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Isolation and Characterization of Amylolytic Yeast Strains Isolated from Indigenous Fruits of Assam, India

Sushree Sangita Senapati[1], Devabrata Saikia*[2] and Prasenjit Bhagawati[3]

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A B S T R A C T

Fruits are natural sources of sugars harbored by different yeast strains. The local fruits were used as sources for the presence of diverse yeast strains with different morphology and biochemical characteristics. Out of the 122 isolates, 9 did not show any production of SO_2 in the media. These 9 strains were evaluated for stress tolerance assays and found that the strains L1 and J7 could tolerate ethanol, pH and temperature stress better than the other strains. These strains were also found to be amylolytic and potential ethanol producers. L1 and J7 were identified to be *Saccharomyces cerevisiae* isolate L1 and *Saccharomyces cerevisiae* isolate J7 and received Genbank accession numbers OK326788 and OK326764 respectively.

***Key words*:** Yeast strains, Stress tolerance, Amylolytic activity, Ethanol producer

Yeasts are ubiquitous microorganisms and mostly appeared in sugar rich environments naturally [8]. Researchers reported that out of 150, 0000 yeast species present on earth, only1% is known [9]. They are heterotrophic in nutrition and their growth is encouraged in moist environment with support of different sugars and amino acids. Diverse group of yeasts dwell in plant preferably for nectars, flowers, fruits, decaying tissues, and tree saps. These attract insects which make the nest for varieties of seasonal yeasts because pollinating insects are seasonal [10]. In nature, fruits, plant leaves, flowers, soil are rich in these sources for the nourishment of yeasts and therefore, they are found abundantly [1]. Yeasts from fruits like lemon, mango, and guava were identified and reported yeasts from these fruits were *Candida albicans, Debaryomyces hansenii, Kodamaea ohmeri, Rhodotorula mucilaginosa*. The varieties of yeasts from fruits and blossoms of apple, plum, and pear orchards in Slovakia was being studied. It was also reported that many new yeasts were also identified from different fresh fruits [11]. Yeasts are also present in different fermentation food as raw materials used for this purpose. Because in traditional fermentation process, yeasts are not mixed separately rather added by used raw materials like fruits, flowers etc. These microorganisms e.g., yeasts boost spontaneous uncontrolled fermentation process [2]. Yeasts are known for alcoholic beverage fermentation by utilizing different carbon sources for growth and this is why yeasts are of biotechnologists' interest. But it is also very important to select appropriate yeasts strain for the production and development of food product like beverages. In case of alcoholic beverages, the selected yeasts strain should show maximum alcohol yield as well as maintain sensory quality [3]. Fruits are rich in different fermentable sugars. As yeasts utilize sugars for growth, fruits are used as substrate for fermented beverage. The microflora of the substrate also depends on its pH and as fruits are acidic in nature which again encourage the presence of yeasts in it. Yeasts strains associated with fruit surfaces are capable of converting wide range of sugars into alcohol and they can also tolerate high concentration of alcohol [1], [4]. Yeasts are obtained commercially from foreign suppliers and are costly. Their high cost renders the cost of their products too high for consumers [12]. This study is carried out to isolate yeast from local sources, characterize them, understand their morphology, ethanol tolerance, fermentative ability as well as stress exclusion test in order to have better understanding of the yeasts to see if they can serve as microorganisms of industrial importance.

* **Devabrata Saikia**
✉ devabratasaikia@gmail.com

[1-3] Program of Biotechnology, Faculty of Science, Assam Down Town University, Sankar Madhab Path Gandhi Nagar, Panikhaiti, Guwahati - 781 026, Assam, India

METHODS AND MATERIALS

Fruit samples were purchased from the local markets of Guwahati, Assam. Some of the local fruits were selected for experiment purpose were wood apple (*Aegle marmelos*), papaya (*Carica papaya)*, date palm, (*Phoenix dactylifera*) sapodilla (*Manilkara zapota*), pineapple (*Ananas comosus*), Star fruit (*Averrhoa carambola*), Java palm (*Syzygium cumini*), burmese grape (*Baccaurea ramiflora)* guava (*Psidium guajava*), Litchi (*Litchi chinensis*), bhim kol

