH 9.0, Gelatin 0.1 %, 0.5% Triton-X100 and 0.05% NP₄₀. 25 · g of genomic DNA was used as the template for the standardization of PCR reactions and the PCR conditions were optimized to produce the reproducible and fine fingerprints. PCR reactions were performed in a final volume of 20 µl containing 25·g of template DNA, 3 µl of 2mM dNTPs mix, 2.5µl of 10X Taq buffer, 1µl 1.0 unit of Taq DNA polymerase, 3 µl of 10 pico mole Primer.

Protocol

The following PCR reactions were set up. (For each reaction volume of 20µl) label 0.2 ml PCR tubes for each genomic DNA and tubes were arranged in the rack provided to hold the 0.2 ml tubes.

Prepared the following reaction mixture in a 1.5 ml eppendorf tube for required number of

reactions plus two reactions to compensate the pipetting loss (if more than one primer are used prepare cocktail separately for each primer).

PCR amplification conditions

Added 19 µl of the reaction mixture to the 0.2 ml PCR tube, and then at last added the 1 µl of template DNA making the final volume to 20 µl. (While preparing the PCR reactions, it is important to keep the reactions in ice; add the components in the order as indicated).

Mixed the content and performed the PCR reaction in the thermocycler following the programme given below. Checked the amplified product by running through agarose with Ethidium bromide.

The yeast specific primers used in the present study are obtained from the public domain (Perez *et al.*, 2001). Primer construction was done by Amnion Biosciences Company, Bengaluru.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to resolve the amplification product using 1.2 per cent agarose in 1X TBE buffer, 0.5 µg/ml of Ethidium bromide, and loading buffer (0.25% Bromophenol blue in 40% sucrose). 5 µl of the loading dye was added to 20 µl of PCR products and loaded to the agarose gel. Electrophoresis was carried at 65 V for 4 h. The gel was visualized under UV light and documented using Gel Documentation unit.

Scoring the data

As the SSR markers are co dominant, the bands were scored '1' for the homozygous and 2 for the heterozygous and 0 for the absence and in ADH the bands were scored '1' for presence and '0' for the absence of band was used for statistical analysis. The scored band data (Presence or absence) was subjected to cluster analysis-using STATISTICA software. The dendrogram was

constructed by unweighted pair group arithmetic mean (UPGMA). The dissimilarity matrix was developed using Squared Euclidean Distance (SED), which estimated all the pair wise differences in the amplification product (Sokal and Sneath, 1973). Only clear and unambiguous bands were taken into account and the bands were not scored if they were faint or diffused, as such fragments possess poor reproducibility. The band sizes were determined by comparing with the 1000 bp DNA ladder, which was run along with the amplified products. The Genetic distance was computed as:

$$\Sigma^n=1 \quad dj^2 \quad \text{where } dj = (X_{ik} - X_{jk})$$

Where X_{ik} refers to binary code of i^{th} tree for allele "k" and X_{jk} refers to the binary code of the j^{th} tree for allele "k". Dendrogram was computed based on Ward's method of clustering, using minimum variance algorithm (Ward, 1963).

RESULTS

The experimental results on isolation, identification, ethanol tolerance, SSR and ADH specific- PCR characterization of different sources

Isolation of yeast strains

Isolation of yeasts was made from different sugar rich sources like sugarcane juice, banana, grape juice, apple juice, mosambi, pomegranate, pineapple, watermelon, muskmelon and flower nectar collected from various locations and isolates were made from these samples.

Identification of yeast isolates

All the Yeast isolates were identified upto Genus level by studying colony characters, cell morphology and cell shape.

Colony characters

Colony characters were used for preliminary identification. Yeast strains produced different types of colonies on YEPDA medium.

