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Isolation and Characterization of Yeast Strains for Bio-ethanol Production

H.B. Manoj Kumar*[1], K.M. Harini Kumar[1], S.B. Yogananda[2], H.V. Vijay Kumar Swamy[1], T.E. Nagaraja[2], S.V. Sunil Kumar[3] and Ningaraj Dalawai[1]

[1]Department of Plant Biotechnology, UAS, GKVK, Bangalore - 560 065, India.
[2]Zonal Agricultural Research Station, V. C. Farm, Mandya - 571 405, India.
[3]Department of Genetics & Plant Breeding, UAS, GKVK, Bangalore – 560 065, India.

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Yeast strains are commonly associated with sugar rich environments. Ethanol production by yeasts has been extensively studied, the aimof this study was to isolate and characterize yeast to produce ethanol. The investigation was carried out to isolate and characterization of yeast strains from their natural habitats and to screen them for ethanol tolerance and ethanol production. Out of 40 microbial cultures 10 were identified as *Saccharomyces* strains 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. *Saccharomyces* species were screened for the ability to tolerate different ethanol concentrations from 0-18%. Growth in different ethanol concentrations varied from one strain to another. Yeast strains showed tolerance level from 7-16%. Even though some strains had tolerance at 16% but the growth was less. Yeast from molasses and grapes showed highest tolerance among 10 isolates up to 16. The study was aimed at determining the genetic variability and efficiency among yeast strains. Out of twenty five primers, eight primers were shown polymorphism and successful in amplifying DNA in the sample. Analysis was employed to characterize yeast isolates. Ten *Saccharomyces* strains were subjected to ADH specific primer analysis using eight primers. Cluster diagram was divided into 2 major cluster and 3 sub cluster, two major cluster showing 30% dissimilarity. There was correlation between ethanol tolerance and genetic relatedness shown by ADH specific primer analysis.

Key words: Yeast, Bio-Ethanol, ADH-Specific primers.

Increased interest on alternative fuels has been observed in the past few years, as a result of increasing energy demand and forecasted depletion of fossil resources [Saxena *et al*., 2009]. Global warming and the consequent need to diminish greenhouse gases emissions have encouraged the use of fuels produced from biomass [Gupta. R. *et al.*, 2009], which is the only renewable carbon source that can be efficiently converted into solid, liquid or gaseous fuels [Balat M *et al*., 2008].

In the future, oil resources will be exhausted. This knowledge has stimulated interest in one of mankind's oldest chemical processes: the production of bio-ethanol from sugars by fermentation. (Slaa*et al*., 2009). Ethanol is the most consumed biofuel in the world. Brazil is the country that first introduced this renewable fuel in its energy matrix. A large industry arose from this pioneering initiative, and nowadays detains the most economically feasible process for Bioethanol

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

production (Luiz*et al.*, 2008). Today, the products of yeast impinge on 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 forceable future (Pretorius *et al*., 2003). A more general definition of fermentation is the chemical conversion of carbohydrate into alcohol or acids. The yeast species *Saccharomyces cerevisiae* has been used in baking as well as in brewing industry for production of alcoholic beverages for thousands of years. It is extremely important as a model organism in modern cell biology research for alcohol production and is one of the most thoroughly researched eukaryotic organisms (Dake*et al.*, 2010). Yeast alcohol 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*. Therefore strong economic incentives can be revealed by improving production processes resulting in a substantial growth for the ethanol industry in the near future (Carlos *et al*., 2011).

MATERIALS 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 500-milliliter 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 and sugarcane molasses were selected as sources for isolating yeast cells, Samples were collected from ZARS, Jaggery Park, V. C. Farm, Mandya. Grape juice, Apple juice, Mosambi, Pomegranate, Pineapple, Watermelon, Muskmelon and Honey were also used as sources for isolation of yeast which were procured locally

Protocol for isolation of yeast

The sugarcane juice and molasses 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 from10^{-3} and 10^{-4} were plated on YEPDA medium. The plates were incubated at 30°C for 48 h.

Maintenance of yeast isolates

Purified Yeast isolates were maintained using glycerol stocks and the isolates were preserved at -20°.

Screening of the isolated yeast strains for ethanol tolerance

Ethanol tolerance is 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 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.

Ethanol tolerance of each isolates was studied by allowing the yeasts to grow in YEPD broth having different concentrations of ethanol (Osho, 2005).

Molecular Characterization of Yeast strains using ADH specific primer

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

Isolation of DNA

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

Quantification of DNA

The exact amount of DNA was quantified by taking the spectrophotometer readings at a wavelength of 260 nm.

