

Isolation and Characterization of Temperature and Ethanol Tolerant Strain of *Saccharomyces cerevisiae* Strains From Naturally Fermented Juices

Sonia Kumari¹, Awadhesh K.Jha¹ and Anil K Singh²

¹Asstt. Prof cum Jr. Scientist, Department of Microbiology, SGIDT, BASU, Patna, India.

²Asstt. Prof cum Jr. Scientist, Department of Dairy Economics, SGIDT, Patna, India.,
Principal scientist ICAR RCER, Patna, India.

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Saccharomyces cerevisiae is common yeast of economic importance in food and brewing industries. This study aimed at isolating the yeast from local sources, viz. Toddy, Date palm toddy, Fermented dough, Fermented fruit juice of apple and orange and then characterized on the basis of their morphology, microbial count, temperature, ethanol tolerance, flocculation, and fermentative ability and on stress test, to see if they can serve as a sources for industrial purposes. Some of them were found to possess high temperature and ethanol tolerance ability, some had high flocculation and fermentative capacity and survived when subjected to various stress conditions. *Saccharomyces cerevisiae* isolated from these local sources can be used effectively in the bakery and brewing industries. The yeast isolated from date palm has ability to grow at low temperature, have high ethanol tolerance ability and high flocculation ability. Likewise yeast isolated from toddy has ability to grow at high temperature and also have good ethanol tolerance ability. The ability of yeast to flocculate have benefit of easy separation from the final product at the end of fermentation without filtration/centrifugation and it is a important source for bakers industry while yeast that have good ethanol tolerance ability and high temperature tolerance ability can be use in alcoholic industry from various sources .

Keywords: *Saccharomyces cerevisiae*, Brewing, Flocculation, Toddy, Date Palm, Fermented dough, Fermented fruit Juice.

Fermentation preserve crops and dairy product by creating an unfavorable environment for Microorganism that are responsible for spoilage. The fermented beverages and dairies industry are known since more than 3500 years ¹. The organism responsible for fermentation is “Yeasts.” It was isolated from cereal based foods ^{2,3} from various milk product ^{4,5} or cheese ⁶. Correlation of yeast growth rate metabolism and concentration

of ethanol was noticed by ⁷ and optimum value noted was around 30°C. Ethanol tolerant strain of yeast have ability to increase the efficiency of ethanol production on an industrial scale ⁸. Selection of ethanol tolerant microorganism by allowing fermentation at high temperature ⁹. Ethanol and thermo-tolerant strains can be isolated from natural resources such as soil, water, plants, and animals. So the present investigation aims at

*Corresponding author E-mail:



the isolation, characterization and selection of a suitable *Saccharomyces cerevisiae* strains from local toddy, Date palm and other fermented fruit juices as well as to assess their ability to tolerate the high temperature and high alcohol content prevailed during fermentation process.

MATERIALS AND METHODS

A part of research work was carried out in Microbiological laboratory (Department of Microbiology, FBS&H, R.A.U. Pusa (Samastipur, Bihar) and other in the Department of Horticulture in B.A.U Sabour (Bhagalpur, Bihar).

Collection and preparation of Sample

Toddy from local tree toddy palm (Tp) (Palmyra Palm) liquor from date palm (Dp) (*Phoenix dactylifera*) Fermented dough preparation for Jalebi, (Fd), Fermented Orange Juice (Fo), Fermented Apple juice (Fa) were obtained from local market of Pusa, Samastipur, Bihar and kept in a sterile glass bottle at 4° C for further process. One gm Fermented dough was weighed and mixed in 9 ml of distilled water. Similarly Spoiled apple and orange was smashed properly with the help of spatula and about 10 ml liquor was collected for analysis. Then serial dilution was done for the entire liquid sample up to 10⁻⁶ dilution.

Media Preparation

Yeast extract potato dextrose agar media (YPDA) was prepared by mixing yeast extract-10 gm, Peptone-20 gm, Glucose-20 gm and Agar -18-20 gm. The entire component was

taken in a volumetric flask of 1000 ml capacity. The components was thoroughly mixed with small volume of water and heated for proper mixing of agar. Then mixture was cooled at room temperature. Final volume was adjusted up to 1000 ml by adding distilled water into the volumetric flask.

