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A Study on Yeast Micro Flora Isolated From Common Indian Fruits and Cheese

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ABSTRACT

In the present study twenty-five yeast cultures were isolated and purified from common Indian spoiled fruits and cheese, out of which, five yeast cultures were found to be yeasts. These strains were named as Y2, Y3, Y4, Y6 and Y10. These five yeasts strains were characterized and identified by morphological biochemical and physiological identification scheme. The results were compared with reference strain Saccharomyces cerevisiae. The yeast strains were identified by using different parameter and characterized with respect to temperature tolerance, ethanol tolerance, pH tolerance, sugar tolerance, killer strain, invasive growth and osmotolerance. It was found that isolated strains are capable of tolerating 15% (v/v) ethanol, 42ºC temperature, 2 to 5 pH, 19% NaCl etc. Selected yeast strains showed invasive growth Isolated yeast was found to be resistant to sugar upto 10% (w/v) glucose concentration. Growth curve study was done to analyze the actual time of maximum growth. Ethanol production was estimated at 28°C up to 72 h and was found to be higher in case of strains Y2 and Y3 (6.2%). In this study, Superior yeast strains capable of ethanol production were isolated and characterized.

Key words: *Yeast, fruit, cheese, production, fungus and strain.*

INTRODUCTION

Yeast is very common in enviroment. It is a eukaryotic fungus which is unicellular and mostly saprophytic. It is widely present in terrestrial, Ariel and aquatic habitats. Fungi taxonomy divided it into ascomycetes and basidomycetes[20], and it is estimated that there are about 1500 species of yeast exist[22]. Yeast were isolated from natural substances like soil, leaves, flowers, sweet fruits, grains, exudates of trees, insect and dung. Yeast are considered to be sugar loving so, it is it is habitually isolated from sugar rich materials like fruits very high sugar concentration is found in fruits, so yeast microflora can be easily isolated from fruits[41,42]. Fermenting wild yeast species are isolated and applied in fermentation process for over a decade[28]. described various methods for isolation and characterization of yeast from environmental samples. Yeast alcohol technology has undergone considerable improvements and modifications during the last decade still turnover margins are not striking.

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There are so many reasons like contamination, inadequate availability of raw materials and proper plan of fermentation process are the major boundaries due to which alcohol yields and quality get reduced. As the alcohol is an important alternative for liquid fuel, a number of investigations in ethanol fermentations are presently reported. The cost of the sugar source is a significant parameter when considering the overall economy of production and it is of great interest to optimize alcohol yields to ensure an efficient utilization of carbon sources[2]. Some of cost effective substrates for ethanol production include molasses. Molasses a non-crystallizable byproduct of sugarcane or sugar beet processing industries. It contains 60% sucrose and 40% non sucrose substances like raffinose, inorganic salts, organic acids and nitrogen containing compounds. It is most commonly used raw material because it is cost-effective, does not required starch hydrolysis provide value addition to byproduct and easily available. So a commercial substrate and efficient strains of yeasts have been selected for maximum ethanol production[43,6,17]. The most common fermentative yeast is *Saccharomyces cerevisiae which is widely* used in production of ethanol. It is able to use substrates like renewable biomass such as sugar cane or sugar beet molasses as the major carbon source[14,35]. As yeast have very important characteristics of fermentation, scientist are trying to find out the best yeast specifically which can tolerate high concentration of ethanol so that ethanol as a biofuel can be produced at commercial scale[11]. In addition to *S. cerevisiae*, it is urgent need to search for a novel, wild and non-toxic fermentative yeast species so that they can be exploited in industries such as fermentation industry, baking industry, therapeutic production etc[1,23]. The present study was intended to determine the potential of yeast isolated from the waste of ripe banana, orange and apple fruits for alcohol production. The outcome of this study may expand the utility fruit wastes. That would not only ensure a cleaner environment but also create more job opportunities, reduce seasonal losses of the fruits and serve as a substitute for produced alcohol by increasing their production. This research study reports on ethanol production from the over ripen fruits. We also tried to find about some unconventional yeast strains which can tolerate extreme stresses such as *Zygosaccromyces rauxii* which is osmotoelrant and *Brettenomyces like Dekkera bruxellensis considered to be ethanol tolerant*[32], The proposed research would, therefore, be directed towards the isolation of ethanol producing yeast strains from different fruits, with following objectives: a) to isolate yeast from natural enviroment like fruits. b) To investigate physiological characteristics of the isolates. c) To investigate the alcohol producing abilities of the isolates.

