

Screening of Efficient Ethanol Tolerant Yeast Strain for Production of Bio-ethanol

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(Received: 17 September 2015; accepted: 30 November 2015)

Bio-ethanol is a readily available, clean fuel for combustion engines made from plant-based feedstock s. It produces considerably lower gas emissions on combustion and it only releases the same amount of carbon- dioxide (CO₂) as plants bound while growing. With advanced, energy saving production technology bio-ethanol can considerably reduce the climate relevant greenhouse gas emissions from transport and traffic. In recent years, largely in response to uncertain fuel supply and efforts to reduce carbon dioxide emissions, bio-ethanol has become one of the most promising biofuels today and is considered as the only feasible short to medium alternative to fossil transport fuels. Bio-ethanol is seen as a good fuel alternative because the source crops can be grown renewably and in most climates around the world. In addition the use of bioethanol is generally CO₂ neutral. This is achieved because in the growing phase of the source crop, CO₂ is absorbed by the plant and oxygen is released in the same volume that CO₂ is produced in the combustion of the fuel. It balances the carbon cycle on the earth. This creates an obvious advantage over fossil fuels which only emit CO₂ as well as other poisonous emissions. The Ethanol tolerance investigation was carried out to isolate yeast strains from their natural habitats and to screen them for ethanol tolerance and ethanol production. Out of 40 microbial culture¹⁰ were identified as *Saccharomyces* strains based on colony type and budding characters. *Saccharomyces* species were screened for the ability to tolerate 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

Key words: Yeast, *Saccharomyce*, Bio ethanol and Ethanol Tolerance.

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.

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 (Sondari *et al.*, 2006). High ethanol tolerant strains are able to extend the process of fermentation for longer time and produce distinct products in the presence of ethanol (Swiecilo *et al.*, 2000).

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

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produce ethanol. In 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. The main source of sugars for fermentation is starch. Americans obtain starch from corn. In Europe starch is obtained from potatoes whereas Asians use rice as a raw material. Brazil obtains most of its starch from cassava roots, although sugar cane provides much of its ethanol (Slaa *et al.*, 2009). Sugarcane (*Saccharum officinarum*) is a high biomass tropical crop and contains about 12–17% total sugars, of which 90% is sucrose (Wheals *et al.*, 1999). Sugar concentration in cane juice depends upon the variety, maturity and the time of harvest. Sugarcane juice normally has sufficient organic nutrients and minerals in addition to fermentable sugars, and thus could be considered as an ideal substrate for ethanol production. Ethanol from sugarcane juice is being produced commercially in Brazil (Moreira, 2000). 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).

MATERIAL AND METHODS

Preparation of Media and Isolation of yeast from different sources

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.

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, 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

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.

Screening of the isolated yeast strains for ethanol tolerance

Screening of the isolated yeast strains for ethanol tolerance The 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. The ethanol tolerance of each isolates was studied by allowing the

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 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, (2001). The specific amount of DNA was quantified by taking the spectrophotometer readings at a wavelength of 260 nm Primers used in SSR and ADH-specific primer Analysis are SCYOR267C, C5, C11, SC8132X, ADH1, ADH3, ADH4, ADH7

RESULTS

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.

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

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%. Ethanol tolerance of all the ten strains is given in Table 5 which reveals cell density of strains at different ethanol stress. The same has been plotted on graph in Fig-1. and Fig-2 shows selected samples (YBA, YFN, YPA, and YSJ).

PCR The study was aimed at determining 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 ADH7 primers, For all loci, 20µl reaction mixture containing genomic DNA concentration



Yeast strains placed in different concentrations of ethanol for screening them for ethanol tolerance



