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Improvement of Yeast Strains by Mutation for Enhancement of Ethanol Tolerance

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Abstract

The improvement of yeast strains (*Saccharomyces cerevisiae*) is getting more attention due to its high fermentation capacity that can be used as a renewable energy product. Uncovering genetic control of variation in ethanol tolerance in natural populations of yeast *Saccharomyces cerevisiae* is essential for understanding the evolution of fermentation. In order to obtain mutant strains showing higher bioethanol production than wild-type strains, a commercial *Saccharomyces cerevisiae* type was subjected to mutagenesis using ethyl methane sulfonate (EMS). Hence Ethanol tolerance investigation was carried out to isolate yeast strains from their natural habitats and to screen them for ethanol tolerance and ethanol production. *Saccharomyces* spp. was subjected to EMS (Ethylmethanesulphate) mutation of different concentration *i.e.,* 0.1M, 0.2M, 0.3M, 0.4M in which 0.1M and 0.2M EMS (Ethyl methane sulphate) showed good growth of ethanol tolerance. *Saccharomyces* spp. were screened for their ethanol tolerance and showed good growth in medium containing 6-14 per cent ethanol. YPA and YSP isolates showed higher tolerance to ethanol stress and YOR with low tolerance while other isolates showed decreased growth under high ethanol concentration compared to original isolates. SSR profiling reflected polymorphism among *Saccharomyces* spp. However there was correlation between their genetic makeup and ethanol tolerance.

Keywords: Yeast strains, *Saccharomyces cerevisiae,* EMS, Bioethanol

Ethanol production is among the oldest expertise that is being practised. Commercially, it is produced by fermentation of cereal grains, different fruits, molasses or other materials which are rich in sugar and starch content. The yeast genus *Saccharomyces* is principle biological agents of fermentation which is used to catalyses alcoholic fermentation to ethanol making. The fermentation method involves change of sugars to alcohol and carbon dioxide by the yeast *Saccharomyces cerevisiae.* In current years, yeast strains of *Saccharomyces cerevisiae* were widely studied for biotechnological property being improved. The important criteria to select *Saccharomyces cerevisiae* is to tolerate various stresses with respect to change in temperature, additional substrate and pH, chemical mutation for efficient ethanol fermentation.

Current industrial production relies on crop-based materials, like sugarcane and corn starch, which are also used for food and fodder. This competition for substrate drives up the price of both food and fuel; in 2008, 23 per cent of the total United States corn crop was used to produce ethanol, yet this resulted in supplying only 2 per cent of the total transportation requirement (Demain *et al*., 2009). According to EIA, the industry churned out 15.84 billion gallons of ethanol, up 3% from the 2017 total and a four-fold increase over the 3.91 billion gallons produced in 2005 when the original RFS (Renewable fuel standards) was adopted. The data also preliminarily indicated record domestic ethanol blending, with 14.4 billion gallons blended into 142.9 billion gallons of finished gasoline, equating to a record average blend rate of 10.08%.

At present, about 90 per cent of energy is generated from fossil fuels and only about 10 per cent is produced from renewable energy sources. The forecasted energy demand increases make evident that the conventional oil reserves that can be commercially exploited will be vanished after 2050.

The combustion of coal, oil and natural gas emits more than 6 billion tones of CO_2 annually in the atmosphere.

According to the Inter governmental Panel on Climate Change, the average concentration of CO_2 increased from 315 ppm in 1960 to 380 ppm in the year 2007 and there has been a 35 per cent increase in CO_2 emission worldwide since 1990. In this regard, mitigation of CO_2 by biological means has been gaining the momentum because it leads to the generation of energy from biomass grown by CO_2 fixation through photosynthesis (Kondili *et al.,* 2007). According to the Intergovernmental Panel on Climate Change the average concentration of CO_2 increased from 408.84 ppm in 2017 to 410.79 ppm in 2018 (Ekwurzel *et al*., 2018).

Being the most important bioethanol producer, the yeast *Saccharomyces cerevisiae* has obtained main place amongst biofuel-producing organisms. However, unprecedented difficulties and challenges for yeast biotechnology are needed to be positioned ahead, as upcoming future biofuels will have to be formed on a huge scale from sustainable kind of feedstock, so that it should not interfere with food assembly and are usually not the traditional carbon basis intended for *S. cerevisiae.* Moreover, the recent tendency in the growth of biofuels is to create molecules that capable to be used as drop-in fuel for active engines. Their properties should subsequently be further linked to those of oil-derived fuel than those of ethanol. Current challenges and developments lying ahead for price-effective making of such designed biofuels, using *S. Cerevisiae* based cell factories (Petrovic, 2015).