Reagents used in the PCR reaction		PCR amplification condition			
Constituent	Quantity required to make volume upto 20µl		Temperature (°C)	Time (minutes)	Cycles (No.)
Sterile water	9.50	Initial denaturing	94	0.5	One
PCR buffer	2.50	Denaturing	94	0.5	30
DNTPs	3.00	Annealing	53	1.0	
ADH primer (F+R)	3.00	Extension	72	2.0	
Taq polymerase	1.00	Final Extension	72	2	One
Template DNA	1.00	Storage	4		

Some colonies spread all over the plate with dull yellow colour and these colonies were not isolated and others produced isolated, raised and were creamy with white colour. Genus *Saccharomyces* are known to produce raised creamy white with isolated colonies. Based on these characters *Saccharomyces* colonies were selected and further purified on YEPDA plates. The isolates which were identified as *Saccharomyces*.

Yeast DNA Extraction and optimization of PCR condition for yeast DNA.

Yeast DNA was extracted as described in materials and methods from overnight yeast cultures. Extraction method yielded a good amount of DNA. The amount of DNA present was determined and quantified by taking the spectrophotometer readings at a wavelength of

260nm and quality of DNA was determined using 0.8% agarose gel.

PCR products were amplified for informative and reproducible fingerprint profiles for yeast strains

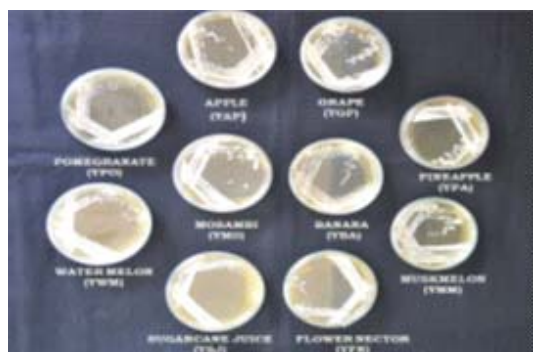
PCR amplification was carried out using Four SSR and four ADH specific primers with optimal amplification condition mentioned in the chapter 3. It was found that the reaction components used were optimal and yielded scorable amplification product. The amplification product were separated in 1.2% agarose gel and stained with Ethidium bromide. Agarose gel showed good resolution to score the band.

PCR analysis

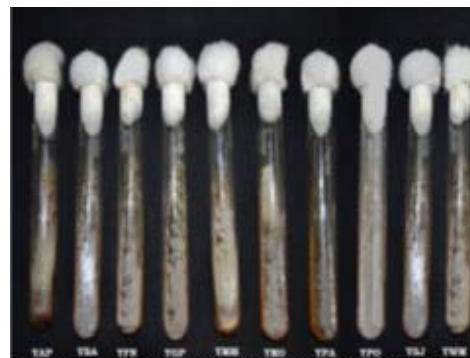
The study was aimed at determining the genetic variability and efficiency among yeast strains. Eight primers were used to amplify the

Primers used in SSR and ADH-specific primer Analysis

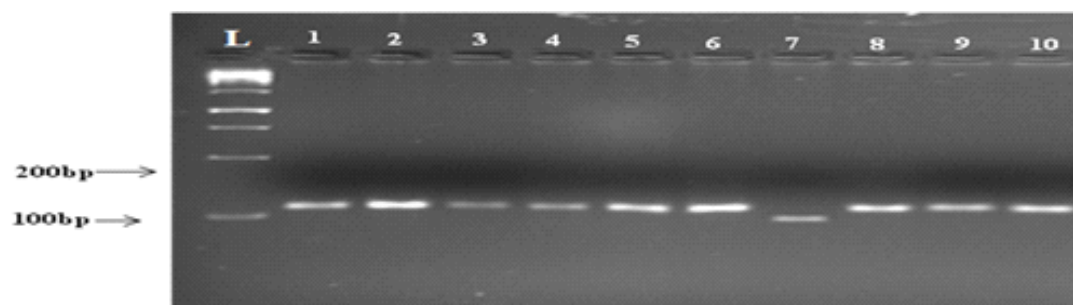
Sl. No	Locus	Primers
1	SCYOR267C	TAC TAA CGT CAA CAC TGC TGC CAA GGA TCT ACT TGC AGT ATA CGG G
2	C5	TGA CAC AAT AGC AAT GGC CTT CA GCA AGC GAC TAG AAC AAC AAT CAC A
3	C11	TTC CAT CAT AAC CGT CGT GGA TT TGC CTT TTT CTT AGA TGG GCT TTC
4	SC8132X	CTG CTC AAC TTG TGA TGG GTT TTG G CCT CGT TAC TAT CGT CTT CAT CTT GC
5	ADH1	ATG TCT ATT CCA GAA ACT CAA AA TTA TTT AGA AGT GTC AAC AAC GT
6	ADH3	ATG TTG AGA ACG TCA ACA TTG TTA TTT ACT AGT ATC GAC GAC G
7	ADH4	TTA ATA TTC ATA GGC TTT C ATG TCT TCC GTT ACT GGG TT
8	ADH7	CTA TTT ATG GAA TTT CTT ATC ATG GGG TAG CCA CTC GAC AAA