MATERIALS AND METHODS

Sampling site and sample collection: The micro organisms are derived from their natural habitat. Micro organism generally found on the raw materials which are having available carbon source and nutrient for their growth and multiplication. Therefore, overripe fruits (tomato, papaya, and banana) and cheese procured from the local market of Hisar (Haryana) was used for the isolation.

Isolation of Yeast from fruits and Cheese: Slightly over ripened fruits and cheese were crushed using sterile glass rods in a test tube containing sterilized distilled water. A thoroughly crushed raw material was subjected to vortexing and diluted. One ml of each of the sample was transferred to nine ml of sterile distilled water to be successively diluted to 10^{-1}up to 10^{-6}. Aliquots of 0.1 ml from final dilutions were spread plated on YEPDA[30]. YEPD broth was used as Inoculum medium. YEPD plates were streaked with a loopful of suspension and followed by incubation at 28^0C for 24-28 h. In natural habitats micro organisms usually grow in complex, mixed population containing several species. Therefore, multiple streaking steps were applied on YEPD plates to obtain a pure culture. The level of purity was investigated microscopically using wet mount technique. Finally, purified cultures were transferred to

YEPD slants in screw cap glass tubes and preserved at 4°C under refrigeration conditions

Maintenance of strain: The culture was grown at 28°C for 48 h in a medium containing 1% yeast extract, 2% peptone, 2% dextrose and 2% agar. The culture was stored at 4°C in a slant form and sub cultured at regular intervals in order to maintain viability. For long–term storage, stock cultures were maintained in 20% glycerol at -80°C.

Identification and characterization of yeast

Identification and characterization of isolated yeast strains was done by studying classical, morphological and biochemical characteristics[21]. and[20]. The morphology of the vegetative cells of yeast was grown in liquid and on solid media.

Identification scheme:

Morphological identification: Morphological identification was done by observing the colony, color, texture, shape, elevation[26].

Biochemical characterization: different tests were used to study the yeast biochemically. These includes carbohydrate fermentation test, urease test, 1% acetic acid test etc.

Physiological characterization: Physiological characterization was done by observing thermotolrence, pH tolerance, ethanol tolerance, sugar tolerance, oxidative stress, osmotolerance, killer capabilities and invasive growth by selected yeast strains.

Growth curve studies

Yeast strain was inoculated in YEPD inoculums broth and was incubated under stationary conditions at 28ºC for six days and samples were drawn at incremental time intervals spread over several days. Growth pattern was investigated using two different ways by spectrometric analysis and cell biomass studies.

Bioethanol production

Bioethanol production capacity of selected yeast strains was tested using molasses as a carbon substrate. Fermentation media was prepared using molasses (250g), Urea (0.1), conc. H_2SO_4 (3.30) in 500 ml Erlenmeyer flasks. The experiment was done in triplicates. After adding Inoculum of different yeasty in different flasks, flasks were incubated for 72 h at 28^{o}C. After incubation bioethanol production was estimated. Ethanol was determined with good precision by oxidation with acid dichromate solution[9]. and absorbance was measured at 660 nm.

RESULTS AND DISCUSSION

Isolation of Yeast strains: A total of twenty isolates were purified and out of which only five isolates were found to be yeast. Apple, orange, banana and other fruits locally available and thus serve as readily available raw materials for the separation of ethanol yeasts. Eghafona (1999) isolated various strains of indigenous yeasts capable of producing ethanol from local fermented pineapple juice[3]. and[18]. did comparative study on ethanol production from molasses using *Saccharomyces cerevisiae* and *Zymomonas mobilis.*

Morphological characterization: The cultures were identified as yeasts based on colony characters, microscopic examination and budding formation.

- **Colony characteristics:** Growth was observed on solid medium. Different type of colonies was formed by selected yeast strains. Colonies shape differ from circular to elongated, colony size varies from small to large as shown in table 1. The appearance of colonies was rough and smooth white to cream and color varies from in the young stage, the individual cells of yeast were oval, elongated, and ovoid to spherical but become hexagonal with when aged. Cells also showed different type of budding pattern like oval, globose, spherical, segmented and ellipsoidal. These yeast strains were designated as Y2, Y3, Y4, Y6 and Y10 (table 1) based on differences in colony color, appearance, morphology, color, appearance, shape size and margin.
- **Microscopic examination:** The isolated yeast strains were analyzed microscopically under 40X resolution of compound microscope using wet mount. Besides culture purity, the strain was also observed for cell size, shape and budding frequency. The strains were completely free of contamination and found to have adequate level of budding. Growth and pellicle formation was observed in liquid YEPD medium (table 2).