(*Musa balbisiana*) and Indian jejube (*Ziziphus mauritiana*). The fruits were transported to the laboratory in zip-lock pouches [21]. Dirt was removed by washing, rinsing thoroughly several times in distilled water. The peels of the fruits removed and then chopped into small pieces with a sterile blade in a laminar airflow bench where asepsis was maintained. The experiment was carried out in the Microbiology Laboratory of Department of Microbiology, Faculty of Science, Assam down town University, Guwahati. The chemicals required for the study were procured from Hi Media and glasswares were procured from Borosil. For routine subculturing and maintenance, the strains were grown on Yeast Extract Mannitol Agar Medium (YEMA) at 28°C ± 2.

Isolation of yeasts

One gram of each chopped sample was soaked in distilled water in 250 ml conical flask for 72 h at 28 ± 2°C. Serial dilution of the sample of each was carried out up to 10^{-5}. An aliquot of 0.1 ml of each dilution was plated on Yeast Extract Mannitol Agar Medium (YEMA) (5 g/100 ml) using spread plate technique. The inoculated plates were incubated for 48 h at 28 ± 2°C. 30 µg/ml Amoxyclav was added as an antimicrobial agent to inhibit all bacteria growth. Isolates were sub-cultured on YEMA to check for purity and incubated at 28 ± 2°C for 48 h. Purified cultures were routinely maintained on YEMA slants and kept at 4°C. Morphological and biochemical tests of the selected yeast isolates were carried out by the means of fermentation of different carbon sources using the modified method of [18].

Morphological and biochemical Characterization

The morphology of the vegetative cells of yeast was determined according to the method of Kreger-Van Riij [22] and Kurtzman and Fell [23] by growing in yeast extract mannitol agar (YEMA) media. The biochemical tests like amylase reduction test, catalase test, urease test, cellulase production test, Hydrogen Sulfide production test, Casein hydrolysis test, Citrate utilization test, Lipase test, Carbohydrate fermentation tests (glucose, lactose, sucrose, dextrose, mannitol) were done for each of the isolates.

Ethanol tolerance of isolates

Screening of the nine isolates (W5, W7, P8, D1, L7, J7, Pa10, L1, K1) for ethanol tolerance was carried out following the procedure by Ekunsanmi and Odunfa [5]; Nowak *et al*. [6]. To 50ml of autoclaved Yeast Extract Rose Bengal Broth (YERB) medium composed of (g/l): Yeast extract 5, Disodium Phosphate 17.25, Bile salts 2.00, Sodium chloride1.00, Magnesium sulphate 0.01, Sodium pyruvate1.00, Rose Bengal 0.04, pH 7.9 ± 0.2 in 250ml conical flasks. Absolute ethanol was added constituting varying percentage of ethanol 5%, 10%, 12%, 15%, 18%, 20% (v/v). A control flask was run parallel containing no ethanol. The flasks were inoculated with 100µl of actively growing (24 h±2) cell suspension containing about 10^6cells/ml of respective cultures and incubated at 28°C for 72hrs. The optical density (OD) at 615nm of each flask was measured calorimetrically using sterile YERB medium as the blank. Increase in the optical density with the increasing incubation time of the culture was considered as the evidence of growth. The concentration at which the growth of the yeast was just inhibited was considered as the highest concentration of ethanol that the strain could tolerate. Strains that showed growth in medium containing 8% ethanol (v/v) or more were selected for the further study (All the experiments were performed in triplicates) [7].

Thermo-tolerance of isolates

The selected yeast strains (W5, W7, P8, D1, L7, J7, Pa10, L1, K1) 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 colorimeter at 615 nm against the medium as blank. All cultures were incubated at 15°C, 28°C, 30°C, 32°C and 37°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 [1].

pH tolerance of isolates

YEPD liquid medium was used for detecting the ability to grow in different pH of selected yeast strains (W5, W7, P8, D1, L7, J7, Pa10, L1, K1). 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 615 nm. All the tubes were incubated at 28°C for 48 hours. Growth was analysed after a period of 48 hours by recording the cell density in colorimeter at 615 nm [1].

