

Research Article

Isolation and Characterization of Yeast as Potential Probiotics from Fermented Cereals Dough

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Abstract

Fermented foods are sources of yeasts, which have various beneficial effects in animal health and are used as probiotics. Therefore, the objective of this study was to isolate and characterize the probiotic potential of yeast from local cereal sourdoughs. The six samples flour (Barley, Maize, Finger millet, teff, sorghum, and Degussa) were blended in equal amount then mixed with distilled water and kept at room temperature for 6 days to allow spontaneous fermentation to take place. Sample from fermented dough was inoculated into potato dextrose agar and yeasts were isolated based on colony morphology and microscopic structure. The isolated yeasts were screened for their antimicrobial activity against the common pathogens *E. coli* and *Salmonella*. Those isolates with the best antimicrobial activity were selected and subjected to potential probiotic screening tests such as sugars fermentation ability, growth at 37°C, and low pH tolerance. Fifty Colonies, suspected to be yeasts, had unique earthy smells and color ranging from cream to white cream, and form of an oval, circular, irregular, and occurring singly was isolated. Six isolated namely (Tf5, Mz5, B3, Sr3, D1, and Fm2) with higher antimicrobial activity against the test organisms were selected. All the six yeast isolates were able to ferment the given all sugar (Glucose, fructose, galactose, maltose, sucrose, and sorbitol) except lactose. The selected yeast isolates grew favorably at 37°C (body temperature) when compared to a conventional growth temperature at 30°C. All the selected yeasts isolate exhibited a markedly higher growth at pH 3 and 5.5 and a good growth was also recorded at pH 2 except for isolate B3. Yeast isolates B3, Fm2, and D1 produce H₂S which is undesirable for livestock feed additives. The yeast isolates Sr3, Tf5, and Mz5, which did not produce hydrogen sulfide (H₂S), showed promising probiotic activities, and possessed comparable attributes with other reported probiotic yeasts. Supplementation of feeds with these yeasts to improve the quality of animal production can benefit. However, before therapeutic application, further research should be done to ensure the safety and efficiency of the potential of this probiotic yeast.

Keywords

- Antimicrobial activities
- Fermented foods
- Hydrogen sulfide production
- Probiotics
- pH tolerance
- Yeasts

INTRODUCTION

Probiotics are traditionally used as a term to describe the use of live microorganisms as food supplements. More precisely, probiotics can be defined as, "live microorganisms which when administered in a sufficient number provides a health benefit to the host" [1]. These friendly living micro-organisms are widely used for several therapeutic purposes and their beneficial effects are rooted in history. Usually, certain strains of lactic acid bacteria and bifidobacteria are prevalently applied in pharmaceutical preparations, feed additives, and in functional foods. Similarly, yeasts (*Saccharomyces cerevisiae*) are used as well, which is one of the most familiar microorganisms for animals [2].

The yeast percentage in the microbiota is approximately 0.1% and they are detectable in the gut of about 70% of healthy adults [3]. *In vitro* and *in vivo* studies have shown that probiotics could have beneficial physiological effects on the body such as the production of antioxidants and lowering of blood cholesterol level [4].

Isolation and characterization of yeasts as probiotics from natural sources required special considerations [5]. Yeasts can live in different niches such as plants, animals, soil, and water and they are associated with the skin, the gastrointestinal tract

of animals, including aquatic animals, as well as fermented foods. FAO has published a guide to systematically evaluating probiotic yeast. This evaluation includes the identification, *in vitro* test, safety, and finally *in vivo* tests [6].

In this study, the characterized yeasts isolated during the spontaneous fermentation of Ethiopian traditional foods (cereals) for probiotic potential [7]. In general, the current study determines the antimicrobial activity test of yeast isolates as one probiotic property, Characterize sugars fermentation ability and Hydrogen sulfide production of the isolates, and determines the temperature, pH tolerance of the yeast isolates. Therefore, the objective of the study was to isolate probiotic yeast from fermented cereal sourdoughs.

MATERIALS AND METHODS**Study Area**

The study was conducted at Mekelle city, University of Mekelle College of Veterinary Medicine at Molecular and Microbiology Laboratory from November 2019 to May 2019. Mekelle is the capital city and commercial center of the Tigray National Regional State located 785 km north of the capital city of Ethiopia, Addis Ababa. Mekelle is located at geographic coordinates of 13° 32'N

latitude and 39° 33'E longitude.