The two mainly common types of bio fuels in make use of today are biodiesel and ethanol. Ethanol is an alcohol, the similar as in beer, wine and alcoholic drink (although ethanol used the same as a fuel is customized to craft it undrinkable). It is most generally ready by fermenting several biomasses high in sugar compound (carbohydrates) through a process related to beer brewing.

Now a days, yeast cell mutants are extensively applied in molecular and cellular studies. In order to change the genome structure in different microorganisms, various methods have already been applied, such as random and site-directed mutagenesis . Ultraviolet ray, transposons, and ethyl methane sulfonate (EMS) are used to induce random mutagenesis. In addition, transposons are used to identify genes responsible for ethanol tolerance and cell wall biosynthesis in *S. cerevisiae*. EMS is an alkylating agent that induces point mutagenesis by A-T transition to G-C.

Material and Methods

The experiments on isolation and molecular characterization of natural and mutated yeast strains for high ethanol tolerance were carried out at Biofuel laboratory in the Department of Plant Biotechnology, GKVK, UAS, Bengaluru.

Isolation of yeast strains

Preparation of media

Yeast Extract Peptone Dextrose Agar (YEPDA) medium (Sambrook and Russell, 2001) was used intended for isolation of yeast strain. Media for petriplates were prepared in 500ml conical flasks. All components were separately weighed and mixed. Then the pH was adjusted to 5.6 before adding up of agar. Agar was melted earlier to autoclaving. Medium was autoclaved together for 15 min. at 121°C temperature and 15 psi pressure.

Isolation of yeast from different sources

Yeasts are naturally linked with sugar rich environment. Samples were collected from local market in Shakarnagar, and GKVK, Bengaluru. Apple, Orange, Papaya, Sapota, Pomegranate, Watermelon, Pineapple, Muskmelon fruits were used as source for separation of yeast strains which were procured locally on 21/9/2017.

Procedure for isolation of yeast

The fruits juices were collected in sterilized containers and kept back at room temperature. Fruit samples were rinsed and washed numerous times in distilled water to take away other contamination. They were then cut into pieces, squeezed and the juice was collected in separate sterile bottles.

Samples of the juice were in sequence diluted. From 10^{-3} and 10^{-4} of diluted samples of 0.1 ml of the samples were placed on YEPDA medium. The plates were incubated for 48 h at 30°C.

Microscopic observation

Different samples of isolated yeast cultures were considered for their development characteristics on cell morphology and YEPDA. Crystal violet stain was used for simple staining techniques for microscopic observation by means of 24 h old culture. The stained cells were observed by microscope with under oil immersion. Oblong shaped cell with budding character of yeast isolates observed under microscopic field were purified that was maintained on YEPDA slants. The budding characters and cell characters of yeast isolates was studied by microphotograph and subsequently microphotographs were recorded. The isolates were known with specific names for further experimentation and identification.

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*.

Yeast mutation

Mutation by EMS (Ethylmethanesulphate)

A comparative mutational study was executed by mutating the yeast isolates with inter-chelating mutagen Ethylmethanesulphate. The yeast isolates were mutated with different concentrations i.e. 0.1 M, 0.2M, 0.3M, 0.4M of Ethylmethanesulphate. The above concentrations were weighed and added to the culture medium to which the isolates gave high ethanol tolerance strain and low ethanol tolerance strain were inoculated and incubated at 30°C for 24 hrs.

Isolation of DNA

Yeast DNA was isolated by using procedure given in Sambrook and Russell, (2001)

Molecular characterization of yeast strains using SSR specific primer

Saccharomyces spp. isolated from 'diverse samples were used for SSR specific-PCR characterization.

Results and Discussion

Isolated *Saccharomyces* spp. were subjected to EMS (Ethylmethanesulphate) mutation and screened in the direction of evaluating their ethanol tolerance of both mutated strains as well as wild yeast.

The different yeast strains were mutated with different concentrations *i.e.,* 0.1 M, 0.2 M, 0.3 M, and 0.4 M of Ethylmethanesulphate. Among all these concentration 0.1 M and 0.2 M of Ethylmethanesulphate showed significantly good growth in different concentration of ethanol (Table 1, Table 2, Table 3, Table 4 and Table 5).

