

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2016.511.033>

Isolation and Characterization of Native Yeast from *Mahua* Flowers

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ABSTRACT

Keywords

Mahua flowers, Yeasts, Saccharomyces, Ethanol.

Article Info

Accepted:
16 October 2016
Available Online:
10 November 2016

Mahua flowers are rich source of sugars and hence harbors natural yeast flora which brings about natural fermentation process by metabolizing sugars to alcohol. The current study was aimed at isolating wild yeasts from the mahua flowers and testing the strains to screen strains having high productivity and high tolerance characteristics. Total seven strains were isolated and after screening only three isolates were showed ethanol tolerance of and osmotolerance. The IL 2 tolerated 20% ethanol whereas IL 1 and IL 2 tolerated ethanol between 7 to 12%. All the strains preferred pH 5 for growth. Osmotic stress tolerance was carried out using varying concentrations of salt and sugar it was found that IL 2 is a better strain than IL 1 and IL 3 with regards to stress tolerance. These strains can therefore, be further explored to test their potential of producing ethanol which can be used as a Biofuel.

Introduction

Ayurvedic formulations belonging to the category of asavas and arishtare prepared by traditional fermentation processes using the flowers of *Madhu calongifolia*. Traditionally, the flowers of *Mahua* are used as inoculums for the induction and maintenance of fermentation process. Apart from this *Mahua* flowers are basically used in manufacturing of *Mahua* liquor by tribal people and commercial distillers.

Mahua flowers are naturally rich in sugars hence a probable substrate for growth of natural yeasts which are the natural mycoflora of the flowers and hence these flowers are used in generation of liquor by the tribals. This naturally harbored yeast flora can be an ideal source for inoculum in

bioethanol production as they are sturdier to ever changing environmental conditions.

Generally, yeasts are heterotrophic with relatively simple nutritional needs and live as facultative anaerobe. Therefore, they are widely distributed in natural habitats such as in flowers and fruits, cereals, and plant debris. However, yeasts are exclusively isolated from various fermentation foods or their raw materials. In Traditional fermentations, microorganisms are brought by raw materials and produce a spontaneous uncontrolled fermentation (Ameyapoh *et al.*, 2006).

Also a limited number of studies investigating yeast diversity from flowers

from a handful of regions around the World including India have resulted in identification of a variety of species. There is a need to isolate wild yeasts which can be novel yeast strains having high productivity and high tolerance characteristics so as to aid in production of higher amounts of bioethanol.

Mahua flowers are rich in sugar content (40-70%) and can act as excellent natural habitat for a variety of yeast. Therefore, these flowers can be used for screening of the different yeast species that harbour on it. It is necessary to isolate wild yeasts from natural sources and screen for the yeast mycoflora.

Literature survey revealed the presence of yeast species on the flower (Rao *et al.*, 1961) and six genera were reported to be found on *Mahua* flowers namely *Kloeckera*, *Candida*, *Torulopsis*, *Pichia*, *Saccharomyces* and unidentified genera (Ethiraj *et al.*, 1980). since then no reports have been documented with reference to yeast diversity on *Mahua* flowers. Also there are no reports on evaluation of alcohol-producing ability in quantitative way amongst this naturally identified wild yeast. Since the yeasts on the flowers vary depending on the source location there may be some other species of yeasts that are yet to reveal but are responsible for the alcohol generation. Therefore, the current study was aimed at screening and isolation of yeasts from the *Madhuca longifolia* (*Mahua*) flowers with high ethanol tolerance and better ethanol productivity.

Materials and Methods

Name of the Plant: *Madhuca longifolia* var. *latifolia*

Sample collection: Flowers were collected from Uttarpradesh and Dahanu.

Enrichment

Five flowers were inoculated in each of the Erlenmeyer flask containing 200 ml of sterile MGYB broth (3 g malt extract, 10 g glucose, 3 g yeast extract, 5 g peptone per liter of distilled water, pH 4.5) and YEPD broth (0.3% yeast extract, 1% peptone, 2% glucose per liter, pH 5), for a period of 72 hours on Orbital incubator shaker (LabTop) at 25°C at 180 rpm.

Isolation

The enriched sample from the broth was serially diluted and then about 0.5 ml was spread on MGYB agar plates and YEPD agar plates. The plates were incubated for 24 hours at 32.5°C.