The climatic condition of the Mekelle city is semi-arid with an altitude of 1800 a.s.l. The annual minimum and maximum temperatures of the city is 20°C and 30°C, respectively. The average annual rainfall is 400-800 mm. The rainy season is bimodal, with a short rainy season occur from March to May and the long rainy season is occur from June to August. The soil type is clay type (85% vertisols). The vegetation type that cultivated in the area is both indigenous and exotic plant species. Example; acacia species, coniferous, suspense, elephant grass, etc. [8].

Sample Collection

The samples were collected randomly from the local markets. Each 150g of *maize*, *barley*, *finger millet*, *Degussa*, *Teff*, and *sorghum* cereals were purchased from the local markets. The collected samples were placed aseptically in sterile plastic bags and brought to Mekelle University, College of Veterinary Medicine Molecular Biotechnology Laboratory.

Sample Preparation

Samples preparation was done following the method of Procter and Meullenet [9] and Thais *et al.* [10]. The six samples of flour (*Barley*, *Maize*, *Finger millet*, *teff*, *sorghum*, and *Degussa*) were mixed in an equal amount and used to prepared dough. The Cereal flour was mixed with distilled water and kept at room temperature for 6 days to allow spontaneous fermentation to take place. The fermented dough was used as a source of yeasts for the current study.

Isolation of Yeast

One ml of the dough samples was transferred to 9 ml of sterile normal saline water and mixed thoroughly. It was inoculated on to potato dextrose agar (PDA) plates containing 1µl/ml Kanamycin. The samples were incubated at 30°C for 48 hrs. Then different colonies were picked based on their colony shape and color [11]. The colonies were purified by repeated subculturing using the streak plate method on freshly prepared PDA. The purified isolates were transferred to the PDA slant and preserved at 4°C for further study.

Identification of Yeast Isolates

Identification of yeast isolates to the genus level was carried based on cultural, morphological, and biochemical tests as described by Barnett *et al.* [11] and De Maristela *et al.* [12].

Morphological characterization of yeast isolates

To determine the morphology of yeast cells and reproduction type, the cultures was examined microscopically [11].

Staining and microscopic observation of yeast isolates: Vegetative cells were observed after 3 days of incubation in YEPD broth at 30°C. A sample of yeast was smeared then stained with diluted methylene blue and observed under a light microscope at (X100) magnification using oil immersion objective.

Observation of ascospores: Induction of ascospores formation from yeast was performed as per the methods described by Lodder [13], and Kirsop and Kurtzman [14]. Accordingly, sporulation media (Macclary acetate agar) was prepared and

loopful of yeast samples (24 hrs. culture) were inoculated into sporulation agar. Yeast samples were wet mounted on a glass slide; the smears were heat-fixed and spores stained according to Lodder [13]. Accordingly, the heat-fixed smears were flooded with 5% aqueous malachite green for 30 seconds and then counterstained with 0.5% safranin red for about 30 seconds. The excess stain was gently washed with running tap water and the preparations were observed both under high power (40X) and oil immersion objectives (100X).

Screening of Yeast Isolates

Hydrogen Sulphide (H₂S) production test: The selected yeast isolates were streaked on Bismuth Sulfite Agar (BSA) plates and incubated at 30°C for 3 days. Colonies that exhibited black color on BSA plates considered as positives for H₂S production [15].

Probiotics Screening Tests

Antimicrobial activity test of yeast isolates as one probiotic property: Yeast colonies were subcultured into 15 ml Glucose yeast extract broth (GYEB) and incubated at 27°C for 48 hrs. From the incubated broth, a loopful of yeast was cultivated aseptically on the SDA plate, and then the inoculated plates were incubated at 37°C for 48 hrs. **E. coli** and **Salmonella** test isolates were provided by Ethiopian Public Health Institute. They were swept separately onto nutrient agar (NA) plates using sterile swabs. Yeast colonies were carefully picked up using a platinum loop and placed in the center of inoculated Nutrient Agar plates with **E. coli** and **Salmonella**. Inoculated plates were incubated at 37°C for 24 hrs, and then clear inhibition zones were measured using a digital caliper [16].