Fermentation setup for different yeast samples

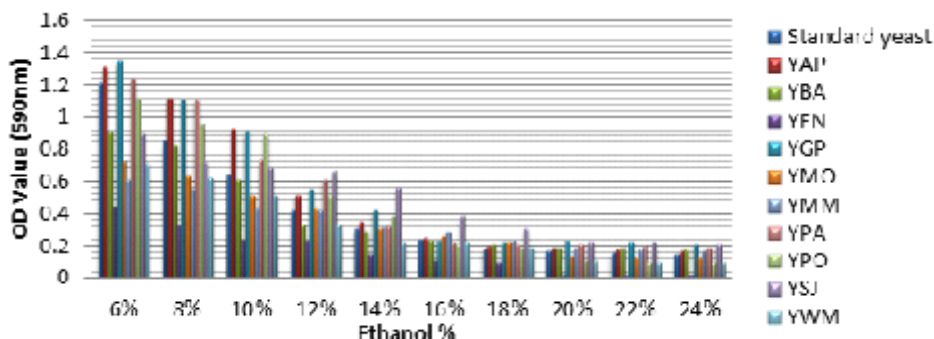


Fig. 1. Yeast strains with very high tolerance

of 25 ng/ml, primer concentration of 10 pM/μl and dNTPs 2.5mM, 10X buffer and 1 unit of *Taq* polymerase were used.

The cluster diagram was constructed for ten yeast strains using eight primers. 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). 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

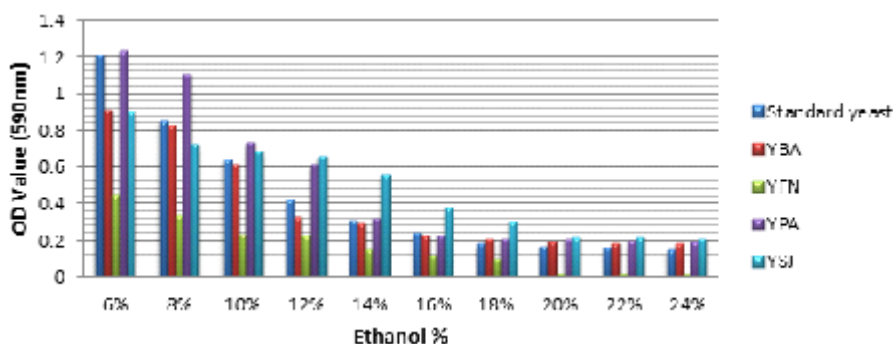


Fig. 2. yeast selected strains with tolerance level to ethanol

Table 1. Cell density of the yeast isolates at various levels of Ethanol concentration (absorbance at 595nm)

Strains	6%	8%	10%	12%	14%	16%	18%	20%	22%	24%
Standard yeast	1.21	0.852	0.641	0.416	0.306	0.244	0.18	0.169	0.158	0.145
YAP	1.31	1.12	0.922	0.513	0.347	0.253	0.203	0.186	0.175	0.166
YBA	0.91	0.825	0.614	0.328	0.294	0.225	0.205	0.191	0.185	0.182
YFN	0.445	0.335	0.237	0.227	0.15	0.113	0.098	0.021	0.019	0.018
YGP	1.345	1.11	0.915	0.557	0.425	0.232	0.218	0.228	0.215	0.211
YMO	0.725	0.628	0.512	0.428	0.305	0.261	0.215	0.137	0.13	0.125
YMM	0.614	0.553	0.432	0.424	0.315	0.291	0.231	0.192	0.175	0.169
YPA	1.235	1.11	0.732	0.613	0.318	0.221	0.212	0.211	0.198	0.193
YPO	1.115	0.952	0.893	0.511	0.377	0.212	0.187	0.113	0.102	0.101
YSJ	0.899	0.723	0.679	0.66	0.559	0.377	0.305	0.218	0.214	0.21
YWM	0.709	0.623	0.51	0.329	0.217	0.221	0.189	0.108	0.101	0.098

P-Value is Less than 0.05, Conclude That Ethanol, Yeast, (Ethanol*Yeast) Interaction Effects Have Shown Significant. 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.

Table 2. Ethanol tolerance of yeast strains

Ethanol tolerance	Strains Isolated
Below 8%	NIL
08%-10%	YPA
10%-12%	YAP, YBA, YWM, YPO
12%-14%	YFN, YMO, YMM
14%-16%	YGP, YSJ
>16%	NIL

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

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.

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 (Versavaud *et al.*, 1995).

CONCLUSION

Yeast strains were from sugar rich sources 10 were identified as *Saccharomyces spp.* *Saccharomyces spp.* were evaluated for their ethanol tolerance and showed good growth in

medium containing 8-15% ethanol. YSJ isolate which showed high tolerance to ethanol stress and YPA with low tolerance were showed decreased growth under high ethanol concentration compared to original isolates. 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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