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# Screening of Efficient Ethanol Tolerant Yeast Strain for Production of Ethanol

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**ABSTRACT**

*An efficient method for conversion of biomass into fuel is by ethanol production because ethanol is an economically as well as environmentally friendly fuel. Ethanol has the advantages of being renewable, cleaner burning and produces no greenhouse gases. Hence Ethanol tolerance investigation was carried out with yeast strains by isolating from their natural habitats and screened them for ethanol tolerance and ethanol production. Out of 40 microbial culture 10 were identified as Saccharomyces strains based on colony type and budding characters. Saccharomyces species were screened for their ability to tolerate different ethanol concentrations from 0-24 %. Growth in different ethanol concentrations varied from one strain to another. Some 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, whereas from pineapple least tolerance was observed 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:*** *Bioethanol, S. cerevisiae, Bioenergy, Sustainable, Renewable, Biomass*

## INTRODUCTION

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. Bio-ethanol can considerably reduce the climate relevant greenhouse gas emissions from transport and traffic. An efficient method for conversion of biomass into fuel is by ethanol production because ethanol is an economical as well as environmentally friendly fuel. Ethanol has the advantages of being renewable, cleaner burning and produces no greenhouse gases.

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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 production of bio-ethanol first uses enzyme amylases to convert a feedstock crop into fermentable sugars. Yeast is then added to the 'mash' to ferment the sugars to alcohol and carbon dioxide, the liquid fraction being distilled to produce ethanol.

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*[5]. *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[1].

Microsatellites or Simple Sequence Repeats (SSR) consist of direct tandem repeats of a short DNA motif, usually less than 10 bp[1]. These repetitive sequences are a major component of higher organism DNAs. They are hyper variable in length[13] as a result of DNA-replication errors, such as slipped-strand mispairing[11]. Thus, microsatellites show a substantial level of polymorphism between individuals of the same species and are extensively used for paternity exclusion tests[4], Field and Wills[3] 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.

As low yield of ethanol produced, the separation process by distillation and absorption might have some problems. Especially in the high utilization of energy can make this process inefficient. Therefore some effort should be taken to increase the ethanol yield. One way is by increasing the capability of yeast to tolerate ethanol content in the media[10]. High ethanol tolerant strains are able to extend the process of fermentation for longer time and produce distinct products in the presence of ethanol[12].

## MATERIAL AND METHODS

The experiments were carried out at bio fuel laboratory in the Department of Plant Biotechnology, UAS, GKVK, Bengaluru.

**Isolation of yeast from different sources**

Yeasts are naturally associated with sugar rich environments. In the present study sugarcane juice was selected as sources for isolating yeast cells, these samples were collected from ZARS, Jaggery 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 and samples were mentioned as YAP: Yeast Apple, YBA: Yeast Banana, YFN: Yeast Flower nectar, YMO: Yeast Mosambi, YMM; Yeast Musk melon, YPA: Yeast Pineapple, YPO: Yeast Pomegranate, YSJ: Yeast Sugarcane juice, YWM: Yeast Water melon, YGP: Yeast Grape.

**Protocol for isolation of yeast**

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 hr.

**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 hr. 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.

**Screening of the isolated yeast strains for ethanol tolerance**

Ethanol has three major effects on yeast. It decreases the rates of growth and of fermentation, and reduces overall levels of cell viability. In this experiment, an attempt was made to check the viability of yeast cells under different concentrations of ethanol.

The ethanol tolerance of each isolates was studied by allowing the yeasts to grow in YEPD medium having different concentrations of ethanol[6].

**Procedure:** Yeasts were inoculated into 10ml tubes containing 5ml portion of YEPD broth, incubated for 24 hr. at 30 °C, 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 hr. at 30 °C, Growth expressed as generation time, it was determined by measuring the optical density of cultures at 595 nm, The initial optical density of each tube was read off on a spectrophotometer at 595 nm against the medium as blank, The inoculated tubes were transferred to a shaker set at 30 °C for 48 hr. The increase in optical density in a tube was recorded as evidence for growth of the yeast and 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. Yeast DNA was isolated by using protocol provided in Sambrook and Russell[8]. 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. PCR reactions were (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 1µl 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 thermo cycler. Checked the amplified product by running through agarose with Ethidium bromide. The yeast specific primers (Table 1) used in the present study are obtained from the public domain[7]. Primer construction was done by Amnion Biosciences Company, Bengaluru.