Fermentation and qualitative test for alcohol production

The production process was carried out in the month of April and a temperature of 28-30°C was maintained during the fermentation process. Mass production of promising yeasts isolates was done using potato dextrose broth and incubated at 28±2°C for 36-48 hours. Starter culture was prepared using the following protocol. Rice was mixed with water, heated at 60°C for 20 min, cooled and the excess water was filtered using muslin cloth and then autoclaved at 121°C 15 psi for 20 min and it was allowed to cool. Starter medium was then inoculated with 10% of 36-48 hours old mother culture and incubated at room temperature for 5 days. It was then filtered, and the alcohol was estimated by potassium dichromate method [25].

Estimation of alcohol was performed by potassium dichromate method which is based on the formation of green coloured chromate ions resulting from treatment of ethanol and potassium dichromate in presence of sulfuric acid and acetate buffer. To an aliquot of standard stock solution, 5 ml of potassium dichromate solution (40mg/ml), 5 ml of acetate buffer (pH 4.3) and 25 ml of 1 N sulfuric acid (54 ml/1000ml) was added in a conical flask. The mixture was shaken gently for 1 min and allowed to stand for 2 hours at room temperature and observed for the formation of green coloured reaction product. The absorbance was then read off at 578 nm [24].

Molecular identification of promising isolates

The 5.8S internal transcribed spacer (ITS) rDNAs of yeast isolates J7 and L1 were amplified using the primers ITS-1(5´-TCCGTAGGTGAACCTGCGG-3´) andITS-4(5´-TCCTCCGCTTATTGATATGC-3´). The polymerase chain reaction (PCR) was performed according to previously reported work. The amplified product was purified and subjected to automated DNA sequencing. The sequence obtained was submitted to NCBI (National Centre of Biotechnology information) BLAST search tool in order to

retrieve the homologous sequences and then submitted to GenBank as per the required protocol (https://www.ncbi.nlm.nih.gov/genbank/). The phylogenetic tree was constructed using MEGA 11.0.8 software [15].

RESULTS AND DISCUSSION

Isolation and characterization of yeast isolates

A total of 122 strains were isolated from 12 fruit samples. Most of the colonies were observed to be smooth, creamy, opaque and convex. The cells were ovoid or elongated Under microscope. The cells were observed to be present as single and budding under microscope.

Out of 122 isolates, those showing negative for hydrogen sulphide production were selected for further studies. [20]. All the selected 12 isolates were shown to produce amylase enzyme, having amylolytic activity. These isolates were also showing enzymatic activity for catalase, urease and citrate. But they are not able to produce casein, lipase and cellulase enzyme as shown in (Table 1).

Table 1 Biochemical tests performed in different strains

Isolates	Amylase production test	Catalase test	Urease test	Cellulase production test	Hydrogen sulfide production test	Casein hydrolysis test	Citrate utilization test	Lipase test
W5	+	+	+	-	-	-	+	-
W7	+	+	+	-	-	-	+	-
P8	+	-	+	+	-	-	-	+
D1	+	+	+	-	-	-	+	-
L7	+	+	+	-	-	-	-	-
Pa10	+	+	+	-	-	-	+	-
J7	+	+	+	-	-	-	+	-
L1	+	+	+	-	-	-	+	-
K1	+	+	+	-	-	-	+	-

*+represents positive and – represents negative

As shown in the (Table 2), most of the isolates utilized hexose sugars like glucose, disaccharides like lactose and sucrose and sugar alcohol like mannitol. Mannitol is not readily fermented by most of the yeasts and selected strains for further study, showed a promising trend in utilizing mannitol, the sugar alcohol [19]. The isolates were tested on their carbohydrate utilization ability to ferment different sugars namely glucose, lactose and mannitol. The table shows that all strains were able to ferment glucose and mannitol. These isolates may be used as starter culture in bakery industries. The breakdown of sugars indicates that the isolates have enzymes responsible for fermentation of glucose, sucrose and mannitol. This could be an important feature for strains used in fermentation industry [18].