Table 1: Colony Characteristics of Yeast isolates

Yeast Isolate	Colony Color	Colony Nature	Appearance and Size	Elevation	Margin
Y2	Cream	Smooth	Circular & Small	Raised	Entire
Y3	Cream	Smooth	Yeast like & medium	Convex	Entire
Y4	Cream	Rough	Circular & Medium	Convex	Entire
Y6	Cream	Smooth	Ovoid & Small	Raised	Entire
Y10	Cream	Rough	Circular & Small	Convex	Entire

Table 2: Growth characteristics of Yeast isolates

Yeast Strains	Budding characters	Pellicle Formation	Sedimentation in YEPD medium	Ascospores		Tentatative genera designated
				Shape	Number	
Y2	Ellipsoidal	Yes	Yes	Ovoidal	4	Brettenomyces
Y3	Oval	No	Yes	Round	4	Saccromyces
Y4	Ellipsoidal	Yes	Yes	Ovoidal	2	Brettenomyces
Y6	Oval	No	Yes	Round	3	Saccromyces
Y10	Oval	No	Yes	Round	3	Saccromyces

- **Growth in liquid medium:** After 72 hr of incubation dry pellicles formation and white layer above the media surface was formed on the surface of YEPD broth medium. The isolated and purified culture was transferred to YEPD slants, preserved at 4ºC in the refrigerator and maintained by continuous sub culturing after every 8-10 weeks. For further study, only five out of twelve strains were selected.

Biochemical characterization:

- **Fermentation of carbohydrates:** In this study, five different yeast strains showed variation in terms of utilization of five different sugars. The strain utilized Glucose, sucrose, lactose, dextrose and fructose. The following results after 48 hours (table 3).

Table 3: Fermentation of different carbohydrates by isolated yeast strains

	Carbohydrate	Color of media before fermentation	After fermentation	
			Color of media	Gas Production
Yeast Strain (Y2)	Glucose	Pink	Yellow	Yes
	Fructose	Pink	Yellow	No
	Sucrose	Pink	No color change	No
	Dextrose	Pink	Yellow	Yes
	Starch	Pink	No color change	No
Yeast Strain (Y3)	Glucose	Pink	Yellow	Yes
	Fructose	Pink	Yellow	Yes
	Lactose	Pink	No color change	No
	Sucrose	Pink	Yellow	Yes
	Starch	Pink	No color change	No
Yeast Strain (Y4)	Glucose	Pink	Yellow	Yes
	Fructose	Pink	Yellow	Yes
	Lactose	Pink	No color change	No
	Dextrose	Pink	No color change	No
	Starch	Pink	No color change	No
Yeast Strain (Y6)	Glucose	Pink	Yellow	Yes
	Fructose	Pink	Yellow	Yes
	Lactose	Pink	No color change	No
	Sucrose	Pink	No color change	No
	Starch	Pink	No color change	No
Yeast Strain (Y10)	Glucose	Pink	Yellow	Yes
	Fructose	Pink	Yellow	Yes
	Lactose	Pink	No color change	No
	Sucrose	Pink	No color change	No
	Starch	Pink	No color change	No

Microbes show different behavior towards utilization of energy source in the medium. This specificity of using different substrates depends upon the specific enzymes present in the medium. The enzymatic system of some microbes is able to oxidize and utilize simple sugars like glucose while some bacteria can degrade complex carbohydrates like sucrose and polysaccrides. The utilization of different sugar by the microbe's enzyme system, aid in identification of unknown microbes.

- **Urease test:** urease test was performed in broth. All five strains were found to be negative for this test. This test established the presence of urease and its role as the major enzyme concerned in the utilization of urea by yeasts as studied by Booth and Vishniac[7].

- **Tolerance of 1% of Acetic Acid:** Yeast strains can also be differentiated on the basis of resistance to acetic and benzoic acid (Sand, 1973). All five selected yeast strains were negative for this test. 1% acetic acid was used to discriminate *Zygosaccharomyces rouxii* from *Z. bailii* and *Z. bisporus*.

Physiological characterization:

- **Effects of temperature on growth of selected yeast isolate (thermotolrence):** Selected five yeast strains were spreaded on five YEPD Agar containing plates and incubated for 48 hours at 28°C, 30°C, 35°C, 37°C, 40°C, 42°C and 45°C and 47°C. All the five selected yeast isolates were able to grow at 25°C-42°C. Growth behavior of different yeast strains in solid media is shown in table 4.