Among eight wild yeast strains, YPA showed highest optical density 1.025 and 0.845with 45.50 per cent and 37.55 per cent growth decline at 12 per cent and 14 per cent concentration of alcohol respectively. YOR and YPI yeast strains showed lowest ethanol tolerance of 0.357 and 0.358 with decline growth 46.12 per cent and 41.62 per cent at ethanol concentration of 14 per cent (Table 1).

Optimization of the fermentation performance of *S. cerevisiae*, regardless of the type of substrate, includes increasing the microorganism's tolerance to high ethanol concentrations. Wild types of yeast strains are more prone to contamination by bacteria. Normally traditional *S. cerevisiae* produced 8 to 12 per cent of ethanol tolerance. There optical density is less at 12 per cent and 14 per cent as compare to mutants due to intolerance of high ethanol. Traditional *S. cerevisiae* cannot survive at higher concentration of ethanol and pH environment.

Among eight strains, 0.1M Ethylmethanesulphate mutants yeast strains, YPA showed highest optical density 2.303 and 1.955 with 88.27 per cent and 74.93 per cent growth decline at 12 per cent and 14 per cent concentration of alcohol respectively (Table 2). YOR and YPI strains showed 0.288 and 0.456 with 39.66 per cent and 36.24 per cent growth decline at 14 per cent ethanol concentration (Table 2).

EMS is a mutagenic agent that induces point mutations in a DNA molecule by A-T transition to G-C. In the presence of EMS, native sequences of affected genes

TABLE 1

Growth of the wild yeast strains at different ethanol concentration (OD measured at 600nm)

Strains	Ethanol concentration (%)					
	0 %	6%	8%	10%	12%	14%
YPO	1.256 ^{b} (100)	0.985 ^{b} (78.17)	0.886 ^{b} (70.32)	0.654 ^{c} (52.00)	0.546 ^{c} (43.33)	0.456 ^{c} (36.19)
YPA	2.245 ^{a} (100)	1.899 ^{a} (84.40)	1.625 ^{a} (72.20)	1.256 ^{a} (55.50)	1.025 ^{a} (45.5)	0.845 ^{a} (37.55)
YOR	0.774 ^{e} (100)	0.623 ^{e} (80.50)	0.629 ^{f} (81.26)	0.544 ^{ef} (70.28)	0.428 ^{de} (55.30)	0.357 ^{d} (46.12)
YPI	0.860 ^{e} (100)	0.767 ^{e} (89.18)	0.765 ^{e} (88.96)	0.751 ^{d} (87.32)	0.531 ^{d} (61.44)	0.358 ^{d} (41.62)
YMU	1.192 ^{d} (100)	0.628 ^{d} (52.68)	0.531 ^{g} (44.55)	0.463 ^{f} (38.84)	0.356 ^{e} (29.86)	0.244 ^{e} (20.47)
YWM	1.434 ^{c} (100)	0.724 ^{de} (50.50)	0.639 ^{f} (44.56)	0.560 ^{e} (39.26)	0.333 ^{e} (23.22)	0.242 ^{e} (16.87)
YAP	1.564 ^{c} (100)	1.243 ^{c} (79.47)	1.032 ^{d} (66.11)	0.845 ^{d} (54.03)	0.432 ^{de} (27.62)	0.381 ^{d} (24.36)
YSP	1.896 ^{b} (100)	1.562 ^{b} (82.38)	1.473 ^{c} (77.69)	1.241 ^{b} (65.45)	0.995 ^{b} (52.48)	0.685 ^{b} (36.12)
Means	1.403	1.054	0.947	0.789	0.580	0.446
C.D	0.187	0.141	0.058	0.135	0.169	0.052
C.V at 1%	2.759	4.894	2.230	6.074	2.351	3.656

(Same alphabets within column represent not significant differences but different alphabets represent significant differences).
(Values in bracket () represent growth reduction with increase in concentration of ethanol)
Legend : YPO: Pomegranate, YPA: Papaya, YPI: Pineapple, YMU: Muskmelon,
YWM: Watermelon, YAP: Apple,YSP: Sapota, YOR: Orange

TABLE 2

Growth of the 0.1M Ethylmethanesulphate mutants yeast strains at different ethanol concentration (OD measured at 600nm)

