Evaluation of probiotic attributes of isolated yeast strains from fruits and bread

ABSTRACT

Aims: To characterize the probiotic attributes of yeasts obtained from fruit and bread samples

Study design: Isolation, characterization amd performance evaluation for probiotic attributes

Place and Duration of Study: Department of Dairy Microbiology, Dairy Science College, Hebbal, Bengaluru, Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar,
Karnataka, India, between June 2022 and March 2023.

Methodology: 3 samples (Litchi, orange and bread) were taken to isolate yeasts using pour plate method. The obtainedcolonies were isolated, purified and cultured on YMM (Yeast maintenance media) and checked for prelimenary identification, biochemical tests followed by probiotic characterization using acid and bile test.

Results: The yeast counts were only obtained from litchi and orange but not in bread sample. The counts were 4.20, 3.3 and 0 $\log_{10}$cfu/g for litchi, orange and bread respectively with significant difference (P=.05). Isolated yeasts of 5 numbers were identified as Saccharomyces species and those were subjected for acid and bile tolerance test to check probiotic attributes. All the isolates showed tolerance to both acid and bile with significant difference.

Conclusion: The isolated yeast strain possess some probiotic characteristics and use of probiotic yeasts by humans through food gives biotherapeutic value.

Keywords: Probiotics, Acid and Bile tolerance, Saccharomyces, Biotherapeutic

1. INTRODUCTION

Yeast constitutes a diverse group of single-celled fungi, playing pivotal roles in various biological processes. Classified under the kingdom Fungi, yeast species are widespread in nature, thriving in environments ranging from soil and water to the surfaces of plants and animals (Borkar*et al.*, 2023). *Saccharomyces cerevisiae* is one of the most well-known yeast species and is extensively used in food and

beverage fermentation. The characteristics of yeast encompass a broad array of features that define its biology and behavior. One notable trait is its reproductive versatility (Wallen and Perlin, 2018).

Yeast cells can reproduce both sexually and asexually, providing them with flexibility in different environments. Asexual reproduction often occurs through budding, where a smaller cell (bud) forms on the parent cell, eventually detaching to become an independent organism. Genetic plasticity is another significant characteristic (Ansari *et al*., 2023) .

Different yeast species exhibit preferences for specific temperature ranges, impacting their performance in various environments. Most yeast strains thrive in a moderate temperature range, approximately 25-30 □. This mesophilic preference aligns with the typical conditions found in many ecological niches, including fermenting fruits and the human body. The appearance of yeast is a diverse and fascinating aspect that varies among species and can be observed at both macroscopic and microscopic levels. Under a microscope, typical yeast cells exhibit distinctive shapes, commonly appearing as ovals or spheres.

Probiotics, often associated with bacteria, also include certain types of yeast that confer health benefits when consumed in adequate amounts. *Saccharomyces boulardii*s a prominent example of a probiotic yeast known for its therapeutic properties. This yeast has gained attention for its potential positive impact on gastrointestinal health and overall well-being (Fakruddin*et al*., 2017).

Gut microbiota is a complex community of microorganisms that live in the digestive tracts of humans and animals, including insects. In people, the gut microbiota has the biggest quantities of microorganisms, and the greatest number of species compared to other parts of the body (Gomaa, 2020). One of the primary role of probiotic yeast is in maintaining a balanced microbial community in the gut. The human gastrointestinal tract is home to a complex ecosystem of microorganisms, collectively known as the gut microbiota. This microbiota plays a crucial role in digestion, nutrient absorption, and the overall health of the digestive system. Probiotics, including certain yeast strains, contribute to the diversity and balance of this microbial community (Roto*et al*., 2015).

2. MATERIAL AND METHODS

All the experiments were conducted at the post graduate laboratory of Dairy Microbiology Department, Dairy Science College, Hebbal, Bengaluru, Karnataka Veterinary, Animal and Fisheries Sciences University, Regional Campus.

2.1 Materials

2.1.1 Fruit and bread samples- Litchi, organe and bread samples were purchased from the local market, Hebbal, Bangalore, Karnataka for isolating yeast.

2.1.2 Yeast maintenance media(YMM): Media used for isolating yeast from sample.

2.1.3 Physiological saline- Diluent used for serial dilution of samples

2.1.4. For biochemical tests: Hugh and Leifson's medium, Carbohydrate fermentation broth, Christensen's Urea Agar, Nitrate broth is used.