Table 4: temperature tolerance by selected yeast isolate

Strains	Temperature (°C)							
	28	30	35	37	40	42	45	47
Y2	+	+	+	+	+	+	-	-
Y3	+	+	+	+	+	+	-	-
Y4	+	+	+	+	+	+	-	-
Y6	+	+	+	+	+	+	-	-
Y10	+	+	+	+	+	+	-	-

Note: + for growth, -for no growth

As the cooling cost during the ethanol production process is very costly, attention has given to find out the thermotolerant yeast so that cooling and distillation can be decreased.

Effect of ethanol concentration in the media on growth of selected yeast isolates: The isolate was selected for screening of yeasts tolerant to ethanol and the results are shown in table 5. It can be observed from table 5 that the yeast isolates can grow up to 15% ethanol containing liquid YEPD media. Maximum growth was seen in 5% ethanol containing media. Growth were recorded at 5%, 7%, 10%, 15%, 18% and 20% of ethanol containing liquid media and the Table shows the change in O.D at 48 hours with ethanol concentration in the media.

Table 5: Ethanol tolerance of isolated yeast strains (Optical density (600nm)

Strains	**Ethanol percentage (%)**					
	5	**7**	**10**	**15**	**18**	**20**
Y2	1.22	1.10	0.48	0.8	00	00
Y3	1.25	1.17	0.51	0.11	00	00
Y4	1.19	1.13	0.35	0.3	00	00
Y6	1.32	1.21	0.27	0.12	00	00
Y10	1.27	1.19	0.25	0.5	00	00

Ethanol is an inhibitor of yeast growth at relatively low concentrations, inhibiting cell division, decreasing cell volume and specific growth rate, while high ethanol concentrations reduce cell vitality and increase cell death. Ethanol also influences cell metabolism and macromolecular biosynthesis by inducing the production of heat shock like proteins, lowering the rate of RNA and protein accumulation, enhancing the frequency of petite mutations, altering metabolism, denaturing intracellular proteins and glycolytic

enzymes and reducing their activity. The main sites for ethanol effects in yeast are cellular membranes, hydrophobic and hydrophilic proteins and the endoplasmic reticulum. For both ethanol stress and heat shock, vacuole morphology is altered from segregated structures to a single, large organelle. Membrane structure and function appear to be a predominant target of ethanol. Exposure of yeast to ethanol results in increased membrane fluidity and consequential decrease in membrane integrity. A decrease in water availability due to the presence of ethanol causes the inhibition of key glycolytic enzymes and these proteins may be denatured.

Effect of pH on growth of selected yeast isolates: pH tolerance of selected yeast isolates was studied. The behavior of different yeast isolates in the form of Optical Density has shown in fig. 1. The selected isolate is able to grow lower to higher pH. The isolates can grow up to pH 5. Maximum growth was seen at pH 5. After 48 h, cell density was recorded at 600 nm and given gradually for evidence of growth. Control of pH during ethanol fermentation is important for two reasons.

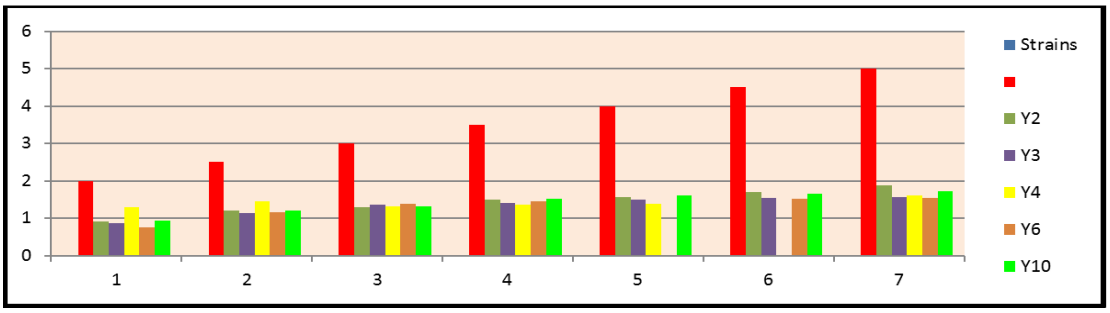

Fig. 1: pH tolerance in selected yeast strains

First, the growth of harmful bacteria is retarded by acidic solution. Second, Yeast grows well in acidic conditions. With increase in pH yeast produces acid rather than alcohol. Molasses has naturally alkaline pH and must be acidified prior to fermentation. The rate of ethanol production by yeast cells is highly affected by the pH of the fermentation medium. More acidic and basic conditions, both retard the yeast metabolic pathways and hence the growth of cells. Productivity may decrease by increase and decrease in pH due to the lower metabolic rate of the yeast cells. It may also be due to the growth of other microbes with the increase in pH, as the fermentation carried out without sterilization. In addition, pH of the surrounding medium change the configuration and permeability of the cell membrane thus reduced the rate of sugar fermented enzymes.