**Table 1: SSR and ADH-specific primer used for Molecular characterization of Yeast strain Analysis**

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

**Agarose gel electrophoresis**

Agarose gel electrophoresis was performed to resolve the amplification product using 1.2 per cent agarose in 1X TBE buffer, 0.5µg/ml of Ethidium bromide, and loading buffer (0.25 % Bromophenol blue in 40 % sucrose). 5µl of the loading dye was added to 20µl of PCR products and loaded to the agarose gel. Electrophoresis was carried at 65 V for 4 hr. 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[9]. 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:

$\Sigma^{n}=1dj^{2}$ 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.

## RESULTS

**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 (Plate 1). Yeast strains produced different types of colonies on YEPDA medium. 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.


**Plate 1: Petriplates showing yeast strains isolated from different samples Ethanol tolerance of yeast strains**

Ten strains which were identified as *Saccharomyces spp*. were screened for Ethanol tolerance at various levels of ethanol stress from 6 % to 24 %. Strains had tolerance levels from 7 % to 15 %. Strains like YGP and YSJ showed highest tolerance among 10 isolates up to 15 % and strain YPA had least with 10 %. In mutation physical mutant strain had tolerance level from 7 % to 14 %. Cell density of the yeast isolates at various levels of Ethanol concentration was checked by taking absorbance at 595 nm (Plate 2). Ethanol tolerance of all the ten strains is given in Table 1 and 2 which reveals cell density of strains at different ethanol stress. The same has been plotted on graph in Fig-1.