Strains	Ethanol concentration (%)					
	0 %	6%	8%	10%	12%	14%
YPO	1.020 ^{cd}(100)	1.006 ^{f} (98.62)	0.885 ^{ef}(86.76)	0.890 ^{d} (87.25)	0.755 ^{d} (74.01)	0.558 ^{b} (54.70)
YPA	2.609 ^{a} (100)	2.606 ^{a} (99.88)	2.415 ^{a} (92.56)	2.408 ^{a} (92.29)	2.303 ^{a} (88.27)	1.955 ^{ab} (74.93)
YOR	0.726 ^{d} (100)	0.714 ^{g} (98.34)	0.654 ^{g} (90.08)	0.536 ^{g} (73.82)	0.402 ^{g} (55.37)	0.288 ^{c} (39.66)
YPI	1.258 ^{c} (100)	0.956 ^{f}(75.993)	0.856 ^{f} (68.04)	0.756 ^{f} (60.09)	0.654 ^{f} (51.98)	0.456 ^{bc} (36.24)
YMU	1.702 ^{b} (100)	1.356 ^{d} (79.67)	1.263 ^{d} (74.20)	0.872 ^{e} (51.23)	0.714 ^{e} (41.95)	0.544 ^{bc} (31.96)
YWM	1.750 ^{b} (100)	1.208 ^{e} (69.02)	0.909 ^{e} (51.94)	0.854 ^{e} (48.8)	0.622 ^{f} (35.54)	0.395 ^{bc} (22.57)
YAP	1.922 ^{b} (100)	1.707 ^{c} (88.81)	1.412 ^{c} (73.46)	1.125 ^{c} (58.53)	0.962 ^{e} (50.05)	0.576 ^{ab} (29.96)
YSP	2.144 ^{b} (100)	2.125 ^{b} (99.11)	1.913 ^{b} (89.22)	1.628 ^{b} (75.93)	1.439 ^{b} (67.11)	0.833 ^{a} (38.85)
Means	1.629	1.460	1.288	1.133	0.981	0.701
C.D	0.570	0.091	0.058	0.025	0.035	0.291
C.V at 1 %	13.16	2.149	2.293	0.765	1.132	25.043

(Same alphabets within column represent not significant differences but different alphabets represent significant differences).
(Values in bracket () represent growth reduction with increase in concentration of ethanol)
Legend : YPO: Pomegranate, YPA: Papaya, YPI: Pineapple, YMU: Muskmelon,
YWM: Watermelon, YAP: Apple,YSP: Sapota, YOR: Orange

are changed and their related products are modified structurally, causing inactivation of functional proteins. It is evident that fruits are very high in carbohydrates which includes reducing sugar present in papaya fruit. This high carbohydrate content shows that the papaya juice may be good source for ethanol tolerance. Higher and lower concentration of optical density of yeast depends upon the variety of fruits juice and environmental condition of fruits.

Wahlbom *et al*. (2010) and Khattab *et al.* (2009) reported that EMS is a suitable mutagen for related purposes. In addition, French et al., (2006) reported that EMS is a powerful chemical mutagen and its effect on cells is related to its concentration in a medium.

Mobini-Dehkordi *et al.* (2011) reported that the growth of wild-type cells was inhibited in the presence of ethanol at concentrations higher than 10 per cent (v/v), mutant cells grow in solid medium with 12 per cent (v/v) or higher concentrations of ethanol. The reason for the ability of a few yeast mutant cells to grow in the presence of 14 per cent (v/v) ethanol due the presence of an additional tolerance mechanism (s) or mutations in their non-vital genes. The findings about mutagenesis induced by EMS were in good agreement with the above reports and confirmed the usefulness of this potent mutagenic agent for inducing mutagenesis in *S. cerevisiae.*

Growth of 0.2M Ethylmethanesulphate mutants yeast strains growth declined with 74.66 per cent with increased in ethanol concentration of 14 per cent at 0.985 optical densities of YPA yeast strains. YOR yeast strains showed lowest value 0.454 with decline growth of 39.52 per cent at 14 per cent ethanol concentration (Table 3).

