

Isolation and characterization of ethanol tolerant yeast strains

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Received March 31, 2013; Accepted April 01, 2013; Published April 30, 2013

Abstract:

Yeast strains are commonly associated with sugar rich environments. Various fruit samples were selected as source for isolating yeast cells. The isolated cultures were identified at Genus level by colony morphology, biochemical characteristics and cell morphological characters. An attempt has been made to check the viability of yeast cells under different concentrations of ethanol. Ethanol tolerance of each strain was studied by allowing the yeast to grow in liquid YEPD (Yeast Extract Peptone Dextrose) medium having different concentrations of ethanol. A total of fifteen yeast strains isolated from different samples were used for the study. Seven strains of *Saccharomyces cerevisiae* obtained from different fruit sources were screened for ethanol tolerance. The results obtained in this study show a range of tolerance levels between 7%-12% in all the stains. Further, the cluster analysis based on 22 RAPD (Random Amplified polymorphic DNA) bands revealed polymorphisms in these seven *Saccharomyces* strains.

Key words: Yeast, Ethanol, RAPD, Polymorphism

Background:

Saccharomyces are the safest and most effective microorganisms for fermenting sugars to ethanol and traditionally have been used in industry to ferment glucose based agricultural products to ethanol [1]. Yeast is ubiquitous in the environment, but is most frequently isolated from sugar rich samples. Some good examples include fruits berries and exudates from plants. Some yeast strains are found in association with soil and insects. In assessing a yeast strain for industrial use, specific physiological properties are required [2]. Ethanol tolerance, sugar tolerance and invertase activities are some of the important properties for use in industrial ethanol production (Jimenez and Benetez, 1986). Yeast has also been isolated from many fermenting sources including fermenting cassava tubers [3]. Many research workers found yeast in large numbers in a wide variety of natural habitats as different as leaves, flowers, sweet fruits, tree exudates, grains, roots fleshy fungi, insects, dung, soil [4].

Recently they have been used in the production of bio fuels, a potentially important alternative energy source. Renewable energy is one of the most efficient ways to achieve sustainable development. Increasing its share in the world matrix will help prolong the existence of fossil fuel reserves, address the threats posed by climate change, and enable better security of the energy supply on a global scale [5]. Successful fermentations to produce ethanol using yeast require tolerance to high concentrations of both glucose and ethanol. These cellular characteristics are important because of high gravity (VHG) fermentations, which are common in the ethanol industry, give rise to high sugar concentrations, at the beginning of the process, and high ethanol concentration at the end of the fermentation. *Saccharomyces cerevisiae* is an important micro organism in bio- industry and its tolerance to ethanol is one of main characteristics to decide whether it can be used as bio-fermentation resources. Molecular Genetic techniques can be

used to discriminate between yeast strains that have similar physiological characteristics. RAPD analysis is faster, technically less demanding and more economical than the other genomic typing methods like RFLP, AFLP. Unlike conventional PCR data on DNA sequences of the organisms are not a prerequisite for RAPD analysis. Further, this technique elucidates the biodiversity in a group of isolates [6]. The present study is an attempt for isolation and identification of yeast strains from natural habitats, Screening of those yeast strains for ethanol tolerance and Molecular characterization of yeast strains using RAPD marker.

Methodology:

Isolation of yeast strains from different sources

Yeast strains are commonly associated with sugar rich environments. Various fruit samples were selected as sources for isolating yeast cells. Fruits such as grapes, molasses, mosambi, cashew apple, sugarcane, sorghum, and distillery effluents were used for isolation of yeast and named as Yeast Grape strain (YGP), Yeast Molasses strain (YMO), Yeast Mosambi strain (YMI), Yeast Cashew apple strain (YCA), Yeast Sorghum strain (YSM) and Yeast Distillery effluent strain (YDE). The collected fruits were washed and rinsed in distilled water. They were then cut, squeezed and the juice was extracted in separate sterile flasks and allowed for seven days of fermentation. After fermentation they were diluted serially and 0.1 ml of the diluted samples (10^{-4}) was plated on Yeast Extract Peptone Dextrose Agar (YEPDA) medium and incubated at 30°C for 24 to 48 h.