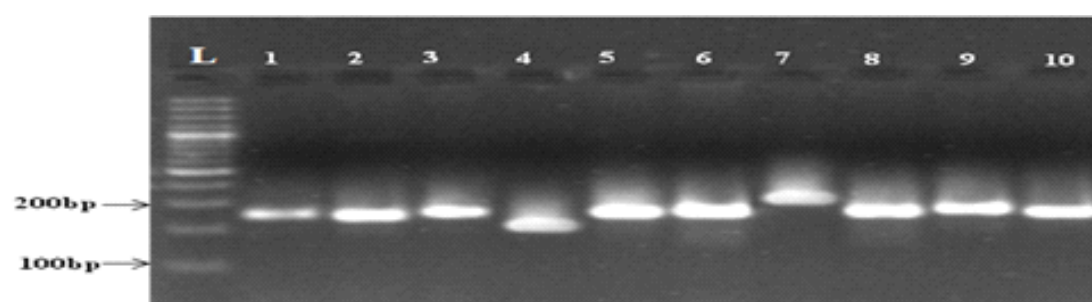
Petri plates showing yeast strains isolated from different samples



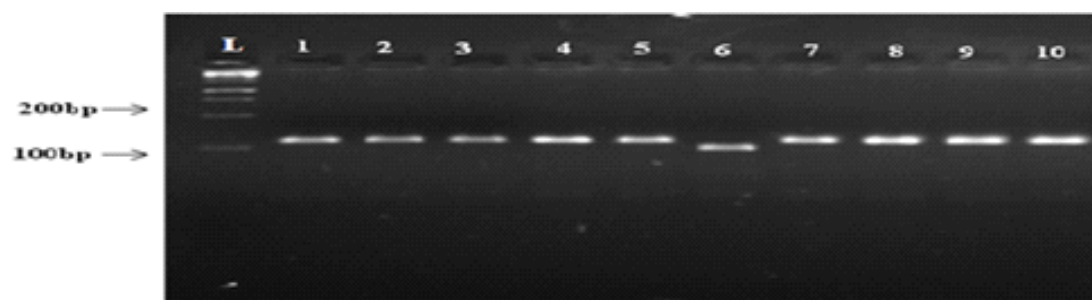
Yeast strains maintained in (YEPDA) slants



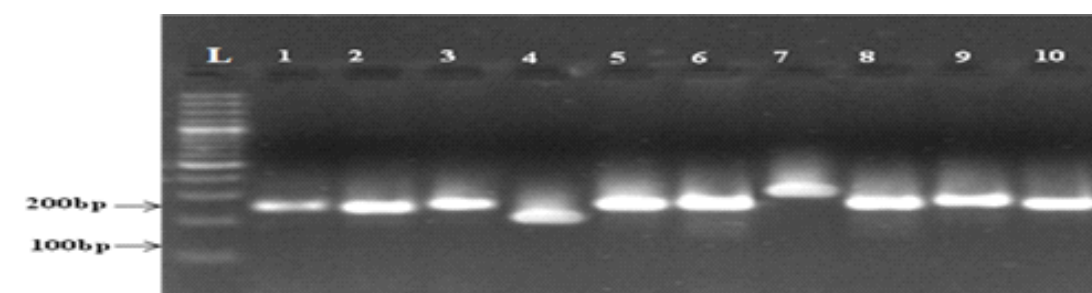
SSR gel profile of yeast isolates generated using SCYOR267C primer. Lane 1: YFN, 2: YMO, 3: YMM, 4: YYPO, 5: YGP, 6: YSJ, 7: YPA, 8: YWM, 9: YAP, 10: YBA, L-Ladder.