2.2 Methods

2.2.1: Enumeration and characterization of yeast from fruits and bread

2.2.1.1 Enumeration of yeast

Enumeration was performed by using plate count method. From the collected orange and litchi samples, peel the outer layer of the fruit and pieces of bread sample were transferred to the prepared yeast maintenance media (YMM broth) of 11g and leave it for enumeration overnight. On next day serial dilution was performed using physiological saline serially like 1:10, 1:100, 1:1000. 1ml of each serially diluted samples were transferred to sterile petri plates and 10ml of sterile molten YMM agar were transferred and kept for incubation at 30°C for 3-5 days. After the completion of the incubation period, the colonies on YMM agar were counted. The countable plates ranging between 30- 300 were counted and average count was expressed as $\log_{10}$cfu/g. From selected countable plates,

colonies were selected, colony morphology noted and purified by streaking on to YMM agar plates three times and maintained on YMM agar slants. Once in a month, the isolates were sub cultured and maintained in refrigerator at 5 °C.

2.2.1.2 Characterization of obtained isolates

Purified isolates were subjected for preliminary test to confirm the genus and specific tests to speciate.

2.2.1.2.1 Preliminary identification of isolates

Maintained isolates were subjected for preliminary test like simple staining as per the standard procedures of Harrigan (1998).

2.2.1.2.2 Specific tests for the isolates

After confirming the status of isolates through the preliminary tests, all the isolates were made to carry out oxidative and fermentative test, Urease test, Nitrate reduction test, carbohydrate fermentation test as per the standard procedures of Harrigan (1998).

2.2.1.2.2.1 OF (Oxidative- Fermentative) test

Prepared 2 ml of HL medium in test tubes was sterilized by autoclaving at 121 °C for 15 min. Inoculated the test culture into HL medium tubes by stabing. To one of the tubes layer sterile paraffin wax as a sealant to cover ½ to 1 cm. Incubate the tubes at 37 °C for 48 h to 72 hrs. change in colour from blue to yellow indicates acid production and positive for test. Blue as such or darkened blue indicate no acid production thus negative for the test.

2.2.1.2.2.2 Urea hydrolysis

Actively grown young broth culture was inoculated on the slope part of Christensen's urea agar slants and incubated at 37 °C for 24-48 h. Urease production and subsequent hydrolysis of urea resulted in the production of ammonia which increased the pH and this indicated by a change in colour of the medium from yellow to pink was considered as positive.

2.2.1.2.2.3 Nitrate reduction test

Inoculate the glucose nitrate broth with the fresh culture and incubate at 37°C for 24 to48 hrs. Add 1 ml of each test reagent (Griess-Ilosvay's reagent) to the test culture and look for colour change. A red colour development within few minutes indicates the presenceof nitrite and hence the ability of the microorganisms to reduce nitrate to nitrite.

2.2.1.2.2.4 Carbohydrate fermentation test

Prepare Carbohydrate fermentation broth and transfer 10ml quantity in sterilize testtube, sterilize by autoclaving. Prepare the sugars (Glucose and Lactose) at 10% concentration each and filter sterilize. Transfer 1 ml of sterile sugar to 10 ml of broth Inoculate the culture and incubate, observe for colour which has pH indicator with gasproduction in Durham's tube. Change in colour from purple to yellow with turbidity indicatespositive for the test with homofermentation. In heterofermentationgas production is observedin Durham's tube. No change in colour indicates negative for test

2.2.2 Evaluation of probiotic attributes of yeast isolates

2.2.2.1 Acid tolerance test

With slight modification, the method of Dixon *et al* (2022) YMM broth was prepared in 3 different test tubes with 3 different pH like 1.0, 2.0, 3.0. Yeast isolates are inoculated into 3 test tubes, after inoculation the culture were inoculated onto the YMM agar plates and checked for the growth of an organism which is incubated at 30°C for 72 hours.

2.2.2.2 Bile tolerance test

For testing the bile tolerance, YMM broth is taken into 3 test tubes.To this ox-bile is prepared and added in a different concentration like 0.1, 0.2, 0.3% (Ragavan and Das, 2017). Active cultures of yeast isolates are inoculated and the culture are immediately inoculated onto the YMM agar plates and checked for the growth of an organism which is incubated at 30°C for 72 hours.

3. RESULTS AND DISCUSSION

3.1 Enumeration and characterization of yeast from fruits and bread

Samples when plated for enumeration on selective media for yeast counts were ranged from 0 to 4.2 log_{10}cfu/g. Among the 3 samples litchi showed highest count of 4.2 log_{10}cfu/g followed by orange of 3.3 log_{10}cfu/g and bread sample showed no yeast counts. A total of 5 isolates (Table 1) were obtained, among that 3 isolates from litchi and 2 isolates from orange were selected from colonies of countable plates (Fig 1).