EMS application to isolation of mutant strains of *S. cerevisiae* that were able to tolerate high concentrations of ethanol and showed higher bioethanol production at 14 percent than the wild type due to transition effect of ethymethanesulphate. This observation is in agreement with Sharma's finding that

TABLE 3
Growth of the 0.2M Ethylmethanesulphate mutants yeast strains at different ethanol concentration (OD measured at 600nm)

Strains	Ethanol concentration (%)					
	0%	6%	8%	10%	12%	14%
YPO	1.241 ^{f} (100)	1.111 ^{g} (89.52)	0.855 ^{cd} (68.89)	0.850 ^{c} (68.89)	0.750 ^{c} (60.43)	0.665 ^{f} (53.58)
YPA	2.333 ^{b} (100)	2.323 ^{a} (99.57)	2.141 ^{a} (91.88)	2.165 ^{a} (90.11)	1.738 ^{a} (74.55)	0.985 ^{a} (42.27)
YOR	0.826 ^{g} (100)	0.739 ^{h} (89.46)	0.631 ^{e} (76.39)	0.522 ^{e} (63.19)	0.588 ^{d} (62.13)	0.454 ^{g} (54.96)
YPI	1.358 ^{a} (100)	0.986 ^{e} (72.61)	0.834 ^{c} (61.41)	0.765 ^{cd} (56.33)	0.608 ^{a} (44.77)	0.489 ^{b} (36.00)
YMU	1.423 ^{e} (100)	1.443 ^{d} (99.12)	0.761 ^{de} (53.47)	0.669 ^{d} (47.61)	0.568 ^{d} (39.91)	0.431 ^{g} (30.28)
YWM	1.868 ^{d} (100)	1.274 ^{f} (68.20)	1.666 ^{b} (89.18)	1.462 ^{b} (78.26)	1.259 ^{b} (67.39)	1.265 ^{c} (67.71)
YAP	2.136 ^{c} (100)	1.752 ^{c} (82.02)	1.584 ^{b} (74.15)	1.570 ^{b} (73.50)	1.208 ^{b} (56.55)	0.869 ^{e} (40.68)
YSP	2.252 ^{bc}(100)	2.116 ^{b} (93.96)	2.151 ^{a} (95.51)	1.554 ^{b} (69.00)	1.199 ^{b} (53.28)	0.856 ^{d} (38.01)
Means	1.681	1.468	1.328	1.194	0.990	0.752
C.D	0.169	0.036	0.224	0.163	0.097	0.040
C.V	3.811	0.949	6.192	5.002	3.588	1.727

(Same alphabets within column represent not significant differences but different alphabets represent significant differences).
(Values in bracket () represent growth reduction with increase in concentration of ethanol)
Legend : YPO: Pomegranate, YPA: Papaya, YPI: Pineapple, YMU: Muskmelon, YWM: Watermelon, YAP: Apple,YSP: Sapota, YOR: Orange

chemical mutants (EMS) have a role in ethanol tolerance. Sharma (2011) reported Mut1, mut2, and mut3 from yeast obtained from fruit juices were more resistant to ethanol and showed higher growth rates in the presence of 14 per cent (v/v) ethanol.

Among eight 0.3 M mutated yeast strains, YPA showed highest optical density 1.256 and 0.946 with 62.85 per cent and 47.33 per cent growth decline at 12 per cent and 14 per cent concentration of alcohol respectively. YOR and YPI yeast strains showed lowest ethanol tolerance of 0.145 and 0.124 with decline growth of 27.62 per cent and 10.71 per cent at 14 per cent ethanol concentration (Table 4).

Increases in the concentration of EMS have deleterious effect on the *S. cerevisiae.* So with 0.3M Ethylmethanesulphate optical density of YPA is decreased as compare to 0.1 M Ethylmethanesulphate due to overdose of chemical mutants. Dehkordi *et al*., 2008 reported deteterious effect of EMS chemical mutants on living organisms.

Growth of 0.4M Ethylmethanesulphate mutants yeast declined with 29.79 per cent with increased in ethanol concentration of 14per cent at 0.553 optical densities of YPA yeast strains. YOR yeast strains showed lowest value 0.162 with decline growth of 30.62 per cent at 14 per cent ethanol concentration (Table 5).

At 12 per cent ethanol concentration 0.1M EMS mutants YPA (2.303) , YSP (1.439) and 0.2M EMS mutants YPA (1.73) , YSP (1.199) showed highest ethanol tolerance than that of wild type whereas 0.1M and 0.2M EMS mutant YOR (0.588) showed lowest ethanol tolerance among eight strains.