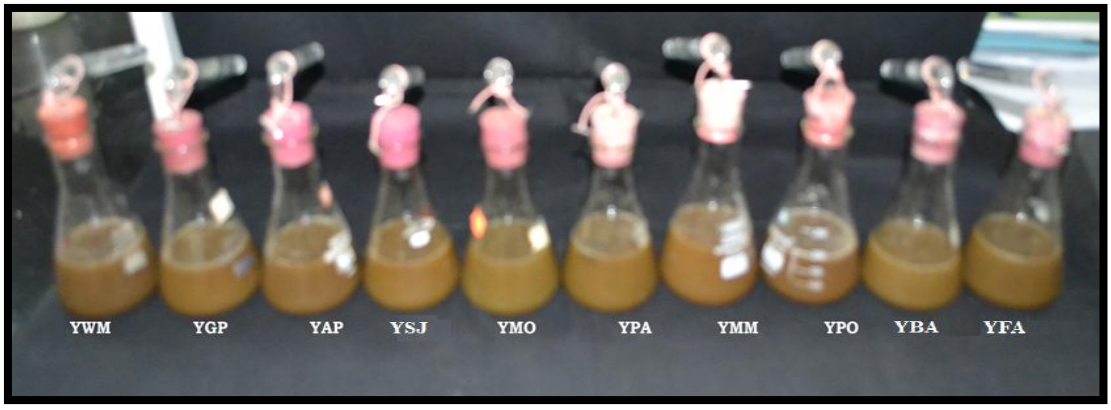



**Plate 2: Fermentation setup for different yeast samples**

**Table 1: Population of the yeast cell isolates at various levels of Ethanol concentration (absorbance at 595nm)**

| **Strains** | 0% | 6% | 8% | 10% | 12% | 14% | 16% | 18% | 20% | 22% | 24% |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Standard yeast | 1.564 | 1.210 | 0.852 | 0.641 | 0.416 | 0.306 | 0.244 | 0.18 | 0.169 | 0.158 | 0.145 |
| YAP | 1.658 | 1.310 | 1.120 | 0.922 | 0.513 | 0.347 | 0.253 | 0.203 | 0.186 | 0.175 | 0.166 |
| YBA | 1.358 | 0.910 | 0.825 | 0.614 | 0.328 | 0.294 | 0.225 | 0.205 | 0.191 | 0.185 | 0.182 |
| YFN | 1.081 | 0.445 | 0.335 | 0.237 | 0.227 | 0.150 | 0.113 | 0.098 | 0.021 | 0.019 | 0.018 |
| YGP | 1.707 | 1.345 | 1.110 | 0.915 | 0.557 | 0.425 | 0.232 | 0.218 | 0.228 | 0.215 | 0.211 |
| YMO | 1.533 | 0.725 | 0.628 | 0.512 | 0.428 | 0.305 | 0.261 | 0.215 | 0.137 | 0.130 | 0.125 |
| YMM | 1.030 | 0.614 | 0.553 | 0.432 | 0.424 | 0.315 | 0.291 | 0.231 | 0.192 | 0.175 | 0.169 |
| YPA | 1.785 | 1.235 | 1.110 | 0.732 | 0.613 | 0.318 | 0.221 | 0.212 | 0.211 | 0.198 | 0.193 |
| YPO | 1.629 | 1.115 | 0.952 | 0.893 | 0.511 | 0.377 | 0.212 | 0.187 | 0.113 | 0.102 | 0.101 |
| YSJ | 1.617 | 0.899 | 0.723 | 0.679 | 0.660 | 0.559 | 0.377 | 0.305 | 0.218 | 0.214 | 0.210 |
| YWM | 1.487 | 0.709 | 0.623 | 0.510 | 0.329 | 0.217 | 0.221 | 0.189 | 0.108 | 0.101 | 0.098 |

**Table 2: Ethanol tolerance of yeast strains**

| **Ethanol Tolerance** | |
|---|---|
| Below 8% | - |
| 08%-10% | YPA |
| 10%-12% | YAP, YBA, YWM, YPO |
| 12%-14% | YFN, YMO, YMM |

**YAP**: Yeast Apple, **YBA**: Yeast Banana, **YFN**: Yeast Flower nectar, YMO: Yeast Mosambi, YMM; Yeast Musk melon, YPA: Yeast Pineapple, YPO: Yeast Pomegranate, **YSJ**: Yeast Sugarcane juice, **YWM**: Yeast Water melon, **YGP**: Yeast Grape

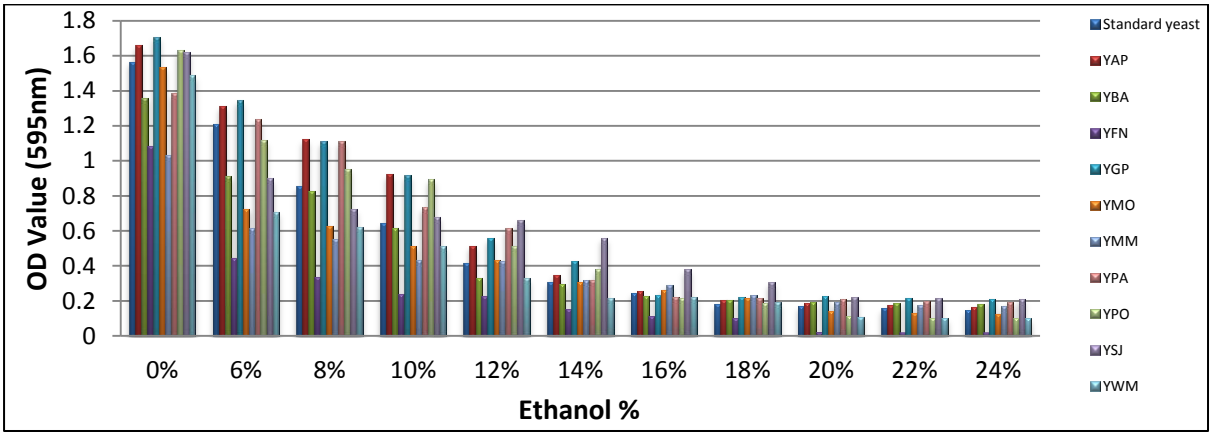



**Fig. 1: Yeast strains with tolerance level to ethanol**

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

The amount of DNA present was determined and quantified by taking the spectrophotometer readings at a wavelength of 260 nm and quality of DNA was determined using 0.8 % agarose gel.