Colonies with distinct morphological differences such as color, shape and size were picked up and purified by streaking at least three times on YEPD agar. Subsequent streaking of each isolate was done on YEPD agar plate containing 100 ppm of Chloramphenicol.

The well isolated colonies were directly examined and their purity was verified by visualizing yeast cells under microscope.

Inoculum Development

The purified yeasts (10^6 - 10^7 cells/ml) were then further grown on YEPD agar medium. The subcultured yeasts were then inoculated in the inoculum broth (g/l) (0.3% yeast extract, 1% peptone, 2% glucose) and incubated at 30 °C for twenty-four hours in vigorous shaking condition (180 rpm). The optical density of this suspension was checked using colorimeter.

Cell count and viability

The cells were picked from the suspension culture incubated for 24 hours. 1 ml

inoculum broth sample was serially diluted with a sterile isotonic saline solution (0.9% w/v NaCl) to a point where a reasonable number of cells could be counted. The suspension was loaded on the haemocytometer slide and the cells were counted (Alfenore *et al.*, 2007). Most of the time, the cell count recorded is 10^6 cells/ml or a fraction higher.

Maintenance of culture

The culture of yeast was maintained by sub-culturing on slants using YMM (Yeast maintenance Media, Himedia) media, incubating for 48 hours at 30°C and thereafter storing in a refrigerator at 4°C for future use.

Identification of the yeast

Morphological characterization

Yeast isolates were identified based on the morphological characters (Kurtzman and Fell, 1998; Mesa *et al.*, 1999) and physiological characteristics.

Growth on solid medium

The morphology of the cells of the isolates and its appearance on solid YEPD agar media was examined. The features of the appearance of cultures such as, color, nature, elevation, margin and surface of the colonies were recorded.

Growth in liquid medium

Yeast isolates were cultured in YEPD liquid medium. The medium was autoclaved at 121°C and 15 psi and cooled. 10 ml portion of the medium was distributed into Suspension tubes, and then inoculated with 48 hours old selected yeast strain and incubated at 30 °C for 3 days. The culture was examined for the growth visually on the

surface of YEPD liquid medium and the shape of cells by compound microscope.

Physiological characterization

Assimilation of various sugars

Yeast fermentation broth base with Durham tube was used for identification yeasts based on fermentation pattern of specific carbohydrates. The sugars such as glucose (dextrose), galactose, maltose, sucrose, lactose, fructose, fructose and xylose were used for the test. Yeast fermentation broth was modification of media developed by Wickerham for the determination of carbohydrate fermentation by yeasts for fermented carbohydrates by yeasts, the color of the medium changed from red to yellow due to the formation of acids and gas produced (Warren & Shadomy, 1991).

The fermentation capacity for the isolates was tested for the ability to ferment eight different carbohydrates. It was examined anaerobically and was assessed for the formation of gas (CO₂) in inverted Durham tube and color change of the fermentation media.

Stress tolerance characterization

Detection of thermo-tolerance

The selected yeast strains were grown YEPD liquid medium to detect thermo-tolerance and growth in the media. 10 ml portion of the medium was distributed into tubes, and then inoculated with 48 hours old selected yeast strains. The initial optical density of each tube was recorded on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 25°C, 30°C, 37°C, 40°C and 45°C for 2 days (48 hours) for observing thermo- tolerance of yeast strains. The

increase in optical density in a tube was recorded as evidence of growth.

Detection of ethanol tolerance

YEPD liquid medium was used for detecting ethanol tolerance of the yeasts. Concentrations of absolute ethanol was varied from 5% 7% 10% 12% 15% 20% and 25% was added to the liquid broth and incubated at 30°C for 48 hours. Isolated strains grown in YEPD broth O.D. was measured after 48 hours on UV spectrophotometer- JASCO. (Mir Naiman Ali *et al.*, 2014; Manwar *et al.*, 2013).The initial optical density of each tube was read on spectrophotometer at 600 nm against the medium as blank.

Growth in different pH in Liquid Media

YEPD liquid medium was used for detecting the ability to grow in different pH of selected yeast strains. YEPD broth was prepared with different pH. Each test-tube contained 10 ml of YEPD media with different pH and blank media was used as a control. Then each tube was inoculated with the yeast culture and the initial optical density was measured at 600 nm.