Isolation and primary identification of yeasts

From each sample one ml of was taken and spread plated on triplicate sterilized YPDA medium. The inoculated plates were incubated for 48 hr at 30 ° C. Re streaking of representative colonies (confirmed to be yeasts by microscopy) were done on PDA plates. Isolated colony was stored on slopes of the same medium. Isolates were sub cultured as needed for the various experiments conducted in this work. Identification was done on the basis of standard morphological and biochemical test^{10,11} viz. Glucose, Sucrose and Fructose fermentation ability, Ability to survive at high temperature, Measurement of growth at different temperature and fermentation ability of isolated yeast, ethanol tolerance and flocculation ability.

Microscopy examination

Isolates were observed for the following features: Color and elevation of colony, budding etc. Thin smear was prepared for microscopic evaluation¹² by taking an emulsified loopful colony of an isolate under test on a clean slide with a drop of water spread to make a thin film and then air dried. After stained with a Methylene blue dye it was observed with a light microscope under X10 and X40 objective lenses.

Table 1. Morphological Characteristic of Yeast

Sample	Color and appearance	Configuration	Margin and elevation	Budding shape & characteristics
Tp	Light brown jelly like	Irregular and spreading colony	Smooth, raised	Budding, oval and bigger
Dp	White and powdery appearance	Irregular and spreading colony	Wavy, flat	Bigger cell, binary fission
Fd	Milky white powdery	Irregular and spreading colony	Wavy raised	budding cell, ovoid bipolar
Fo	Light brown jelly like	Irregular and spreading colony	Smooth, raised	Budding, oval and bigger
Fa	White and jelly like appearance	Round with raised margin. Production of bubble in the slant	Smooth, convex	Round, binary fission smaller cell

Biochemical tests

Fermentation test for glucose

The isolates were tested for their ability to ferment glucose, fructose and sucrose. Isolate was inoculated into a test tube containing an inverted Durham tube and peptone water containing 1 gm of the sugar (glucose) and a drop of Methyl red. It was incubated for 24-48 hours. Changes in color pink to yellow of the indicator as well as liberation of water bubble in the Durham's tube indicate the fermentative ability of yeast. The presence of water bubble was taken as an evidence of a reasonably high rate of fermentative activity¹³.

Stress exclusive tests: Some stress tests were also done to know the temperature and ethanol tolerance of the yeast.

Temperature test

The ability of the yeast to grow at different temperatures was tested by plating the yeast isolates onto Potato Dextrose Agar medium and incubated at different temperatures i.e. 7-15, 15-20, 20-25, 25-35 °C for 72 hours. The resulting growth was observed and recorded in the results.

Ethanol tolerance test

The ability of the different isolated yeast strains from different sources to grow in higher

ethanol concentrations were tested. For this one ml of culture was inoculated in potato dextrose broth containing different concentrations of ethanol, 5%, 10% and 15% (v/v) respectively and incubated at room temperature for 24 -72 hours¹⁴.

Test for Flocculation

Different Isolates were inoculated in 5 ml of Potato Dextrose broth and incubated at 37°C for three days. Tubes were agitated to observe the flocculation formed after full growth of yeast. The culture supernatant was carefully decanted and the adhesion of the yeast sediment to the bottle was observed and recorded¹⁵.

RESULTS AND DISCUSSIONS

Morphological observations

Morphological characteristics of yeast are presented in Table-1. Colonies of yeast appeared after two days of incubation at 30°C on potato dextrose agar incorporated with Tetracycline. Yeasts had unique earthy smells.