Table 2 Carbohydrate fermentation capability of isolates

Isolates	Glucose	Lactose	Sucrose	Mannitol	Alcohol production
W5	+	-	+	+	+
W7	+	-	+	+	+
P8	+	-	+	+	+/-
D1	+	-	+	+	+/-
L7	+	-	+	+	-
Pa10	+	-	+	+	++
J7	**+/g**	-	**+/g**	+	+
L1	++	-	**++/g**	++	+++
K1	+	-	+	+	+

***+** represents moderate rate of fermentation,
++ represents high rate of fermentation, - represents no fermentation and g - represents gas

Table 3 Growth of different strains ($O.D._{600nm}$) at concentrations of ethanol

	5%	10%	12%	15%	18%	20%
W5	0.255±0.007*	0.185±0.007*	0.155±0.007*	0.115±0.007*	0.100±0.000*	0.060±0.000*
W7	0.220±0.000*	0.235±0.007*	0.165±0.007*	0.175±0.007*	0.095±0.007*	0.030±0.000*
P8	0.205±0.007*	0.115±0.007*	0.075±0.007*	0.040±0.000*	0.030±0.000*	0.015±0.007*
D1	0.145±0.007*	0.130±0.000*	0.115±0.007*	0.070±0.000*	0.015±0.007*	0.000±0.028*
L7	0.270±0.000*	0.235±0.007*	0.200±0.000*	0.205±0.007*	0.150±0.000*	0.025±0.007*
J7	1.340±0.000	1.285±0.007	1.290±0.000	0.205±0.007	0.155±0.007	0.080±0.000
Pa10	0.265±0.007*	0.235±0.007*	0.200±0.000*	0.195±0.007*	0.110±0.000*	0.025±0.007*
L1	1.325±0.007	1.270±0.014	0.210±0.000	0.150±0.000	0.145±0.007	0.030±0.042
K1	0.270±0.000*	0.235±0.007*	0.205±0.007*	0.185±0.007*	0.115±0.007*	0.025±0.007*

N.B. Yeast isolates were grown for 48 hours, Values are represented as mean ± standard deviation, n = 3.
*Represent significant differences ($p < 0.05$) along the same column

Ethanol tolerance

The selected strains were grown in different percentage of ethanol and it was observed that except J7 and L1, other strains did not show any significant growth in 5% ethanol and no strains showed growth in 20% ethanol (Table 3). J7 and L1 were observed to be grown in constant manner

in 5%, 10%, 12%, 15% and 18% ethanol. Isolates were characterized on the ability for different concentrations of alcohol tolerance. This study indicates that the isolates can tolerate ethanol toxicity during fermentation which is a very important character of the isolates to utilize them in fermentation process [18].

Statistical analysis

Results were expressed as mean ± standard deviation and the data were analyzed by GraphPad version 5.00 (San Diego, CA, USA). One-way analysis of variance with Dunnett's multiple comparison tests was used to compare the differences among the control group and the rest of the groups. A value of $p < 0.05$ was considered to be statistically significant.

Temperature tolerance: The strains isolated from different local fruits could only tolerate a temperature up to 37°C. The ability of yeast to tolerate high temperature indicates that the isolates can resist high heat associated with fermentation process and therefore these can be used to accomplish fermentation at wide range of temperature conditions. They may also be used in different bakery and fermentation industry [17]. Selected 12 strains were grown in different temperatures, and it was observed that most of the strains showed good growth in the range of 30°C to 32°C whereas J7 and L1 were showing good growth rate in 15°C, 28°C, 30°C, 32°C and 37°C ranges of temperature.

In (Table 4), the growth of different strains at different temperatures was represented. From the table it was found that the yeast isolates J7 and L1 were able to grow uniformly in all temperatures without any statistical difference. However, for the rest of the strains it was found that there was significant difference in growth patterns.