All the tubes were incubated at 30°C for 48 hours. Growth was analyzed after a period of 48 hours by recording the cell density UV Spectrophotometer (JASCO) at 600 nm.

Salt tolerance

YEPD broth was prepared containing 5%, 7%, 10% 12%, 15%, 18% and 20% of NaCl. Tube contained 10 ml of YEPD media with appropriate concentration of salt and blank media was used as a control. Then each tube was inoculated with Yeast culture. The initial optical density after inoculation was recorded at 600 nm. The tubes were

incubated at 30°C for 48 hours. After 48 hours changes in cell density was recorded at 600 nm using UV Spectrophotometer (JASCO).

Sugar tolerance

The yeast strains were tested for their sugar tolerance by testing their growth in YEPD broth containing 10%, 15%, 20%, 25%, 30% and 35% glucose concentration. Each tube contained 10 ml of YEPD media with appropriate concentration of glucose and blank media was used as a control.

Actively growing yeast cultures were inoculated and the OD was taken at 600nm. The tubes were incubated for 48 hours at 30°C. Samples were taken every 24 hours and optical density was recorded at 600 nm using UV Spectrophotometer (JASCO).

Results and Discussion

Identification of the selected yeast

Morphological characterization

The morphology of the vegetative cells of yeast was determined according to the method of Kreger-Van Rij (1984) and Kurtzman and Fell (1997) by growing in liquid and on solid media.

Growth on solid medium and liquid medium

Yeast isolates formed butyrous and smooth white raised colonies on YEPD medium. The budding stage of the yeast isolates was observed under (40X) microscope and confirmed them to be yeast. The morphological and colony characteristics of the strains isolated are given table 1, and table 2 and Photo plate 1

Microscopic observation

The cell morphology of the yeast strains isolated from *Mahua* flowers was studied under compound microscope. An ovoidal to elongate have single, pairs, or triple budding cells were found. The isolate reproduces vegetatively by budding.

Physiological Characterization

Assimilation of various carbon compounds

In this study, the three isolates IL1, IL2, IL3 showed variation of utilization of eight different sugars. The strains IL1 and IL2 utilized glucose (dextrose), galactose, maltose, sucrose, lactose, fructose, and fructose but failed to grow on Xylose. The strain IL 3 utilized glucose, galactose, sucrose, fructose, lactose, and fructose but failed to grow on maltose and xylose.

Stress tolerance characterization

Thermo- tolerance

The three isolates were grown at various temperatures such as 25°C, 30°C, 37°C, 40°C and 45°C in the liquid medium and the growth was checked after 24 hours and 48 hours. The initial OD prior to incubation was also recorded. It was found that isolate IL 2 showed growth at 25°C, 30°C, 37°C, 40°C and 45°C whereas IL 1 and IL 3 showed growth only at 25°C, 30°C, 37°C. The results showed that IL 2 is a thermo-tolerant strain than IL 1 and IL 3. The results are shown in Figure 2.

Ethanol tolerance

The three isolates were grown in varying concentrations of ethanol such as 5% 7% 10% 12% 15% 20% and 25% in the liquid

medium and the growth was checked after 24 hours and 48 hours. The initial OD prior to incubation was also recorded it was found that isolate IL 2 showed growth up to 20 % ethanol whereas IL 1 showed growth up to 12% and IL 3 showed growth only up to 7%. The results showed that IL 2 is a high ethanol -tolerant strain than IL 1 and IL 3. The results are shown in figure 3.

Growth in different pH in liquid media

The three isolates were grown in varying pH such as 2, 3, 4, 5, 6, 7, 8, 9 and 10 in the liquid medium and the growth was checked after 24 hours and 48 hours. The initial OD prior to incubation was also recorded it was found that isolate IL 2 showed growth up to pH 10 but the optimum growth was seen at pH 5 the strain also survived at pH 2 as well. Whereas IL 1 showed growth up to pH 7 could withstand growth even at pH 2 but no growth beyond neutral pH in alkaline conditions. IL 3 showed poor growth in acidic pH, maximum growth at pH 5 only and showed growth up to pH10. The results showed the pH sensitivity is broader for IL 2 while IL1 could not grow in alkaline conditions and IL 3 showed better sensitivity but with poor growth. All the strains showed optimum growth at pH 5. Thus pH 5 is ideal for growth of the isolates. The results are shown in figure 4.