Effect of salt concentration in the media on growth of selected yeast isolates: the selected yeast strains were tested for the salt tolerance. Liquid media of different salt concentration was taken, inoculated with different strains and then incubated for 48 hrs. After incubation cell density was recorded at 600nm. These isolates can successfully tolerate up to 10% sodium chloride salt in the media and this is an index of osmotolerance. However, as the salt concentration increases, growth was reduced (fig 2).

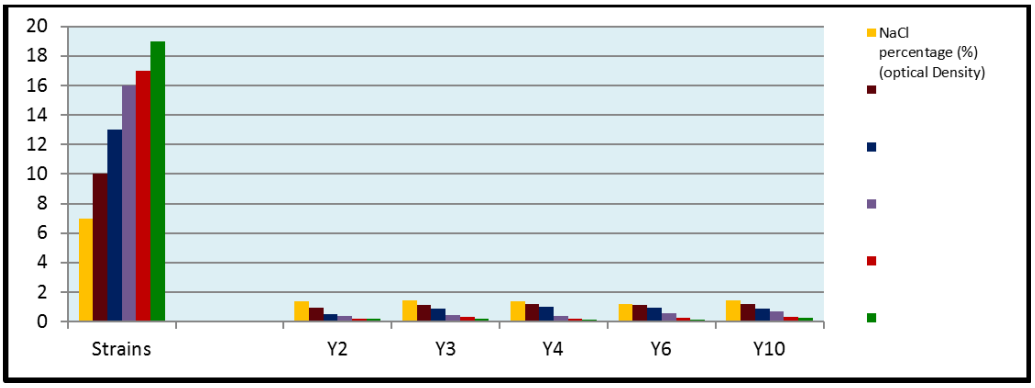

Fig. 2: NaCl tolerance in selected yeast strains

Saccromyces cerevisiae has a system which can neutralize the effect of salt induced stress like NaCl. When yeast cells were exposed in hyper osmotic enviroment, it leads to rapid cell dehydration and cell growth stops. Yeast tackles stress conditions by enhancing accumulation of intracellular osmolytes. Apart from it, yeast cell also accumulates polyols and compatible ions like amino acid and fatty acid in cell membrane.

Effect of different sugar concentration in the media on growth of selected yeast isolates: YEPD medium with varying concentration of glucose 4 to 10 % (w/v) were inoculated with yeast isolate and incubated at 28ºC for 24-48h. Isolated yeast was found to be resistant to sugar up to 10% (w/v) glucose concentration as shown in Table 6.

Table 6: sugar tolerance by selected yeast isolate

Strains	Sugar concentration (%)			
	4	6	8	10
Y2	+	+	+	+
Y3	+	+	+	+
Y4	+	+	+	+
Y6	+	+	+	+
Y10	+	+	+	+

Note: + for growth, -for no growth

The basic source of carbon and energy for yeast is sugar in some form and hence the primary requirement for the fermentation process. The most readily available form of sugar will lead to good growth and therefore, good alcohol production. Yeasts are frequently sensitive to high sugar concentration; therefore, a particular yeast strain should not be expected to give maximum and efficient fermentation with a medium having very high sugar concentration[27]. used glucose with different amounts (50, 10, 30, 50, and 70g/l) and showed that the maximum yeast biomass and maximum ethanol yield was obtained at high glucose concentration. Similar result was shown by[45].

- **Antibiotic resistance test:** Antibiotic resistance test by kanamycin, tetracycline and cycloheximide showed that all the selected strains were resistant to the antibiotics kanamycin, tetracycline and sensitive to different concentration of cycloheximide (table 8). Kanamycin and tetracycline are antibacterial so these were not able to kill yeast cells. Cycloheximide is antifungal as first reported by Whiffen[44].