Table 4 Growth of different strains ($O.D._{600nm}$) at different temperatures

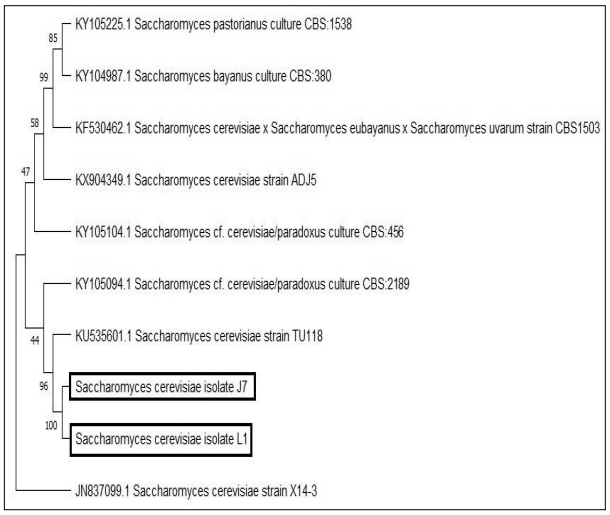
	15°C	28°C	30°C	32°C	37°C
W5	0.025±0.007*	0.910±0.014	1.285±0.007*	1.880±0.014*	1.805±0.007*
W7	0.050±0.000*	1.135±0.0212	1.675±0.007	2.030±0.014*	1.920±0.000*
P8	0.105±0.007*	0.970±0.014*	1.245±0.007*	1.770±0.000*	1.605±0.007*
D1	0.070±0.014*	0.875±0.021	1.425±0.007*	1.970±0.028*	1.915±0.007*
L7	0.030±0.014*	0.205±0.021	1.255±0.035*	1.985±0.007*	1.815±0.007*
J7	1.49±0.056	1.76±0.190	1.86±0.184	1.98±0.063	2.070±0.028
Pa10	0.095±0.007*	1.780±0.14	1.940±0.14	2.235±0.21	2.085±0.7
L1	1.54±0.127*	1.92±0.113	2.13±0.021	2.26±0.311	2.070±0.014
K1	0.050±0.014*	0.250±0.000	1.250±0.014*	1.915±0.021*	1.715±0.007*

N.B. Yeast isolates were grown for 48 hours,
Values are represented as mean ± standard deviation, n = 3.
*Represent significant differences ($p < 0.05$) along the same row

Data depicted in (Table 5) explains pH tolerance of selected isolates. It was observed that isolate J7 and L1 were showing growth in the range of 4 to 8 pH. Other isolates showed good growth rate in the range of 4 – 6 pH.

Table 5 pH tolerance of promising isolates

	2	4	6	7	8	10
W5	0.03±0.007*	0.28±0.007*	0.80±0.007	0.32±0.000	0.23±0.007	0.12±0.007
W7	0.05±0.007*	1.16±0.007	1.69±0.007	0.77±0.014	0.66±0.014	0.37±0.014
P8	0.04±0.014*	0.45±0.007	0.68±0.000	0.30±0.000	0.16±0.035	0.06±0.014
D1	0.06±0.042*	0.19±0.021	0.80±0.014	0.31±0.007	0.24±0.014	0.13±0.014
L7	0.06±0.042*	0.19±0.021	0.80±0.014	0.31±0.007	0.24±0.014	0.13±0.014
Pa10	0.01±0.007*	0.97±0.014	1.41±0.000	1.27±0.014	0.88±0.014	0.3±0.007
J7	0.14±0.014*	2.08±0.007	2.44±0.014	2.27±0.014	1.94±0.007	0.56±0.028
L1	0.03±0.035*	2.47±0.014	2.25±0.000	1.97±0.021	0.99±0.000	0.38±0.000
K1	-0.01±0.007*	0.18±0.000	1.01±0.000	1.27±0.014	0.19±0.007	0.12±0.007



Molecular identification

(Fig 1) represents the phylogenetic tree of the isolates J7 and L1 in comparison with their related strains. After submission to the Genbank, J7 and L1 received accession numbers OK326764 and OK326788 respectively.

CONCLUSION

The present investigation suggests that altogether nine isolates were taken for different biochemical, stress tolerance and enzymatic assays. Out of these, two isolates of *Saccharomyces cerevisiae* isolate L1 and *Saccharomyces cerevisiae* isolate J7 showed better result in comparison with other isolates. These two were found to be potential organism for ethanol production. In future studies, these isolates can further be used for ethanol production from various substrates.

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