Salt tolerance in liquid media for the isolates

The three isolates were grown in varying salt concentrations such as 5%, 7%, 10% 12%, 15%, 18% and 20% in the liquid medium and the growth was checked after 24 hours and 48 hours. The initial OD prior to incubation was also recorded it was found that all the isolates showed the optimum growth at 7 % NaCl concentrations.

Table.1 Morphological features of isolated yeast Isolate

	Budding cells	True mycelium	Pellicle formation	Fragmentation
IL 1	+	-	-	-
IL 2	+	-	-	-
IL 3	+	-	-	-

(+ indicates **present** and – indicates **absence**)

Table.2 Colony characteristics of isolated yeast strains

Isolate	Size	Colour	Colony nature	Appearance	Margin & elevation
IL 1	Big	White	Smooth	Oval	Entire & Convex
IL 2	Big	White	Smooth	Oval	Entire & Convex
IL 3	small	Creamish	Smooth	Oval/spherical	Entire & Convex

Fig.1 Photplate1 Isolated yeast strains from *Mahua* flowers

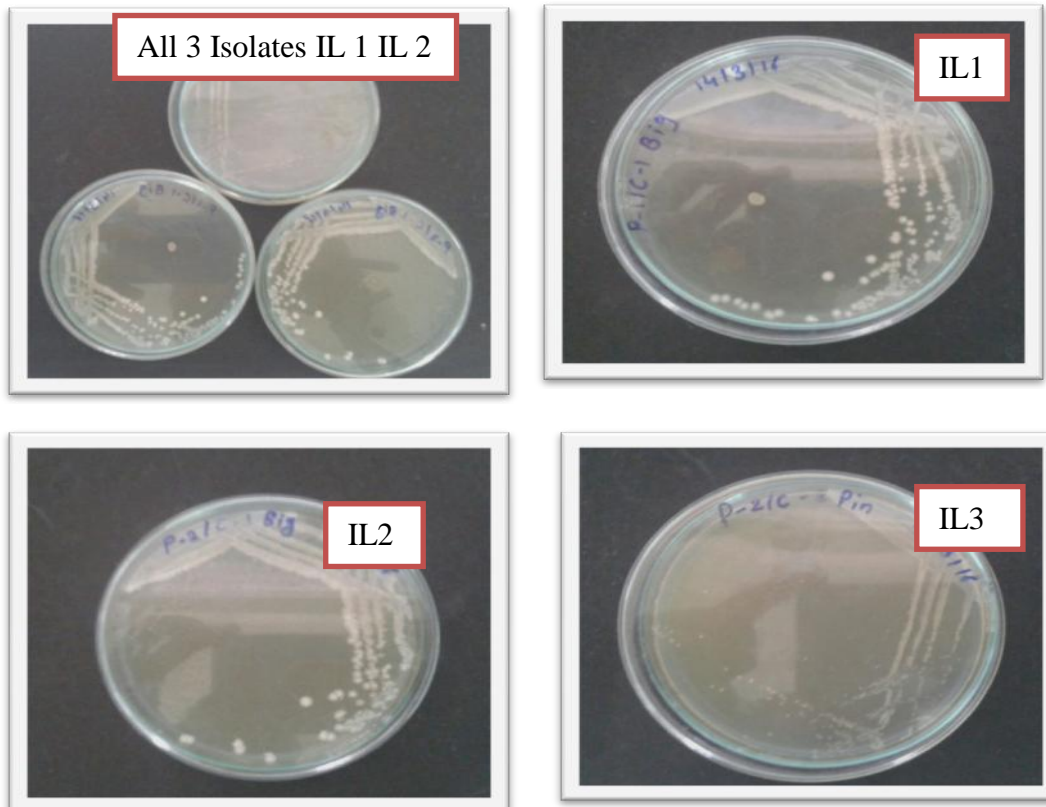


Fig.2 Growth of the three isolates at different temperatures in liquid media.