DNA concentration. (µg/ml) = [OD_{260} x 50 µg/ml]/5

PCR amplification

The genotypic identification of ethanol tolerant yeast was conducted by reading of the nucleotide sequence. The PCR reaction was done in a 20 µl reaction mixture using 1.0 µl of Taq

polymerase (3U), 2.5 µl buffer, $MgCl_2$ (1.5 mM), 3.0 µl dNTPs (0.2 mM) and 1.5 µl eachprimers (500 nM). The reaction was run in a Thermal cycler by heating at 94 °C for 5 min, and then amplification was performed with 30 cycles at 94 °C for 30 second, 53 °C for 30 second and 72 °C for 120 second, followed by elongation at 72 °C for 10 min. The amplification result was detected on 1.2% agarose gel electrophoresis staining with ethidium bromide. The gel was visualized under UV light and documented using Gel Documentation unit.

Specific primers were designed using the *Saccharomyces cerevisiae* genome database **(SGD;www.yeastgenome.org)**.

Scoring the data

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). 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:

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

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 AND DISCUSSION

Recent studies have focused mainly on the genetic modification of *S. cerevisiae* to improve ethanol yields and efficient bioconversion of various substrates to alcohol (Cao *et al.*, 1996). The budding yeast, *Saccharomyces* spp. has enjoyed a long and distinguished history in the fermentation industry

Isolation of yeast

In the present study, 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 *(Slavikova and Vadkertiova, 2003 and Suh et al., 2005);* fluxes of trees, insects and insect frass, flowers (Miller *et al.*, 1962); molasses, fruits, soil, and fermented foods (Bajaj *et al*., 2001); honey (Jermini*et al.,* 1987).

Identification of yeast isolates

Yeast isolates were identified upto 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).

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 5% to 18%. Strains had tolerance levels from 7% to 15%. Even though some strains had tolerance at 16% but the growth was less. Strains like YMS and YGP showed highest tolerance among 10 isolates up to 15% and strain YPA had least with 9%. Ethanol tolerance of all the ten strains is given in Table 2 which reveals cell density of strains at different ethanol stress. Osho (2005),Isolated yeasts from fermenting Cashew apples and reported that isolates had ethanol tolerance level from 9-12%. Unaldi*et al.,* (2002) reported that the maximum alcohol tolerance was found to be 9% (v/v) in yeast strains which were isolated from grapes.

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

Low concentrations of DNA are recommended because abundance of template DNA will favour annealing of the two strands of template. Therefore small quantities of template DNA is sufficient for optimal amplification (Baumforth*et al*., 1991). The method adopted for DNA extraction in the present study was well suitable for amplification of ADH-specific primers

and DNA extraction protocol outlined in material and methods resulted in optimum yield of DNA suitable for PCR.

PCR analysis

The study was aimed at determining the genetic variability and efficiency among yeast strains. Eight primers (Table-1) of yeastwere successful in amplifying DNA in thesample *viz.*, ADH1, ADH2, ADH3, ADH4, ADH6, ADH5, ADH7 and ADH8 primers, *Saccharomyces* sppstrains used on wine production identifying variations at DNA level among the yeasts (Ratón, 2004).

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. Dendogram was plotted using statistica version 2.0 unweighted pair-group arithmetic mean (UPGMA). Dendogram showed that YGP, YMS, YAP, YSC and YMO strains belong to major cluster I and strains like YPO, YMM, YHY, YWM and YPA belong to major cluster II.

YGP, YMS and YAP belong to sub cluster I, hence they are genetically closely related and also they have shown highest ethanol tolerance. YSC and YMO belong to sub cluster II, hence they are genetically closely related and also they have shown moderate ethanol tolerance. YPO, YMM, YHY, YWM and YPA belong to sub cluster III, hence they are genetically closely related and also

Table 1. Polymorphic ADH-specific markers, their flanking primer sequences

S. No	Locus	Primer Sequences (5Œ'-3Œ')
1	ARS304 (ADH1)	F:GCAAAGTTATTATGTTAAAGAAAAAG
		R:ATATTCGTTGTAAACTCATATACTTA
2	ARS310	F:ACGTCTCCTCCAAGCCC
	(ADH2)	R:CATATTCCCTAGAAAAA
3	ARS313(ADH3)	F:GAAAAGATTTGATGAAGACCAA
		R:AAGCTACTTTTAAATAAGTTTT
4	ARS923(ADH4)	F:CTAGTGCTTAAGTTCT
		R:GAGTTTAATTTGTTGT
6	ARS 207(ADH6)	F:GTGTTAGTACGTTAAATGCTACGACT
		R:TTGAGTTTGTACAAAGGAAAGCTGTA
7	ARS111(ADH7)	F:CGGCTATACAACCATATTTAAGTAAA
		R:GCAAAAACTCCTCTTTGTCTT
8	ARS1218	F:TTTTTTTAAGGGAAAATGCAAGCGTTTT
	(ADH8)	R:CACCGATTTTTTGGATAAAATGTATTC

Specific primers were designed using the *Saccharomyces cerevisiae* genome database (SGD;www.yeastgenome.org).