Figure 1: Growth of yeast colonies on various sources on YEPDA medium

Identification and Screening of the isolated yeast strains for ethanol tolerance

Simple staining was performed for of 24 h cultures obtained from different fruit sources plated on Yeast Extract Peptone

Dextrose Agar (YEPDA) and observed under microscope for morphological characters such as shape, size and budding. The obtained isolates were given with specific names for further experimentation and easy recognition. Ethanol tolerance of each strain was studied by allowing the yeast to grow in liquid YEPD having different concentrations of ethanol such as 6%, 7%, 8%, 9%, 10%, 11%, 12%, 12.5% 13%, 13.5%, 14% and 14.5% [7].

Molecular Characterization of Yeast strains using RAPD marker

DNA was isolated from all the strains by following the method as developed by [8]. Isolated DNA samples from all the strains were subjected to RAPD analysis with 4 random primers (Table 1). Agarose gel electrophoresis was performed to resolve the amplified products. The bands were manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis. The scored band data (Presence or absence) was subjected to cluster analysis-using STATISTICA. The dendrogram was constructed by Ward's method of clustering using minimum variance algorithm. The dissimilarity matrix was developed using Squared Euclidean Distance (SED), which estimated all the pair wise differences in the amplification product. 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 100 bp DNA ladder, which was run along with the amplified products. The Genetic distance was computed as:

$$\sum_{i=1}^n d_{ij}^2 \text{ where } d_{ij} = (X_{ik} - X_{jk})$$

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.

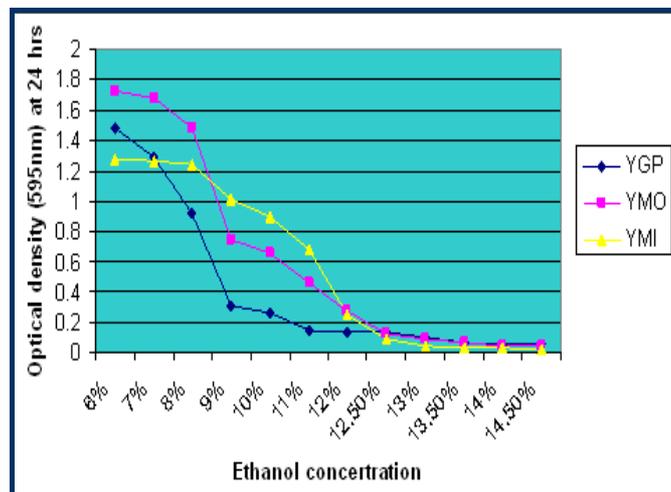


Figure 2: Ethanol tolerance graph of YGP, YMO & YMI

Results:

Colony characters were used for preliminary identification. Yeast strains produced different types of colonies on YEPDA medium such as raised, creamy white color colonies. Microphotographs of different colonies from different samples have shown in (Figure 1). Strains were observed for *Saccharomyces* characteristic oval cell shape and budding characters. Out of fifteen isolates, seven isolates showed oval cell shape with budding character and classified as Yeast Grape

strain (YGP), Yeast Molasses strain (YMO), Yeast Mosambi strain (YMI), Yeast Cashew apple strain (YCA), Yeast Sorghum strain (YSM) and Yeast Distillery effluent strain (YDE). From the ethanol tolerance study the tolerance levels of all the strains were found to be in the range of 7% to 12%. Even though some strains had tolerance at 13%, growth was less. YDE has highest tolerance when compared to other strains up to 12% (Table 2 & Figure 2, 3, 4). A total of 22 RAPD bands produced from the selected 4 primers were used for fingerprinting and for estimation of genetic diversity among seven isolates of *Saccharomyces* species. For the purpose of illustration, the RAPD fingerprints or electrophoretogram generated for seven *Saccharomyces* isolates using 10-mer random primers are presented in (Figure 5 & 6).