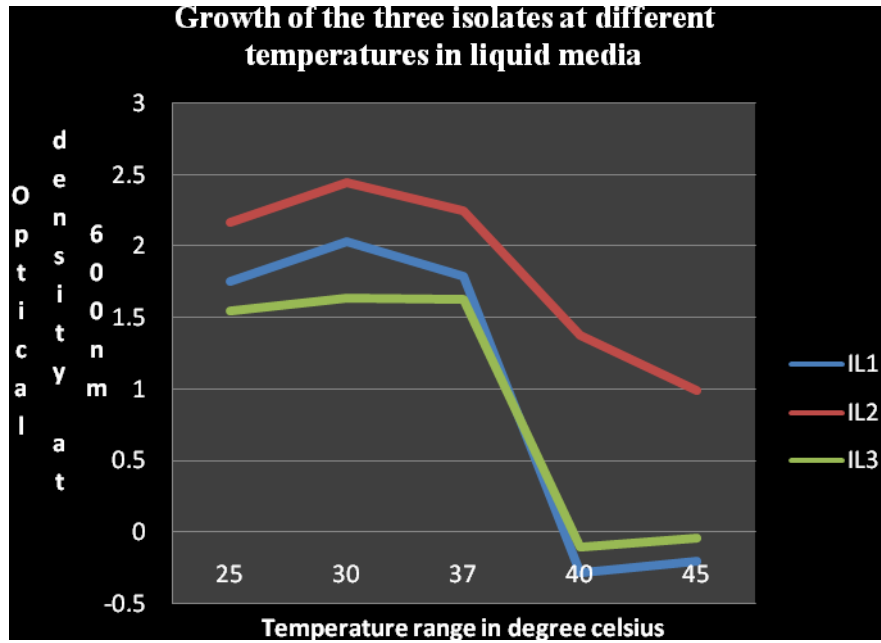


Fig.3 Growth of the three isolates at different concentrations of ethanol in liquid media