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

Strains	0%	5%	7%	9%	10%	12.5%	15%	16%	17%	18%
Standard yeast	2.687	2.468	2.312	2.256	1.568	1.210	1.101	0.632	0.431	0.088
YGP	2.926	2.738	2.718	2.431	1.987	1.726	1.234	0.908	0.395	0.108
YAP	2.721	2.569	2.426	2.356	1.916	1.786	1.187	0.801	0.310	0.096
YMO	2.026	1.936	1.738	1.649	1.601	1.428	1.131	0.326	0.120	0.081
YMS	2.938	2.791	2.686	2.401	2.006	1.861	1.311	0.973	0.685	0.292
YSC	2.325	2.108	2.001	1.910	1.732	1.522	1.227	0.589	0.222	0.086
YWM	1.686	1.538	1.501	1.412	1.382	1.208	0.926	0.401	0.216	0.089
YMM	1.712	1.601	1.589	1.401	1.298	0.906	0.621	0.210	0.086	0.048
YPA	1.426	1.130	1.001	0.812	0.726	0.608	0.422	0.138	0.032	0.015
YPO	2.086	1.831	1.726	1.578	1.311	0.813	0.682	0.411	0.296	0.093
YHY	1.701	1.526	1.395	1.301	0.822	0.686	0.382	0.110	0.061	0.029

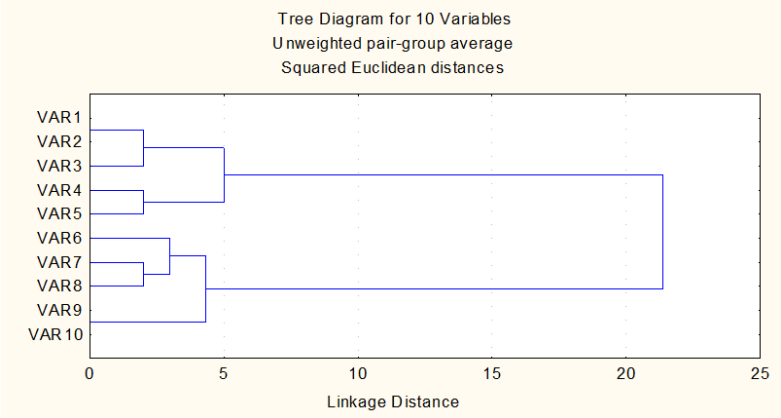
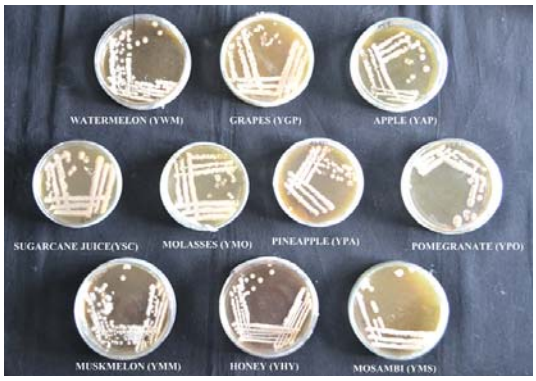


Fig. 1. Dendrogram based on ADH-specific primer profile of ten yeast strainsobtained from different samples (VAR1-YGP, VAR2-YMS, VAR3-YAP, VAR4-YSC, VAR5-YMO, VAR6-YPO, VAR7-YMM, VAR8-YHY, VAR9-YWM & VAR10-YPA)



Isolated yeast colonies

they have shown low ethanol tolerance. The obtained isolated group of *Saccharomyces spp* has similarity ranging from 30 – 100%. These results are similar to with other winery yeasts (Versavaud *et al.*, 1995).

Cluster analysis

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 in to account. This dendrogram were obtained from the binary data deduced from the DNA profiles of the samples analyzed (Fig 1). The tree cluster diagram was constructed for ten yeast strains using eight primers. Cluster diagram was divided into 2 major cluster and 3 sub cluster, two major cluster showing 30% dissimilarity. In first cluster five yeast strains like YGP, YMS, YAP, YSC and YMO were found, the remaining yeast strains like YPO, YMM, YHY, YWM and YPA were found in second cluster.

CONCLUSION

The ability of different yeast strain to produce ethanol was investigated. The data collected from the study concludes, YGP, YMS, YSC and YAP isolates which showed high tolerance to ethanol stress and YPO, YMM, YHY, YWM isolates showed medium tolerance and YPA isolate showed low tolerance. ADH specific primer reflected polymorphism among *Saccharomyces* spp. and however there was a correlation between their genetic makeup and ethanol tolerance.

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