PCR amplification was carried out using Four SSR and four ADH specific primers. 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 to determine the genetic variability and efficiency among yeast strains. Eight primers were used to amplify the 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 ADH7primers (Plates 3-10), For all loci, 20µl 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.

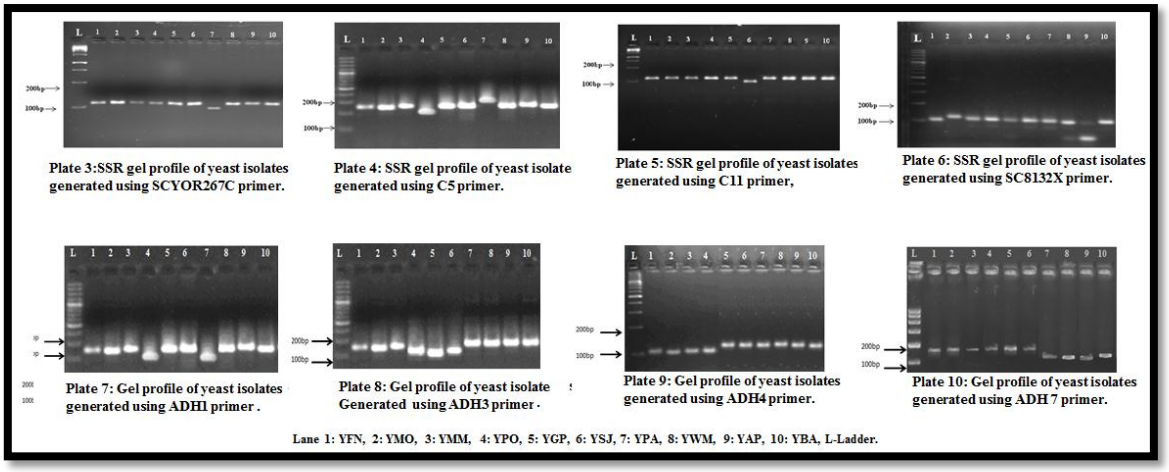


Plate 3:SSR gel profile of yeast isolates generated using SCYOR267C primer.

Plate 4: SSR gel profile of yeast isolate generated using C5 primer.

Plate 5: SSR gel profile of yeast isolates generated using C11 primer,

Plate 6: SSR gel profile of yeast isolates generated using SC8132X primer.

Plate 7: Gel profile of yeast isolates generated using ADH1 primer .

Plate 8: Gel profile of yeast isolate Generated using ADH3 primer .