Color ranging from milky white to brown. Shape was from oval, round and ovoid (Morphological variations of yeast are shown in Fig1, Fig2 and Fig3). The configuration of cell was irregular and spreading (Tp, Fd,). Round with raised margin, and with bubble was appeared in case of fermented apple juice(Fa). Smooth raised convex margin was observed in Tp, Fo, and Fa. Wavy raised and flat margin were observed with Fd. Cell type was budding, oval and bigger in case of Tp, and Fo. Budding ovoid bi polar type appeared in Fd, and round smaller cell appeared in case of Fa. These characteristics had been observed by other researchers like¹⁶. Similar study was done by^{17,18,19,20} by different scientist and reported



Fig. 1.



Fig. 2.

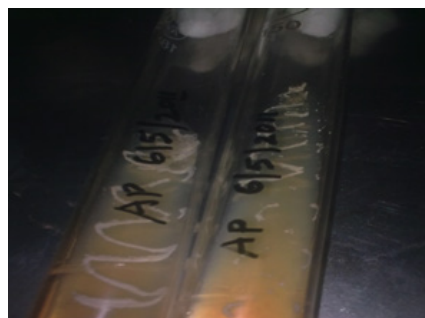


Fig. 3.

different yeast genera as smooth, fluffy, rough and creamy depending on the ability of yeast to form capsule and other extracellular matrix material.

Biochemical test for the identification of the isolates

The isolates were capable of utilizing a wide range of sugars as carbon and energy sources (Table-2). The utilization of sugars of carbohydrate fermentation was indicated by change in the color of the Andrade's indicator from yellow to pink, and by production of gas bubble which was trapped in the inverted Durham's tubes.

As shown in result that all isolates were able to ferment glucose and released CO₂ gas that observed as water bubble in the Durham tube. Most of the yeast ferment glucose in 24-48 hr and CO₂ and media clearance was observed with all the species. Likewise ability to ferment sucrose was also observed with all the yeast strain. So diversity to utilized different carbon sources was observed in all the case. Similar study was done by ²¹.

Ability of Yeast to Ferment Fructose

Change in color was observed Dp and Tp have changes the color of dye from blue to light pinkish, while Fd have changes the color with less intensity. Fo and Fa changes color from blue to dark pinkish. Evolve less CO₂ and slightly color changed (Table-3). The ability to ferment fructose

Table 2. Ability of yeast to ferment Glucose

Yeast	Time	Appearance of Co ₂ bubble	Media Clearance
Tp	24h	+++	++
Dp	24 h	+++	++
Fd	48-72	+++	++
Fo	24h	+++	++
Fa	24h	+++	++

Table 4. Ability of Yeast to tolerate different temperature

Yeast (Strain)	Temp. (7-15 °c)	Temp. (15-20 °c)	Temperature (20-25°c) Inhibition of growth	Temp. (25 -35°c)
Dp	24 h	24h	72h	Growth inhibited
Tp	No growth	No growth	24h	Better survival for 3-4 days
Fd	Slower growth	24h	24h	Better survivability for 3-4 days
Fo	24h-72h	24h	24h	Better survivability at above 35°c for a week
Fa	24h-72h	24h	24h	Better survivability at above 35°c for a week

can be utilized in production of wine from grape and litchi or other fruits having fructose as a reserve food.

Temperature Tolerance test

To know the behavior of yeast different isolates were tested for their temperature and tolerance test and fermentative capacity. The temperature have effect on the fermentation process and the metabolism of the yeast. Date palm yeast (Dp have optimum growth temp at 7-20°C but growth inhibited after 20 °C) that is shown as in table-4. Growth completely inhibited at 20-25 °C.

Yeast isolated from toddy palm has somewhat higher temperature requirement i.e. growth appear only after 25-30 °C but it has less temperature tolerance ability. No growth appears at 25-35 °C. Yeast isolated from fermented dough (Fd) have slow growth rate at 7-15 °C. But as the temperature increases growth appeared after 24 hr of incubation period. Growth was found fairly well at 25-35 °C. Yeast isolated from fermented orange and apple juice (Fo & Fa) have growth optima at 15-25 °C with a duration of 24-72 hr. The isolates which are capable to survive at high temperature signify their effectiveness in bread making as it speed up the proofing by increasing CO₂, that will