Table 8: effect of different antibiotics on selected yeast isolate

Strains	Antibiotics (µg/ml)					
	Kanamycin	Tetracycline	Cyclohexide			
			10	25	100	1000
Y2	-	-	+	++	+++	++++
Y3	-	-	+	++	+++	++++
Y4	-	-	+	++	+++	++++
Y6	-	-	+	++	+++	++++
Y10	-	-	+	++	+++	++++

Note: + means sensitive to antibiotic, -means resistant to antibiotic

- **Oxidative stress tolerance in selected yeast isolates:** All the selected isolates showed sensitivity to H_2O_2 and could not adapt to H_2O_2. Y2 and Y3 were most sensitive, Y4 and Y10 were moderately sensitive and Y6 was least sensitive to H_2O_2. Izawa et al., (1995) investigated that intracellular glutathione plays important role in the response of *Saccharomyces cerevisiae* to H_20_2. Depletion of cellular glutathione or inhibition of glutamyleysteine synthetase enhanced the sensitivity to H_2O_2 and

suppressed the adaptation to H_20_2. These results suggested that intracellular glutathione plays an important role in the adaptive response in *S. cerevisiae* to oxidative damage.

- **Killer toxin test:** Killer toxin test was carried by all the selected strains against *E. coli*, showed negative result i.e., (no clear zone of inhibition by Yeast strains was observed). The capability to produce killer toxin[24]. can give an advantage over more sensitive and competitive yeast strains which grow in a fermentative process. Many studies found that killer phenotype is Investigations revealed that the occurrence of the killer phenotype in yeast is common in alcohol fermentations like in breweries, wine, plants and more recently in sugarcane producing plants[40].

- **Invasive growth assay:** Invasive growth assay was used to observe the penetration of yeast cell in the surface of an agar medium due to carbon limitation when these cells are incubated for a long period in nutrient rich media. When the concentration of carbon source decrease, cells of yeast elongate and invade the agar to look for nutrients. This property of invasion under nutrient limitation is called invasiveness. All the selected yeast strains were tested for their ability to grow invasively into YEPD medium. A total of 4 strains Y2, Y3, Y4 and Y6 showed invasive growth, and this feature was not correlated with a particular colony phenotype[33,4,25,12],