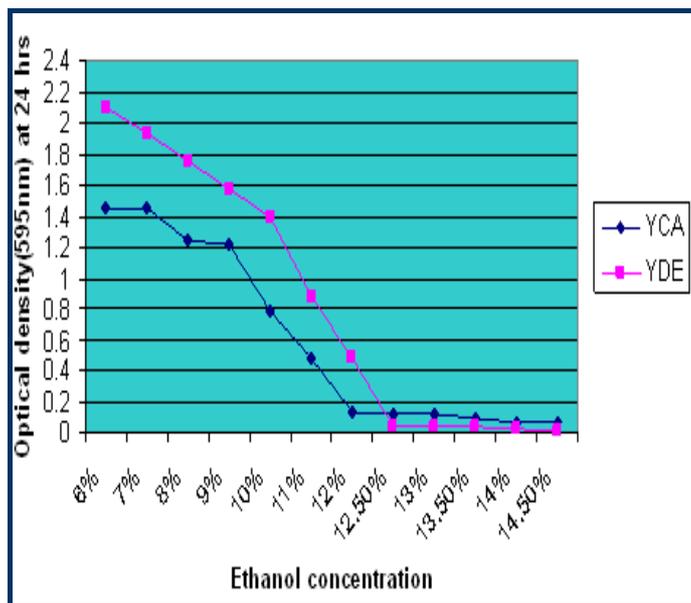


Figure 3: Ethanol tolerance graph of YCA & YDE

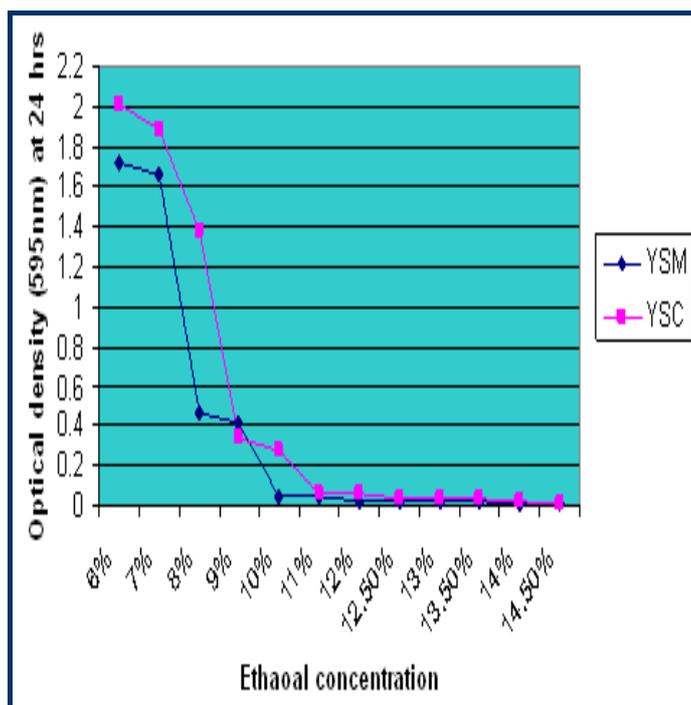


Figure 4: Ethanol tolerance graph of YSM & YSC

The number of bands scored for each primer varied from 1 to 8 with an average of 9.3 bands per primer. Out of 22 different sizes of amplification bands, 2 bands (9.0%) were monomorphic, 4 bands (11.11%) were unique and 18 bands (81.81%) were shared polymorphic, which were informative in revealing the relationship among the genotypes. The Cluster analysis based on 22 RAPD bands revealed that the seven yeast isolates examined. The dendrogram has clearly depicted that all the 7 yeast isolates formed two major clusters. Among the two major groups, there were five sub clusters (Figure 7). Isolates YCA, YDE, formed the first group, the isolates YSM, YMI, YMO, YSC and YGP formed the second group. Linkage distance was almost equal between two clusters. In first group there were no sub clusters and second group two sub clusters with linkage distance from 1.8 to 2.4.