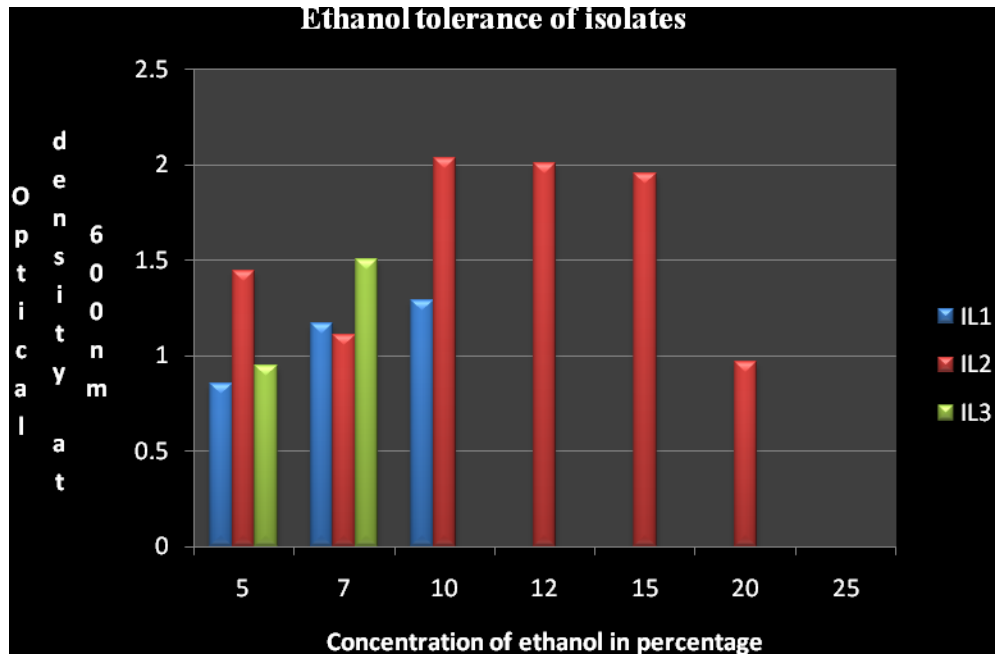


Fig.4 Growth of the three isolates at different pH in liquid media

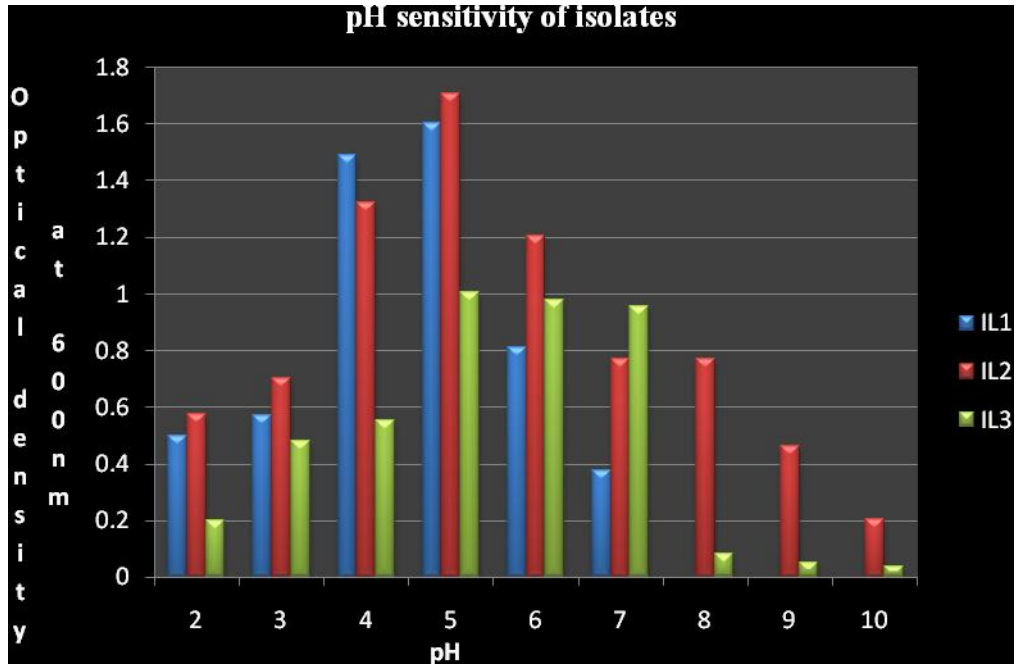


Fig.5 Growth of the three isolates at different salt concentrations in liquid media.

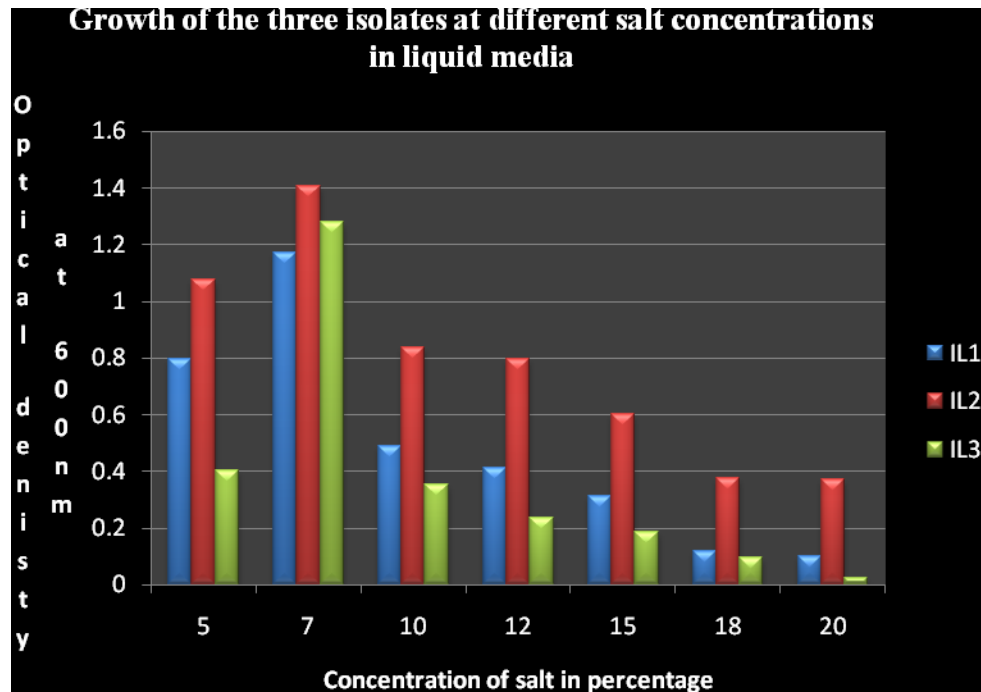
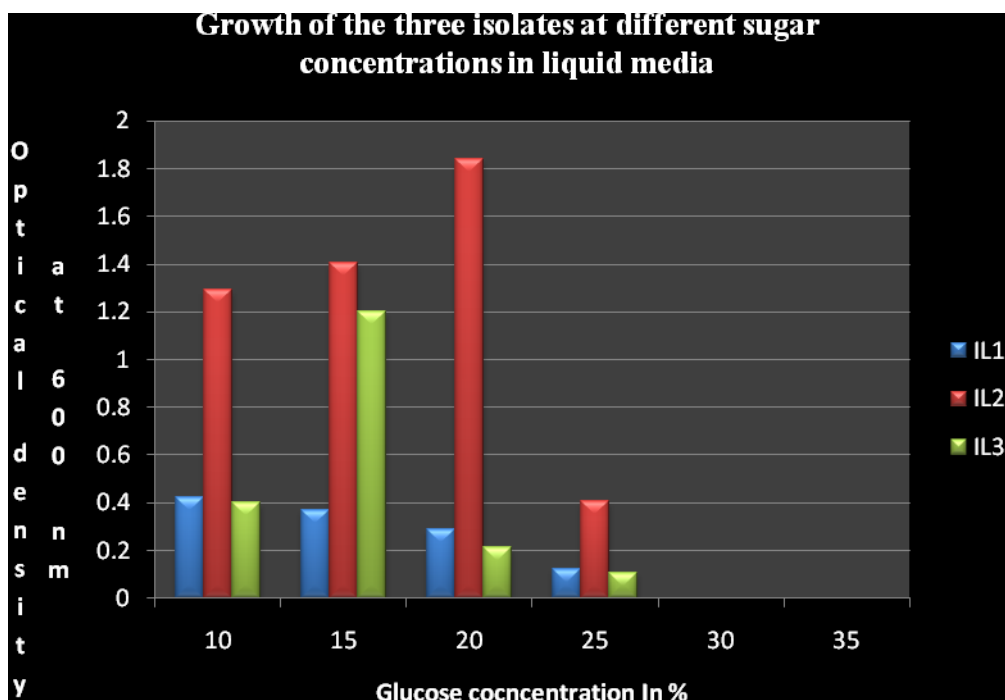