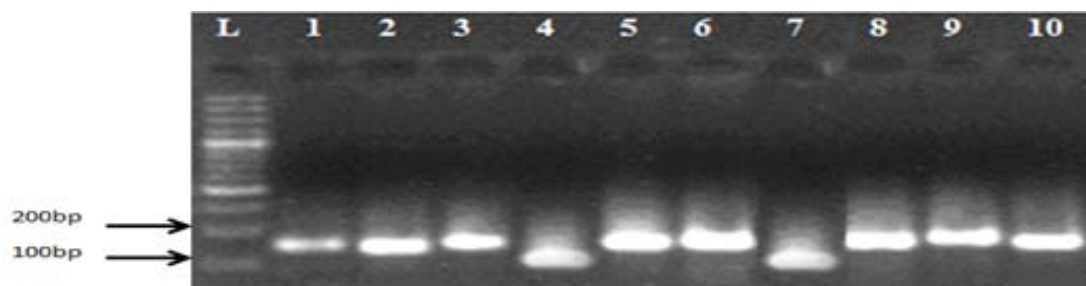
SSR gel profile of yeast isolates generated using C5 primer. Lane 1: YFN, 2: YMO, 3: YMM, 4: YYPO, 5: YGP, 6: YSJ, 7: YPA, 8: YWM, 9: YAP, 10: YBA, L-Ladder.



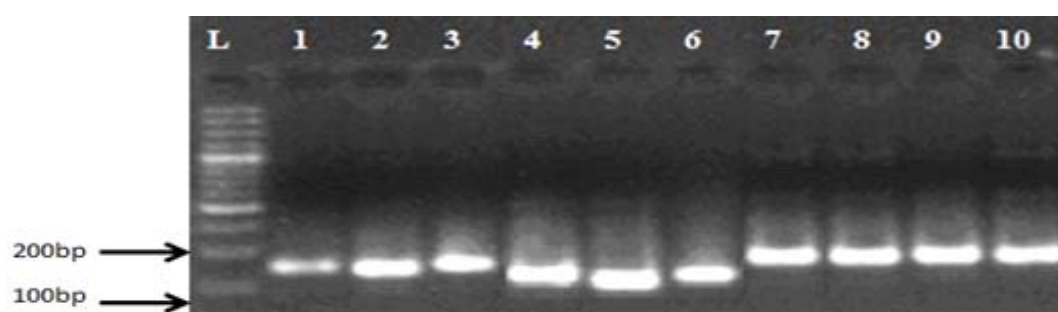
SSR gel profile of yeast isolates generated using C11 primer. Lane 1: YFN, 2: YMO, 3: YMM, 4: YYPO, 5: YGP, 6: YSJ, 7: YPA, 8: YWM, 9: YAP, 10: YBA, L-Ladder.