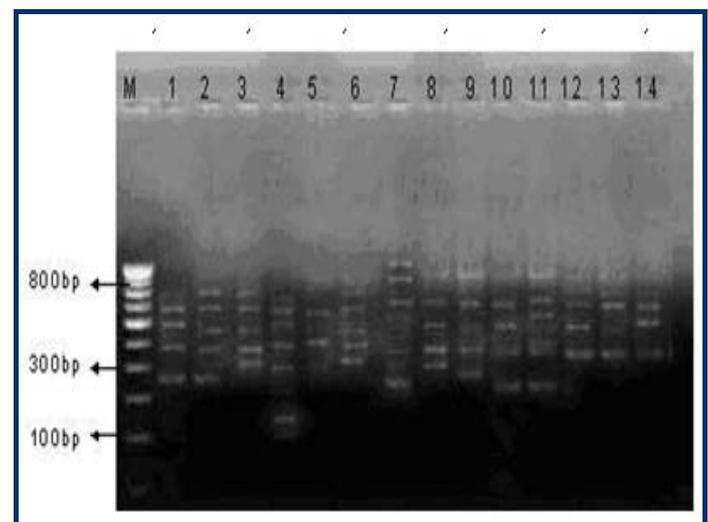


Figure 5: RAPD Gel profile of seven isolates of *Saccharomyces* sp. generated using 10-mer random 2 primers. No.1 primer: Lane1:YGP,2: YMO, 3: YMI,4:YCA,5:YDE,6:YSM,7: YSC. No.2 Primer: Lane 8: YGP, 9: YMO, 10: YMI, 11: YCA, 12: YDE, 13: YSM, 14: YSC.

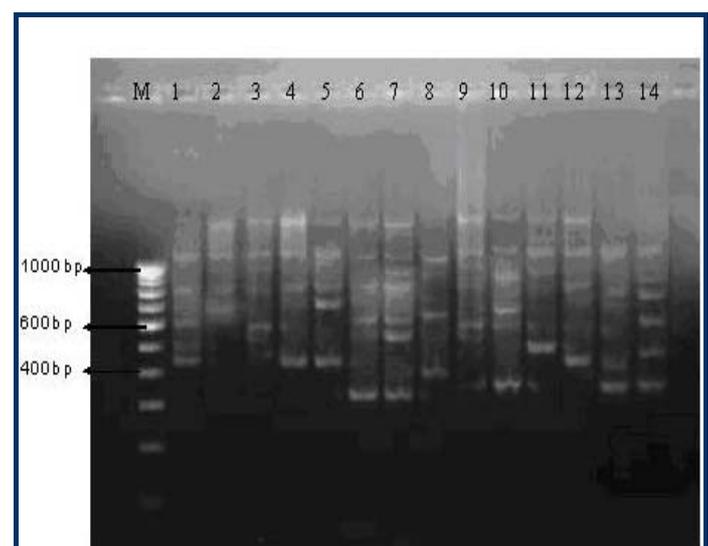


Figure 6: RAPD Gel profile of yeast isolates generated using 10-mer random primer no.3 & no.4; Lane1: YGP, 2: YMS, 3: YMI, 4: YCA, 5: YDE, 6: YSM, 7: YSC. No.4. Primer-8: YGP,9: YMS,10:YMI,11: YCA,12: YDE,13: YSM,14: YSC

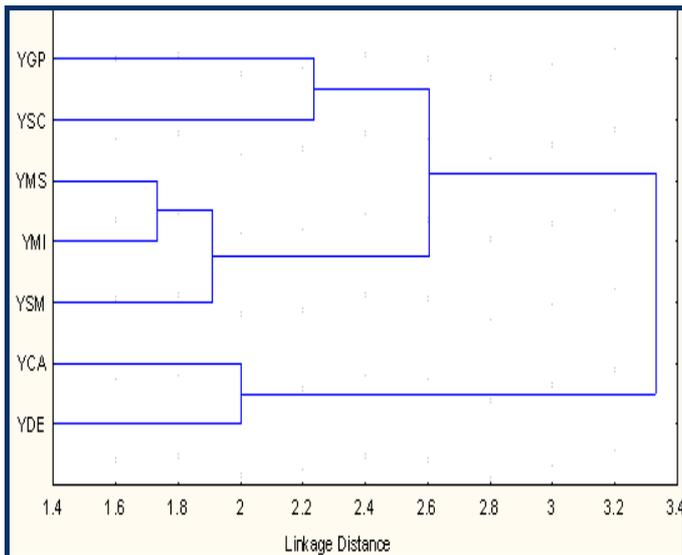


Figure 7: Dendrogram based on RAPD profile of 7 *Saccharomyces* strains obtained from different samples