Table 3. Ability to ferment fructose

Yeast	Time	Appearance of Co ₂ bubble	Change in color of Media
Tp	24h	+++	blue to light pinkish
Dp	24 h	+++	blue to light pinkish
Fd	48-72	+	Pink but with less intensity
Fo	24h	+++	blue to dark pinkish
Fa	24h	+++	blue to dark pinkish

+++Intensive response, **Moderate response, *Low response

ultimately increase flavor and aroma. Those strains are also useful in making alcoholic beverages at high temperature. As the temperature increases yeast growth increases and at a temperature of 20-25 °C. Flourished growth rate was observed that sustained for 2-3 days. At high temperature i.e. 35 °C and above the yeast survivability is increased for a week indicating large temperature tolerance

range both at high as well as at low temperature. Similar study were also done by a group of scientist and reported that *S. cerevisiae* strains isolated from conventional fermentation processes were physiologically adapted to extreme conditions ²².

Table-5 depicts ethanol tolerance and flocculation ability of different isolated strains. Maximum flocculation ability was observed with

Table 5. Ethanol tolerance and flocculation ability of different isolated strains

Yeast (Strain)	Ethanol tolerance V/V (ml)			Flocculation ability
	5 Days	10 Days	15 Days	
Dp	+++	++	-	++++
Tp	+++	++	+	++++
Fd	++	-	-	++
Fo	++	+	-	+
Fa	+++	+	-	+

++++Intensive response, +++Semi-intensive, ++Moderate response, +Low response

Table 6. Growth & Fermentative characteristic of isolated yeast

Source	Growth at 18 °C	Fermentation behavior (Appearance of CO ₂ bubble)	Ability to ferment litchi juice (h)	Ability to ferment 30% fructose
Dp	24-48h	Top	24 hr, high	+++
Tp	No growth at low temp.	-, -, -	-, -, -	-, -, -
Fd	48-72	Top	48h	+, -, -
Fo	72-96 h	Top	24-48h	+, +, +
Fa	48-72h	Top	24-48h	+, +, -
By	48-72h	Top	24-48h	+, -, -

+++ High response, ++Moderate response, +Low response, - - - No response



Fig. Wine produced from Litchi (*Litchi chinensis*) by local isolated strain of yeast

the yeast strain isolated from toddy palm and Date palm. Flocculation is the ability of yeast to stick together that provide them ability to separate easily from the medium.²³ yeast cells which had ability to flocculate can reduce the energy cost involved in biomass centrifugation ²⁴.Higher flocculation rate of *S. cerevisiae* ensure a high cell density and large volume of harvested cells that are able to raise the ethanol productivity during the fermentation process ²⁵.

Growth & fermentative characteristic of isolated yeast

Yeast produces ethanol, carbon dioxide and other secondary products important for flavor, taste and quality of wine during fermentation ²⁶. During the fermentation the yeast faces many adverse environmental condition especially osmotic and ethanol stress ²⁷. During this process yeast gets adapted to the high sugar and other stress condition. For the successful fermentation process yeast should have better survivability in adverse environment else the entire process will be failed.

Yeast were also tested for ethanol and temperature stress that are very crucial factor for wine production. Temperature affects the fermentation and metabolism of the yeasts which defines the quality of wine.

The yeasts are also tested for their flocculation abilities. The flocculation's is an important characteristic that allow an easy separation of the final products at the end of the fermentation without additional filtration/centrifugation steps.

The Dp (yeast isolated from date palm have better ability for fermentation at low temperature while other strain have some high temperature requirement for the fermentation. The ability of Dp to ferment at low temperature may be useful during the winter season where availability of litchi is low.

Also high flocculation rate of the same strain have significance during separation of the product and utilization of the strain as immobilized strain that can increase the economy of the product also. The screened isolated strain can be used for the period when there is off season of fruit. Fruit of litchi may be preserved at low temperature in the form of pulp or in juice and can be utilized during winter season where there is no fruit production. Potential strain for fermentation was

also isolated by other scientist who supported the above statement.²⁸

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