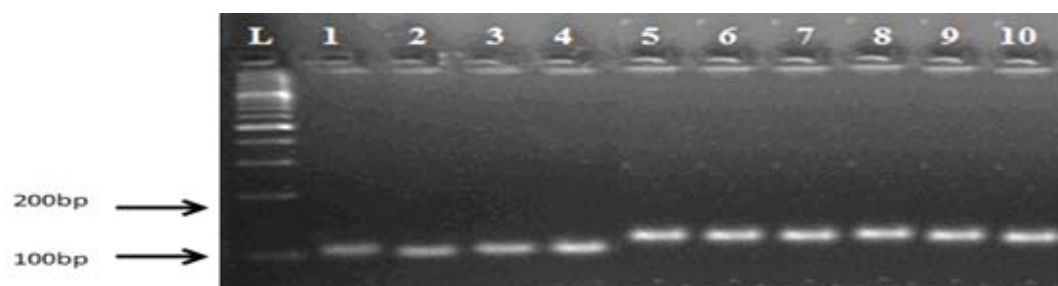
SSR gel profile of yeast isolates generated using SC8132X primer. Lane 1: YFN, 2: YMO, 3: YMM, 4: YYPO, 5: YGP, 6: YSJ, 7: YPA, 8: YWM, 9: YAP, 10: YBA, L-Ladder.



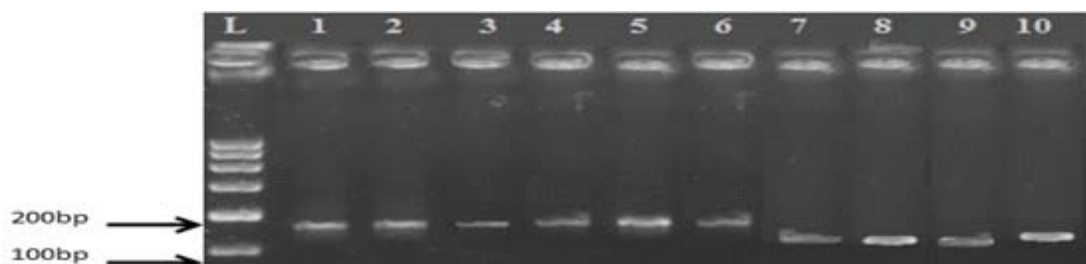
Gel profile of yeast isolates generated using ADH1 primer Lane 1: YFN, 2: YMO, 3: YMM, 4: YYPO, 5: YGP, 6: YSJ, 7: YPA, 8: YWM, 9: YAP, 10: YBA, L-Ladder.



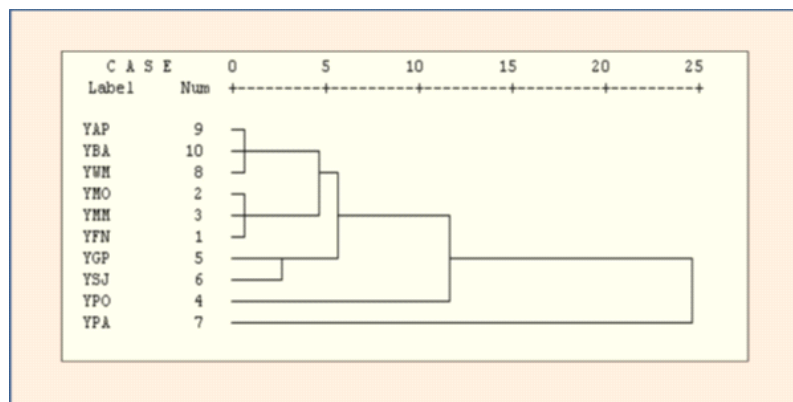
Gel profile of yeast isolates generated using ADH3 primer Lane 1: YFN, 2: YMO, 3: YMM, 4: YYPO, 5: YGP, 6: YSJ, 7: YPA, 8: YWM, 9: YAP, 10: YBA, L-Ladder.



Gel profile of yeast isolates generated using ADH4 primer Lane 1: YFN, 2: YMO, 3: YMM, 4: YYPO, 5: YGP, 6: YSJ, 7: YPA, 8: YWM, 9: YAP, 10: YBA, L-Ladder.