Table 1: Colony and cell morphology of yeast isolates obtained from fruit and bread samples

Sources of isolates	Code of isolates	Number of isolates	Colony morphology	Cell morphology
Litchi	Y1, Y2, Y3	3	Circular shape, creamish white in colour, raised elevation, shiny surface	Oval shaped cells with budding
Orange	Y4, Y5	2		
Bread	--	0		

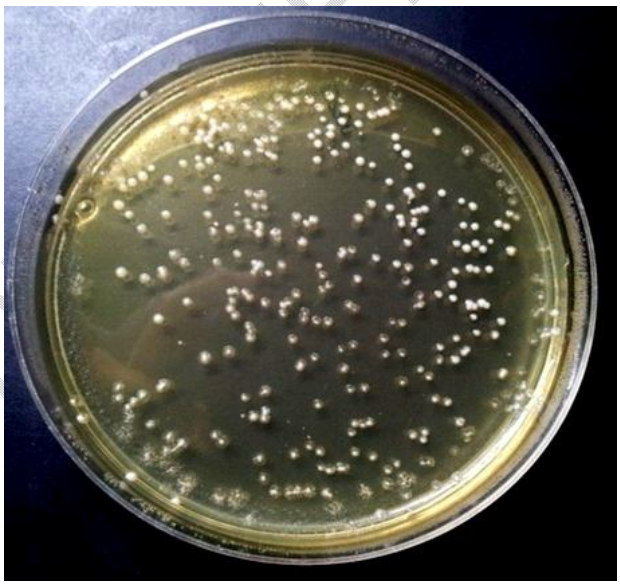

Figure 1: Colonies of yeast on YMM agar

Preliminary identification of the yeast isolates was revealed through simple staining. Smears of yeast isolates are prepared from the slant cultures and are heat fixed and stained with borax methylene and observed under 100x. The following biochemical tests were performed to characterize obtained isolates by

subjecting to oxidative fermentative test, which showed a positive result with color change from bluish green to yellow colour with growth was seen in the HL (Hugh and Leifson's) media followed by urease test displayed a positive result on christensen urease slant agar, i.e., red colour slant changed to pink colour slant. For nitrate reduction test it showed positive with change in colour, which indicated nitrate has been reduced to nitrogen then sugar fermentation test (Sucrose, Fructose, Lactose, and Glucose were the four sugars used). Sucrose, fructose and glucose showed positive results with acid and gas production, whereas lactose showed negative results, and it did not show acid or gas production. Based on these results obtained it showed that yeast isolates were belonged to Saccharomyces family.

In accordance with the study done by Chandimala*et al* (2022), on isolation and characterization of yeast from locally available foods like fruits and vegetables. Yeasts were isolated and enumerated using pour plate technique. Yeast Potato Dextrose (YPD) medium was used for growing yeast and incubation was done for 1-2 days at 25◦C. The discrete isolated colonies were purified by re-streaking on YPD plates and maintained on slants with 2% agar of the same medium at 5◦C in the refrigerator and as frozen stocks in liquid YPD medium with 40% glycerol at -20◦C. A total of 24 isolates were obtained and those were characterizrd by some biochemical tests like catalase test, urease test, sugar fermentation test.

3.2 Screening of yeast isolates for probiotic properties

3.2.1 Screening of yeast isolates for acid tolerance

Sterile pre-adjusted YMM broth to pH 1.0, 2.0 and 3.0 were inoculated individually with 5 yeast isolates and incubated at 30 °C. Samples drawn immediately after inoculation considered as 0 h and after 72 h of incubation were plated. Isolates at 0hr showed viable count ranged between 4.04 to 4.18 $\log_{10}$cfu/ml at pH 1.0, at pH 2.0 the viable count ranges between 4.00 to 4.36 $\log_{10}$cfu/ml and at pH 3.0 the viable count ranges between 4.20 to 4.53 $\log_{10}$cfu/ml whereas the isolates after 72 h ranged with respect to viable count between 5.38 to 5.74 $\log_{10}$cfu/ml at pH 1.0, at pH 2.0 the viable count ranges between 4.54 to 5.39 $\log_{10}$cfu/ml and at pH 3.0 the viable count ranges between 4.30 to 5.17 $\log_{10}$cfu/ml on sterile YMM agar medium.

All the isolates showed growth in different pH of 1.0, 2.0 and 3.0. As the pH increases the viability of each isolate get decreased.