Among eight strains used in experiment 0.1M EMS mutant, YPA (1.955) has shown highly significant ethanol tolerance than that of wild type at 12 per cent ethanol tolerance level. YOR (0.288) mutant of 0.1M

TABLE 4

Growth of the 0.3M Ethylmethanesulphate mutated yeast strains at different ethanol concentration (OD measured at 600nm)

Strains	Ethanol concentration (%)					
	0 %	6%	8%	10%	12%	14%
YPO	0.824 ^{d} (100)	0.751 ^{d} (91.14)	0.655 ^{e} (79.49)	0.569 ^{f} (69.05)	0.658 ^{c} (79.85)	0.149 ^{de} (18.08)
YPA	2.001 ^{a} (100)	1.751 ^{a} (87.55)	1.662 ^{a} (83.11)	1.539 ^{a} (76.95)	1.256 ^{a} (62.81)	0.946 ^{a} (47.3)
YOR	0.524 ^{f} (100)	0.559 ^{e} (99.27)	0.452 ^{f} (86.25)	0.236 ^{h} (45.03)	0.416 ^{e} (80)	0.145 ^{e} (27.6)
YPI	0.865 ^{a} (100)	0.758 ^{a} (87.6)	0.563 ^{b} (65.4)	0.456 ^{b} (52.71)	0.339 ^{b} (39.19)	0.124 ^{b} (10.71)
YMU	0.728 ^{e} (100)	0.531 ^{e} (72.93)	0.448 ^{f} (62.22)	0.337 ^{g} (46.29)	0.439 ^{d} (60.36)	0.189 ^{d} (25.96)
YWM	1.276 ^{c} (100)	1.249 ^{b} (97.63)	1.242 ^{c} (97.61)	1.131 ^{c} (89.05)	1.107 ^{c} (86.75)	0.149 ^{de} (11.73)
YAP	1.532 ^{b} (100)	1.143 ^{c} (74.70)	0.856 ^{d} (55.94)	0.658 ^{e} (43.00)	0.886 ^{c} (57.90)	0.134 ^{e} (8.75)
YSP	1.554 ^{b} (100)	1.272 ^{b} (82.06)	1.260 ^{c} (81.29)	1.046 ^{d} (67.48)	1.193 ^{c} (76.96)	0.733 ^{c} (49.22)
Means	1.125	1.016	0.861	0.627	0.647	0.236
C.D	0.119	0.048	0.172	0.058	0.054	0.055
C.V at 1 %	3.744	1.769	7.173	2.854	4.489	7.892

(Same alphabets within column represent not significant differences but different alphabets represent significant differences).
(Values in bracket () represent growth reduction with increase in concentration of ethanol)
Legend : YPO: Pomegranate, YPA: Papaya, YPI: Pineapple, YMU: Muskmelon, YWM: Watermelon, YAP: Apple,YSP: Sapota, YOR: Orange

TABLE 5

Growth of the 0.4M Ethylmethanesulphate mutated yeast strains at different ethanol concentration (OD measured at 600nm)

Strains	Ethanol concentration (%)					
	0 %	6%	8%	10%	12%	14%
YPO	0.867 ^{c} (100)	0.777 ^{e} (89.61)	0.661 ^{e} (76.23)	0.549 ^{c} (63.32)	0.432 ^{d} (49.82)	0.366 ^{d} (42.21)
YPA	1.856 ^{a} (100)	1.786 ^{a} (96.22)	1.456 ^{a} (78.44)	0.856 ^{a} (46.12)	0.689 ^{a} (37.12)	0.553 ^{a} (29.79)
YOR	0.529 ^{d} (100)	0.451 ^{g} (85.25)	0.346 ^{h} (65.40)	0.337 ^{e} (63.70)	0.276 ^{f} (52.17)	0.162 ^{f} (30.62)
YPI	0.957 ^{c} (100)	0.836 ^{d} (87.35)	0.865 ^{c} (90.38)	0.743 ^{b} (77.63)	0.635 ^{b} (66.35)	0.551 ^{b} (57.57)
YMU	0.857 ^{c} (100)	0.443 ^{g} (51.69)	0.567 ^{f} (66.16)	0.432 ^{d} (50.40)	0.343 ^{e} (40.02)	0.267 ^{e} (31.15)
YWM	0.844 ^{c} (100)	0.626 ^{f} (74.17)	0.454 ^{g} (53.79)	0.346 ^{e} (40.99)	0.263 ^{f} (37.16)	0.254 ^{e} (30.09)
YAP	0.858 ^{c} (100)	0.960 ^{c} (99.41)	0.736 ^{d} (85.78)	0.428 ^{d} (49.88)	0.343 ^{e} (39.97)	0.264 ^{e} (30.76)
YSP	1.393 ^{b} (100)	1.353 ^{b} (97.12)	1.216 ^{b} (87.27)	0.775 ^{b} (55.63)	0.545 ^{c} (39.12)	0.448 ^{c} (32.16)
Means	1.054	0.948	0.842	0.668	0.559	0.395
C.D	0.202	0.060	0.091	0.070	0.049	0.066
C.V at 1 %	8.054	2.718	4.532	4.421	3.702	7.042