Gel profile of yeast isolates generated using ADH7 primer Lane 1: YFN, 2: YMO, 3: YMM, 4: YYPO, 5: YGP, 6: YSJ, 7: YPA, 8: YWM, 9: YAP, 10: YBA, L-Ladder



Dendrogram Based On SSR and ADH-Specific Primer Profile of Ten Yeast Strains Obtained From Different Samples

repeated regions in the yeast strains. Eight primers were successful in amplifying DNA in the sample viz., SCYOR267C, C5, C11, SC8132X, ADH1, ADH3, ADH4 and ADH7 primers. For all loci, 20 µl reaction mixture containing genomic DNA concentration of 25 ng/ml, primer concentration of 10 pM/µl and dNTPs 2.5 mM, 10X buffer and 1 unit of *Taq* polymerase were used.

Cluster analysis

The Dendrogram was constructed by unweighted pair-group arithmetic mean (UPGMA). The dissimilarity matrix was developed using SPSS Software (Version 16.0), which estimated all the pair wise differences in the amplification product (Sokal and Sneath, 1973). Only clear and unambiguous bands were taken into account. This Dendrogram was obtained from the binary data deduced from the DNA profiles of the samples analysed. The tree cluster diagram was constructed for ten yeast strains using eight primers. Cluster diagram was divided into 3 major clusters and 4 sub clusters, in first cluster three yeast strains like YAP, YBA and YWM, In Second cluster also three yeast strains like YWM, YMM and YMO were found, the remaining yeast strains like YGP and YSJ were found in Third cluster. First and second cluster showing 20% dissimilarity, first second and third cluster showing 25% dissimilarity. YGP and YSJ were found in third main cluster, however YPO and YPA did not fall under any of the main clusters.

DISCUSSION

Today, the products of yeast used in many commercially important sectors, including

food, beverages, biofuels, chemicals, industrial enzymes, pharmaceuticals, agriculture and the environment. Ethyl alcohol produced by yeast fermentation is likely to remain the foremost worldwide biotechnological commodity for the foreseeable future (Pretorius *et al.*, 2003).

Yeast isolates were identified up to Genus level through colony characters and cell morphological studies. Out of 40 isolates only 10 were identified as *Saccharomyces* strains. Identification was based on simple microscopic observation. Cells were stained with crystal violet stain and observed under microscope. Elliptical budding cells were identified as *Saccharomyces* strains. Raghavendra Kumar (2006) isolated yeast from surface of beet root and identified based on colony morphology and microscopic studies. Members of the high ethanol tolerant species of *S. cerevisiae* were unique to isolates from natural fruit surface (Martini *et al.*, 1996).

DNA was isolated from all the yeast strains. Quantification was done using Spectrophotometric method and this revealed that good amount of DNA was obtained and PCR was carried out using four SSR and four ADH specific primers. Out of eight different ADH-specific primers six primers showed polymorphism.

Scoring of data

Among the ten different yeast strains isolated from different fruit juices, the banding pattern of the ten yeast strains revealed the genetic diversity. Dendrogram was plotted using SPSS version 16.0 unweighted pair-group arithmetic mean (UPGMA). Dendrogram showed that YAP,

YBA and YWM strains belong to major cluster I, strains like YWM, YMM and YMO belong to major cluster II and strains like YGP and YSJ belong to the major cluster III. YGP and YSJ belong to the major cluster III, hence they are genetically closely related and also they have shown highest ethanol tolerance (15%). YWM, YMM and YMO belong to cluster II, hence they are genetically closely related and also they have shown normal ethanol tolerance (12%-12.5%).

CONCLUSION

Yeast strains were from sugar rich sources 10 were identified as *Saccharomyces* spp. SSR and ADH profiling reflected polymorphism among *Saccharomyces* spp. and however there was no correlation between their genetic makeup Molecular diversity of these isolates was characterized by SSR and ADH profiling. SSR marker analysis clearly depicted that all the 10 yeast isolates formed 3 major clusters and 4 sub clusters, in first cluster three yeast strains like YAP, YBA and YWM, In Second cluster also three yeast strains like YWM, YMM and YMO were found, the remaining yeast strains like YGP and YSJ were found in Third cluster. First and second cluster showing 20% dissimilarity, first second and third cluster showing 25% dissimilarity YGP and YSJ were found in third main cluster, however YPO and YPA did not fall under any of the main clusters.

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