Fig.6 Growth of the three isolates at different sugar concentrations in liquid media



All the strains showed osmo-tolerance till 20% but the growth was gradually reduced. The ideal NaCl concentration for optimum growth for all the isolates is 7%. The results are shown in figure 5.

Sugar tolerance in liquid media for the isolates

The three isolates were grown in varying sugar concentrations such as 10%, 15%, 20%, 25%, 30% and 35% of glucose in the liquid medium and the growth was checked after 24 hours and 48 hours. The initial OD prior to incubation was also recorded it was found that all the isolate IL2 showed the optimum growth at 20 % sugar concentration whereas IL 1 and IL 3 showed optimum growth at 15% and 10% respectively. All the strains showed osmo-tolerance till 25% of sugar concentration but the growth was gradually reduced. There was no growth for all the three isolates at 30% and 35% sugar concentrations. The ideal sugar concentration for optimum

growth for all the isolates is 20%. The results are shown in figure 6.

In conclusion, *Mahua* flowers are rich source of sugars and hence harbors natural yeast flora which brings about natural fermentation process by metabolizing sugars to alcohol this characteristic property of generation of alcohol has made popular amongst the tribals to utilize it for production of liquor. Traditionally in Ayurvedic system arisht are prepared using *Mahua* flowers. In the current study natural yeast flora was isolated from *Mahua* flowers. Total 7 isolates were isolated out of which only three isolates were studied as they were ethanol and osmotolerant. On the basis of microscopy and morphological characteristics the three isolates IL1, IL2, IL3 belonged to the genus *Saccharomyces*. IL2 strain was better ethanol tolerant, thermo- tolerant, osmo-tolerant strain than IL1 and IL3. These strains can be further tested for their potential to produce ethanol. Also the fermentation conditions required by

the strains for maximum yield of ethanol can be optimized. The native strains can further be engineered towards high productivity so as to aid in production of ethanol that can serve as a biofuel resource.

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How to cite this article:

Rohan Gavankar and Maya Chemburkar. 2016. Isolation and Characterization of Native Yeast from *Mahua* Flowers. *Int.J.Curr.Microbiol.App.Sci*. 5(11): 305-314.
doi: <http://dx.doi.org/10.20546/ijcmas.2016.511.033>