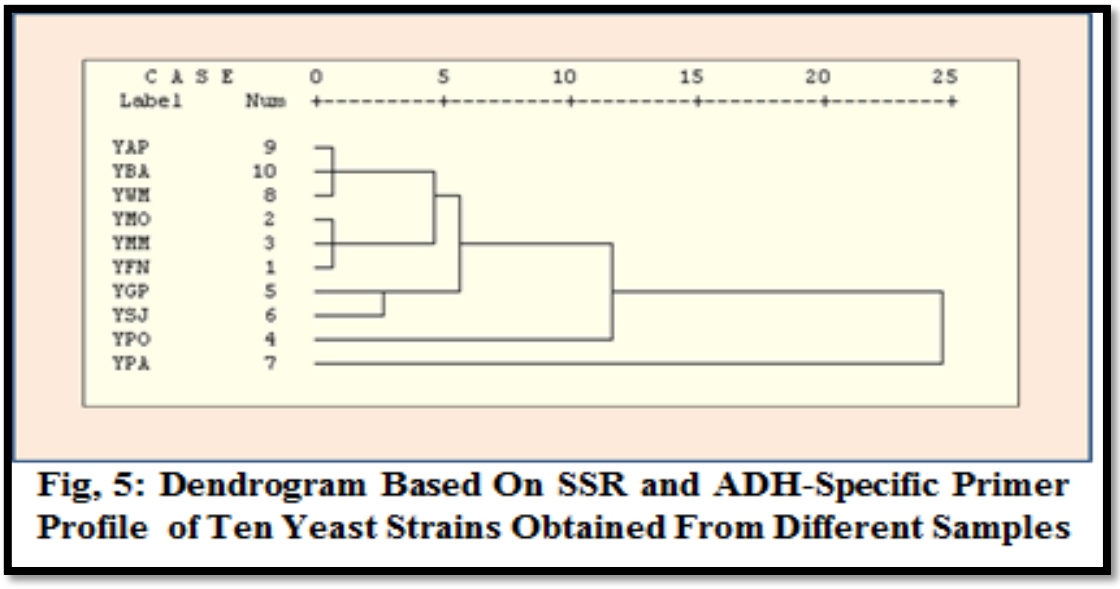
Plate 9: Gel profile of yeast isolates generated using ADH4 primer.

Plate 10: Gel profile of yeast isolates generated using ADH 7 primer.

Lane 1: YFN, 2: YMO, 3: YMM, 4: YPO, 5: YGP, 6: YSJ, 7: YPA, 8: YWM, 9: YAP, 10: YBA, L-Ladder.

**Cluster analysis**

Dendrogram were obtained from the binary data deduced from the DNA profiles of the samples analysed (Fig-2). 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.




**Fig, 5: Dendrogram Based On SSR and ADH-Specific Primer Profile of Ten Yeast Strains Obtained From Different Samples**

## DISCUSSION

Yeast strains were isolated from different sugar rich samples of fruits on YEPDA medium. Totally 40 yeast isolates were obtained from different fruit samples. This shows that the yeast is a common inhabitant of sugar rich environments. Previous studies have shown that the yeasts are naturally associated with sugar rich samples

Ethanol tolerance has yet to be clearly defined, although it has been reported to be reproducible under defined conditions and appears to be under complex genetic control. Ethanol has three major effects on yeast. It decreases the rates of growth and of fermentation, and it reduces overall levels of cell viability. Strains had ethanol tolerance levels from 10 % to 15 %. Osho[6] isolated yeasts from fermenting Cashew apples and reported that isolates had ethanol tolerance level from 9-12 %. Unaldi *et al*[14]., reported that the maximum alcohol tolerance was found to be 9 % (v/v) in yeast strains which were isolated from grapes.

The high ethanol tolerant strains were subjected for ethanol production using the source sugarcane juice. The estimation of ethanol produced confers the efficiency of the selected strains to produce ethanol from the above sources. The strain YSJ and YGP has shown high efficiency in ethanol production when compared with other strains and the standard which showed 15 % in sugarcane juice. The maximum concentration of ethanol fermented from sugarcane juice by YGP was 14 %. YPA was the least fermenting strain with a concentration of 9 % from sugarcane.

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. 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 %). YAP, YBA and YWM belong to major cluster I, hence they are genetically closely related and also they have shown moderate ethanol tolerance. And YPO and YPA did not fall under any of the main clusters they have shown low ethanol tolerance (<9 %). The obtained isolated group of *Saccharomyces spp* has similarity ranging from 30–100 %. These results are similar to with other winery yeasts[15].

## CONCLUSION

Yeast strains from sugar rich sources were identified as *Saccharomyces spp*. Ten identified *Saccharomyces spp*. were evaluated for their ethanol tolerance, which showed good growth in medium containing 8-15 % ethanol. SSR and ADH profiling reflected polymorphism among *Saccharomyces spp*. and however there was no correlation between their genetic makeup and ethanol tolerance. Strain YSJ shows high efficiency in ethanol production based on ethanol tolerance level.

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