(Same alphabets within column represent not significant differences but different alphabets represent significant differences).
(Values in bracket () represent growth reduction with increase in concentration of ethanol)
Legend : YPO: Pomegranate, YPA: Papaya, YPI: Pineapple, YMU: Muskmelon, YWM: Watermelon, YAP: Apple,YSP: Sapota, YOR: Orange

EMS showed lowest ethanol tolerance followed by YPI (0.456).

Dehkordi *et al*., 2008 reported EMS-treated of 0.1M and 0.2 M concentration yeast cells were grown on solid aerobic low-peptone medium containing 2 to 12 per cent (v/v) ethanol. EMS-treated of 0.3 M and 0.4 M concentration yeast cells showed less significant growth due to lethal effect. The mutant strains that tolerated high concentrations of ethanol were selected for bioethanol production in microfuge tubes containing fermentation medium.

Rotten fruit apple produced 9.5 per cent of bioethanol on 2nd day when yeast strains was treated with mutants EMS (Tiwari *et al*., 2014).

Neelakandan and Usharani (2009) also studied maximum ethanol tolerance of EMS mutated yeast strains papaya, apple, Sapota upto 8 to 14 per cent. Rotten papaya obtained 12.5 per cent of bioethanol on 3rd day.

PCR analysis

All SSR primers were successful in amplifying DNA in the sample *viz.*, YSSR-1, ETR-1, ETR-2, ETR-3 primers. SSR specific locus gene related with ethanol tolerance gene found in YPA and YSP Strains.

All gene amplicon had size of 4 180 bp in gene specific marker YSSR-1 which is positively correlated with ethanol tolerance gene specific marker (Fig. 1).

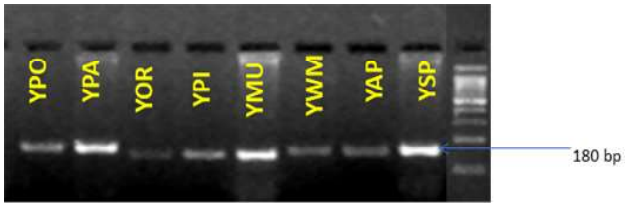

Fig. 1: Gel profile of natural yeast strains generated using YSSR 1 primer

The gene amplicon had a size of 4 100 to 200 bp when amplified using gene specific primer ETR-1 (Fig. 2). According to this result, YPA and YSP which were

shown highest ethanol tolerant activity doesn't have positive correlation with ETR -1 marker.

All the strains had shown positive gene correlation with ETR-2 primer (Fig. 3). All gene amplicon had size of 4 120 bp. None of the strains had shown presence of ETR -3 related gene for ethanol tolerance (Fig 4). This concluded that among all these SSR, markers YSSR-1 reflected polymorphism among Saccharomyces spp. There is correlation between their genetic makeup ethanol tolerance.

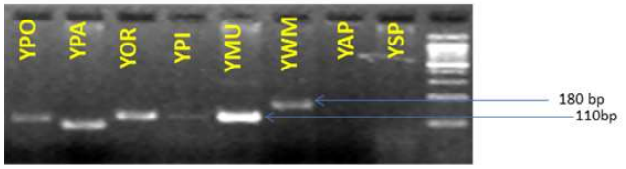

Fig. 2:Gel profile of natural yeast strains generated using ETR 1 primer

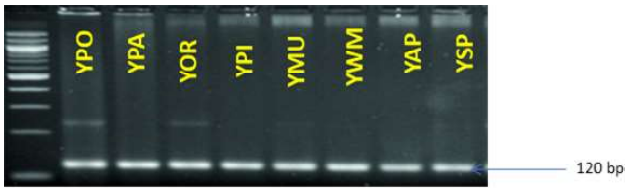

Fig. 3: Gel profile of natural yeast strains generated using ETR 2 primer

Brooks *et al.* (2015) reported that ETR specific primer had shown 180 bp amplicon in ten yeast strains which are specific for ethanol tolerant gene.