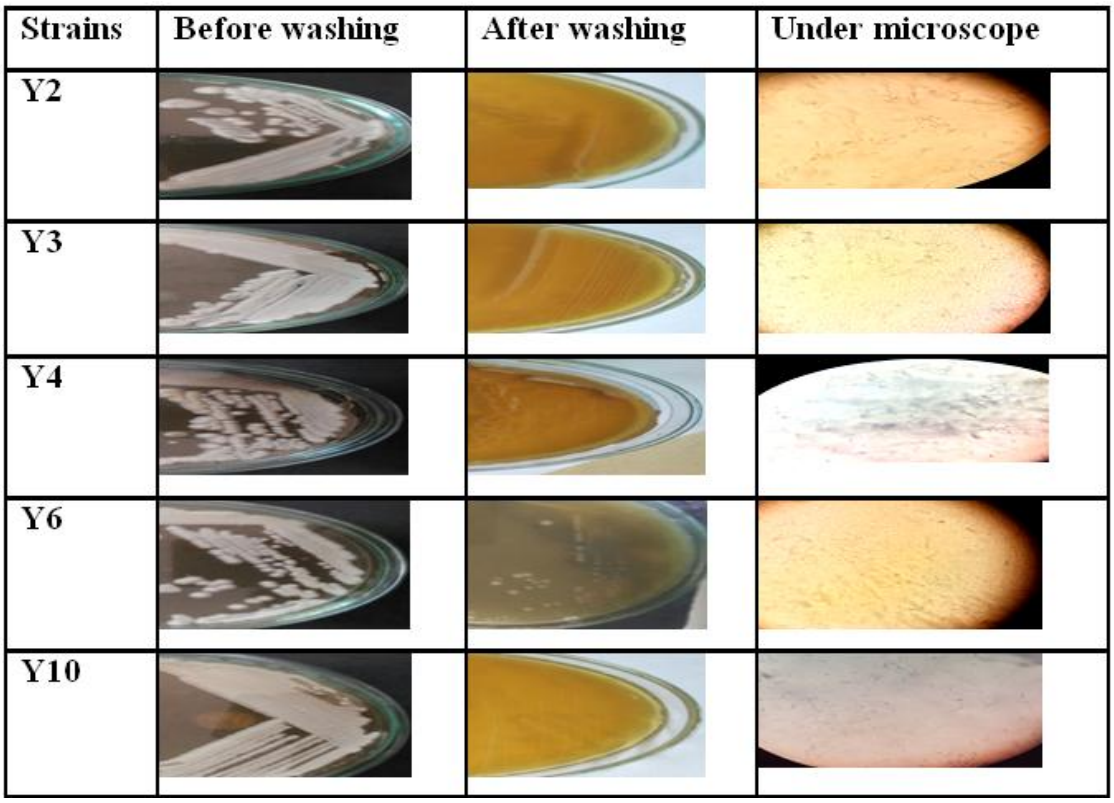

Fig. 3: Plate washing assay for invasive growth of selected yeast strains

Growth curve study of selected yeast stains

The growth curve is defined as growth phases in terms of time after inoculation. Kinetics was studied in yeast isolates Y2, Y3, Y4, Y6 and Y10 using six replicates of 25 ml of YEPD medium for each culture and incubated at 280C. Samples were drawn at incremental time intervals spread over several days and the growth of yeast strains was estimation by dry weight and Spectrophotometric methods. As per the result shown in table 9-10, it was observed that on the basis of dry weight and optical density, strain Y2 and Y4 gave maximum growth that 72 h, while strains Y3, Y6 and Y10 gave maximum growth at 96 h (fig. 4-5).

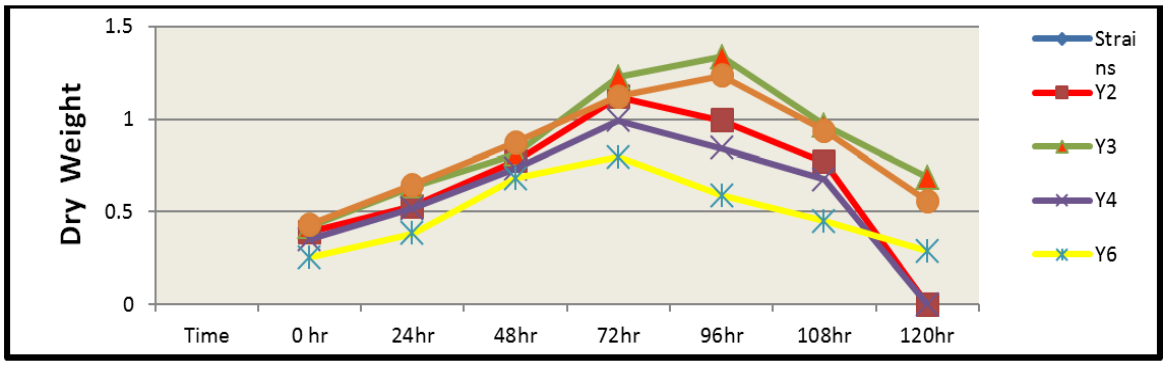

Fig. 4: Graph showing the growth of selected yeast strains on the basis of dry weight

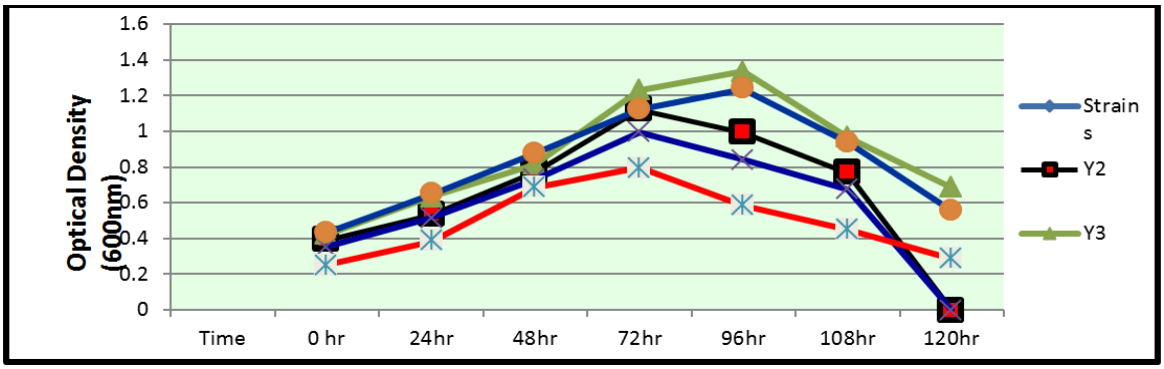

Fig. 5: Graph showing the growth of selected yeast strains on the basis of Optical DensityEthanol production

Yeast isolate was examined for ethanol production using (25% (w/v) molasses, as substrate under the constant set of conditions. Ethanol production was estimated at 28°C and calculated after completion of fermentation. Yeast strains Y2 and Y3 produced maximum ethanol and strain Y10 produced minimum ethanol (fig. 6). Brooks (2008), isolated yeast strains from ripe banana peels for ethanol production and found, that isolates fermented 40% glucose at 30°C to yield 3.6 and 5.8% ethanol respectively.