Table 2: Acid tolerance of yeast isolates obtained from fruit and bread samples

Isolate	Acid tolerance viable count (log10 cfu/ml)					
	pH 1		pH 2		pH3	
	0h	72h	0h	72h	0h	72h
Y1	4.04^{a}	5.38^{a}	4.27^{ab}	5.25^{a}	4.44^{ab}	4.97^{a}
Y2	4.17^{a}	5.47^{a}	4.36^{a}	5.39^{a}	4.53^{a}	5.17^{a}
Y3	3.40^{b}	4.74^{b}	4.00^{b}	4.54^{b}	4.20^{b}	4.30^{b}
Y4	4.18^{a}	5.42^{a}	4.25^{ab}	5.32^{a}	4.51^{a}	5.00^{a}
Y5	4.17^{a}	5.47^{a}	4.36^{a}	5.39^{a}	4.53^{a}	5.17^{a}
CD (*P*=.05)	**0.18**	**0.15**	**0.22**	**0.14**	**0.16**	**0.14**

Note:

- Values are average of three trails
- CD - Critical difference
- Higher value in the table was compared with other values
- Same superscripts within the column indicate no significant difference
- Different superscripts within the column are compared with other values

3.2.2 Screening of yeast isolates for bile tolerance

The bile salt tolerance of all the 5 yeast isolates were tested in the presence of 0.1, 0.2 and 0.3 per cent ox bile. Samples drawn immediately after inoculation (0 h) and after 72h h of incubation at 30 °C were plated using sterile YMM agar. Isolates at 0 h ranged in viable count between 3.3 to 4.39 log_{10}cfu/ml for 0.1%, 3.9 to 4.47 log_{10}cfu/ml for 0.2% and for 0.3% at 0hr ranges between 4.07 to 4.64log_{10}cfu/ml, whereas at 72 hr the viable count ranges between 4.65 to 5.47 log_{10}cfu/ml for 0.1%, 4.34 to 5.44 log_{10}cfu/ml for 0.25 and for 0.3% the viable count at 72h ranges between 4.14 to 5.23 log_{10}cfu/ml.

All the isolates showed growth in different concentration of ox-bile. As the bile concentration increases the viability of each isolate get decreased.

Sunita *et al* (2021) reported acid tolerance property for the six yeast isolates by incubating them at four different pH values i.e. 2, 2.5, 3 and 7 for different time

intervals (1, 3 and 5 hours). It was observed that out of all tested isolates, 1 isolate was highly tolerant to all the acidic pH values tested. 2 isolates showed good tolerance to acidic pH of 2 and 3 when incubated for different duration at these pH values. The other 3 isolates were found to be susceptible to acidic pH. All the isolates showed decrease in growth at pH 7. For testing tolerance to bile salts, the yeast isolates were incubated in the presence of 5% and 10% of bile salts and their tolerance was recorded in terms of their absorbance values. Out of all the six isolates tested, 1 isolate showed maximum tolerance to bile salt followed by next 3 isolates. The other two isolates seem to be susceptible to the presence of bile salts.

Table 3: Bile tolerance of yeast isolates obtained from fruit and bread samples

Isolate	Bile tolerance viable count (log_{10}cfu/ml)					
	0.1%		0.2%		0.3%	
	0h	72h	0h	72h	0h	72h
Y1	4.14^{a}	5.28^{a}	4.33^{a}	5.04^{b}	4.47^{a}	4.91^{b}
Y2	4.39^{a}	5.47^{a}	4.47^{a}	5.44^{a}	4.64^{a}	5.23^{a}
Y3	3.30^{b}	4.65^{b}	3.90^{b}	4.34^{c}	4.07^{b}	4.14^{c}
Y4	4.33^{a}	5.45^{a}	4.37^{a}	5.44^{a}	4.50^{a}	5.22^{a}
Y5	4.39^{a}	5.47^{a}	4.47^{a}	5.44^{a}	4.64^{a}	5.23^{a}
CD (*P*=.05)	**0.19**	**0.14**	**0.12**	**0.12**	**0.17**	**0.10**

Note:

- Values are average of three trails
- CD - Critical difference
- Higher value in the table was compared with other values
- Same superscripts within the column indicate no significant difference
- Different superscripts within the column are compared with other values

4. CONCLUSION

Fermented foods are a source of diverse microflora and the fact that very few types of yeast have been commercially used as probiotic, it is worthwhile to explore the diversity of yeasts in traditional Indian fermented foods and study their

probiotic attributes. Through this research we have isolated yeasts with potential probiotic properties. They could be prescribed to patients on prolonged antibiotic treatment, protect against food borne enteric pathogens. They have the ability to degrade anti-nutrients such as phytic acid and tannic acid, thereby, improving the nutritional status of food. They can hydrolyze lactose, thus prove beneficial to lactose intolerant patients. They also produce L-asparaginase which has anticancer property. These yeast isolates could aid in digestion because of their ability to produce lipase and protease. Enhance vitamin B12 pool due to its production by these isolates. They can be used as immune-stimulants. Their ability to assimilate cholesterol could be of immense value to patients suffering from hypercholesterolemia. Beneficial attributes of *Saccharomyces cerevisiae*, *Candida sp., Pichia sp., and Aureobasidium sp.* indicates them to be promising probiotic agents in future and can be widely used as food and feed supplements.

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