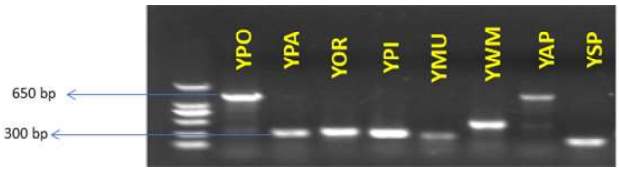

Fig 4: Gel profile of natural yeast strains generated using ETR 3 primer

Cluster analysis with SSR primers

Cluster diagram was divided into 2 major clusters at similarity coefficient, first major cluster includes strains like YPI, YOR, YMU and second major cluster was subdivided into 2 sub cluster at similarity coefficient (Fig. 5). But the first sub cluster included YPA, YSP with 0.8 (80 per cent) and second sub cluster included strains like YWM, YPO, YAP where YPO, YAP with similarity 0.8 (80per cent) (Table 6).

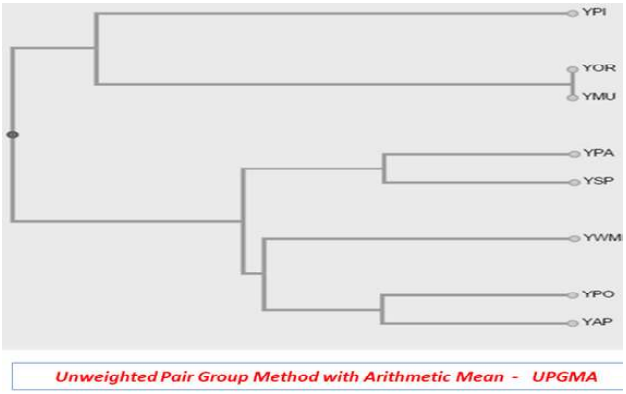

Fig. 5: Dendrogram based on SSR Markers of 8 strains of yeast from different sample

TABLE 6

Similarity Matrix of SSR markers computed with Jaccard coefficient

	YPO	YPA	YOR	YPI	YMU	YWM	YAP	YSP
YPO	1	0.5	0.5	0.25	0.5	0.75	0.8	0.6
YPA		1	0.4	0.2	0.4	0.6	0.667	0.8
YOR			1	0.5	1	0.667	0.4	0.5
YPI				1	0.5	0.333	0.2	0.25
YMU					1	0.667	0.4	0.5
YWM						1	0.6	0.75
YAP							1	0.8
YSP								1

Legend: YPO: Pomegranate, **YPA:** Papaya**, YPI:** Pineapple, **YMU:** Muskmelon, **YWM:** Watermelon, **YAP:** Apple, **YSP:** Sapota, **YOR:** Orange

According to Tikka *et al.,* (2013) the Cluster analysis based on 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**.** Isolates YSP, YPA, formed the first group; the isolates YSM (Yeast sorghum strains), YMI (Yeast mosambi strains), YMU, YWM 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.

Cluster analysis with SSR markers resulted in two cluster group that shows hierarchical relationship between different strains.

The study revealed that

1. Yeast strains were isolated from sugar rich sources and eight isolates were identified as *Saccharomyces* spp.
2. *Saccharomyces* spp. were subjected to EMS ethymethanesulphate) mutation of different concentration in which 0.1M and 0.2M EMS (ethymethanesulphate) showed good growth of ethanol tolerance.
3. *Saccharomyces* spp. were screened for their ethanol tolerance and showed good growth in medium containing 6-14 % ethanol.
4. YPA and YSP isolate which showed higher tolerance to ethanol stress and YOR with low tolerance, other isolates showed decreased growth under high ethanol concentration compared to original isolates.
5. SSR ADH-1 profiling reflected polymorphism among *Saccharomyces* spp. and however there was correlation between their genetic makeup and ethanol tolerance

Future line of work

Further intensive studies are thus required to develop engineered yeast that are capable of efficiently fermenting all sugars including D-xylose found in lignocellulosic hydrolysates to ethanol at a industrial scale.

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