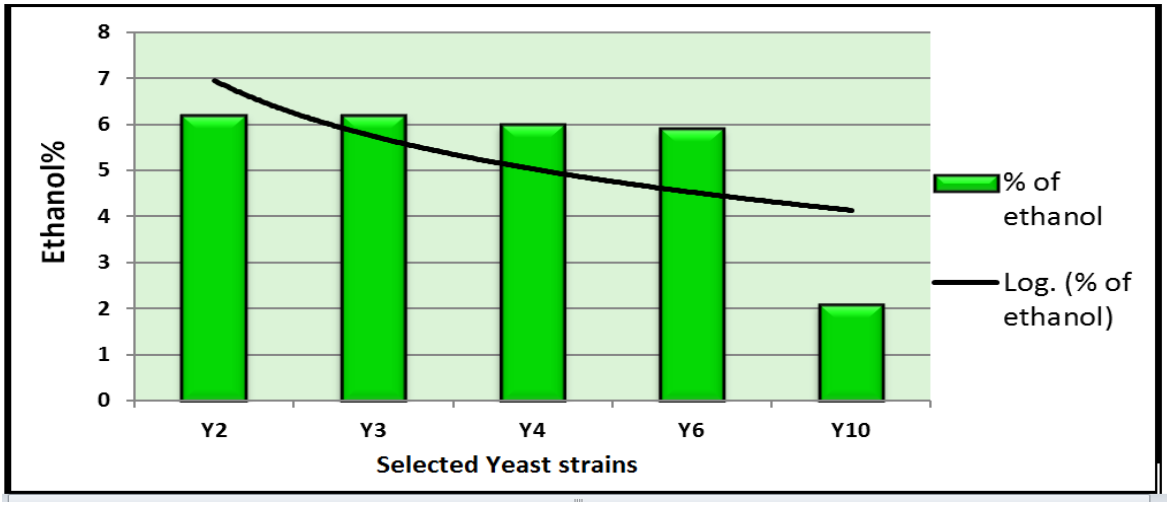

Fig. 6: Ethanol production by selected yeast strains

During identification, Y2 strain was found to be Brettenomyces and Y3 was found to be Saccromyces. Saccromyces is well known for ethanol production but apart from it some wild yeast such as species of Brettenomyces are very good ethanol producer like *Brettanomyces bruxellensis*[13]. Brettanomyces yeast is habitually isolated from the same

niche as Saccharomyces yeasts such as wine and beer. Brettenomyces is generally considered as spoilage yeast because it increases the phenolic off flavors in wine and beer[15]. But, in limbic beer, Brettenomyces brings a characteristic flavor[37]. Over 200 million year ago, Dekkera lineage got separated from Saccromyces clade[31,34]. However, the Dekkera clade shows remarkable similarity with saccromycese in terms of physiological traits. Saccromyces and Brettenomyces are facultative anaerobic and able produce offspring without mitochondrial DNA. Interestingly, both are ethanol tolerant, and able to survive in acidic environments[34,29]. So different studies show that a parallel evolution took place[34], proposed that a parallel evolution took place.

CONCLUSION

Clearly, the results exploring methodologies that allow the isolation of efficient ethanol producing yeast strains including wild strains from fruits. The study on yeast species is still limited and further research is needed. The opportunity exists to use such yeast strains to improve the efficiency of ethanol production which should help reduce the current reliance on petroleum-based fuel. To take advantage of this opportunity, new approaches in the isolation of stable thermo tolerant and wild yeast mutant strains capable of being used during high temperature ethanol production will need to be developed. Therefore, significant and economic conversion of the available substrate into ethanol required an efficient and robust microbial culture. Although, alcohol producing yeast and bacterial cultures are already available in Culture Collections of various research institutes, yet, there is always a possibility to explore natural resources as nature is the richest resource of the novel micro flora. Human being in his quest to find new and high alcohol yielding strains which are adaptable to extreme conditions has led to the discovery and isolation of novel strains and further optimization of these strains might improve their ethanol production efficiencies. On the other hand, in current time the importance of alternative energy source has become even more necessary not only due to the continuous deletion of limited fossil fuel stock but also for the safe and better environment. Rotten fruits may serve as a good substrate as they contain sufficient amount of carbohydrates naturally which can be used for production of bioethanol. Bioethanol consider as a fuel of the future and can solve the problem of pollution and energy crisis.

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