Discussion:

The budding yeast, *Saccharomyces cerevisiae*, has enjoyed a long and distinguished history in the fermentation industry. Owing to its efficiency in producing alcohol, *Saccharomyces cerevisiae* is the most important commercial microorganisms with GRAS (Generally Regarded as Safe) status. Mankind's oldest domesticated organism made possible the world's first biotechnological process with the emergence of modern molecular genetics. *S. cerevisiae* has again been harvested to shift the frontiers of mankind rawest revolution genetic engineering. This yeast represents the prototype for fermentative yeast, responsible for fermentation in foods, such as wine, beer, bread. In the present study, Yeast strains were isolated from different sugar rich samples such as fruits, distillery effluent on YEPDA medium. Totally 7 isolates were obtained from different samples. Previous studies have shown that the yeast are commonly associated with sugar rich samples such as leaves, flowers, sweet fruits, tree exudates, grain, roots, insects, dung, soil [4]. Yeast isolates were identified up to genus level through colony characters and cell morphological studies. Out off 15 isolates only seven were identified as *Saccharomyces cerevisiae*. Identification was based on simple microscopic observation. Ethanol tolerance has yet to be clearly define, although it has been reported to reproducibile 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 cell viability. The range of ethanol tolerance obtained in the present study was 7-12% which correlates with the previous reports by [9]. Even though the highest tolerance level was observed in YDE up to 12% but tolerance rate was found to be very low from 12.5% onwards when compared to the other strains. Same type of results was also observed in case of YSC strain. But based on the above results YCA has optimum tolerance in a wide range up to 14%. RAPD analysis showed the different monomorphic and polymeric bands among these strains and from the dendrogram analysis the 7 strains were divided in to two groups and further in to sub clusters. These studies prove that the substrates have a major impact on the *S. cerevisiae* and can induce some change in the genotype which tends to develop in to different strains. The polymorphism found in these strains may be the reason for this type of results which was yet to be confirmed by further studies.

Conclusion:

The data collected from the study concludes that even though YDE and YSA had highest tolerance up to 12%, YCA showed optimum tolerance throughout the range of ethanol percentage up to 14%. It was also shown that substrate have major impact on the genotype of *S. cerevisiae* using RAPD and dendrogram analysis.

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Edited by P Kanguane

Citation: Tikka *et al.* Bioinformation 9(8): 421-425 (2013)

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Supplementary material:

Table 1: RAPD primers with sequences chosen for analysis

Random primer 1	5'GGG GTG ACG A 3'
Random primer 2	5' GAC GGA TCA G 3'
Random primer 3	5' GGA CCC AAC C 3'
Random primer 4	5' GTG TGC CCC A 3'

Table 2: Cell density of the yeast isolates at various levels of Ethanol concentration (absorbance at 595nm).

Sl.No.	Strains	6%	7%	8%	9%	10%	11%	12%	12.50%	13%	13.50%	14%	14.50%
1	YGP	1.483	1.286	0.92	0.316	0.265	0.152	0.142	0.135	0.101	0.064	0.061	0.054
2	YMO	1.729	1.673	1.488	0.749	0.658	0.456	0.276	0.13	0.097	0.067	0.046	0.044
3	YMI	1.273	1.261	1.242	1.013	0.891	0.674	0.252	0.097	0.047	0.037	0.034	0.025
4	YCA	1.449	1.447	1.239	1.214	0.786	0.484	0.135	0.126	0.121	0.095	0.074	0.07
5	YDE	2.097	1.934	1.754	1.576	1.39	0.882	0.488	0.04	0.038	0.035	0.033	0.011
6	YSM	1.716	1.658	0.464	0.413	0.054	0.05	0.03	0.028	0.022	0.021	0.014	0.009
7	YSC	2.016	1.888	1.378	0.342	0.284	0.064	0.06	0.041	0.04	0.038	0.031	0.016