Sugar fermentation test

Oxidative fermentative basal medium was prepared and an appropriate amount of (1µl/ml) of Kanamycin was added into the autoclaved media. About 5 ml medium was added to each test tube. Following this 0.5 ml sugar (10%) from each sugar type was added into each sterilized test tube. Finally, a single colony (a loopful) of selected yeast isolates taken from the agar plate was inoculated into each test tube and incubated at 30°C for 72 hours [17]. The fermentation was determined visually by monitoring the color change.

Growth characteristic at body temperature (37°C)

Sabouraud Dextrose broth SDB was prepared and sterilized by autoclaving at 121°C for 15 minutes. The media aliquot into to test tube and then inoculated with yeast isolates. The cultures were incubated at 30 and 37 °C for 0, 4, 8, and 24 hrs. Then the OD was recorded [18].

Survival at low pH (acidic environment): Survival at low pH (acidic environment) was determined by using the method of Rajkowska and Kunicka-Styczynska [19] with some modifications. The pH of sterile YPD broth was adjusted to 5.5, 3.0, and 2.0 with 3 mol l⁻¹ HCl. The broth was inoculated with 1% (v/v) of 18 hrs old broth culture of test yeast strains and incubated at 37°C for 3 hrs. Samples were taken at 0 and 3 hrs and colony counts were determined.

RESULTS

Cultural Characterization of Yeast Isolates

The cultural/colonial characteristics of the yeast isolates are given in Table 1. Morphological observations after three days of incubation at 30°C on potato dextrose agar (PDA), colonies suspected to be yeasts had unique earthy smells. Other characteristics were color ranging from cream to white cream, Shape, and form of oval, circular, irregular, and occurring singly.

Morphological Characterization of Yeast Isolates

Microscopic observation of selected yeast isolates: The cell morphology of the yeast isolates under the microscope has ovoidal (Tf5, Mz5, and Cy) to circular (Sr3) microscopic shape (Figure 1). The yeast isolates from, Sr3 and Tf5 had single and paired budding features.

Induction of ascospore formation: In the present study, all the isolates formed spores and contained one to four or more ascospores. These ascospores were globose in shape (Figure 2).

Screening of Yeast Isolates

Hydrogen Sulphide (H₂S) production test: In this experiment, the H₂S producers presented various colony colors that ranged from light brown to black. Some of the isolated yeast strains show hydrogen sulfide production except the three yeast isolates namely Sr3, Mz5, and Tf5 which were found not producing H₂S on BSA. But, the yeast isolates B3, Fm2, D1 and commercial yeast (Cy) produce H₂S on Bismuth Sulfite Agar (BSA) (Figure 3).

Probiotics Screening Test

Antimicrobial activity test of yeast isolates: The selected

Table 1: Cultural or colony characteristics of isolated yeast after 48 hours of incubation.

Substrates	Shape	Color	Elevation	Margin
Sr3	Circular	Creamy	Spread	Rough
D1	Circular	Creamy	Spread	Rough
Fm2	Circular	Creamy	Spread	Rough
B3	Irregular	White creamy	Raised	Rough
Mz5	Irregular	White creamy	Raised	Smooth
Tf5	Circular	Creamy	Spread	Rough

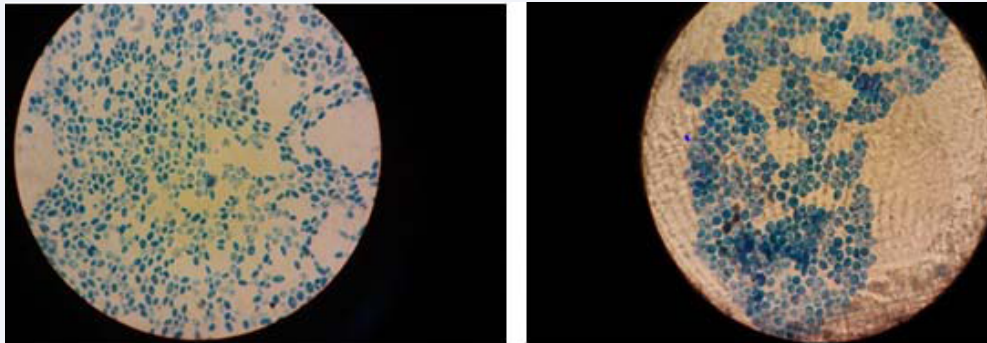


Figure 1 The cell morphology under the microscope (A: ovoidal B: circular shape).

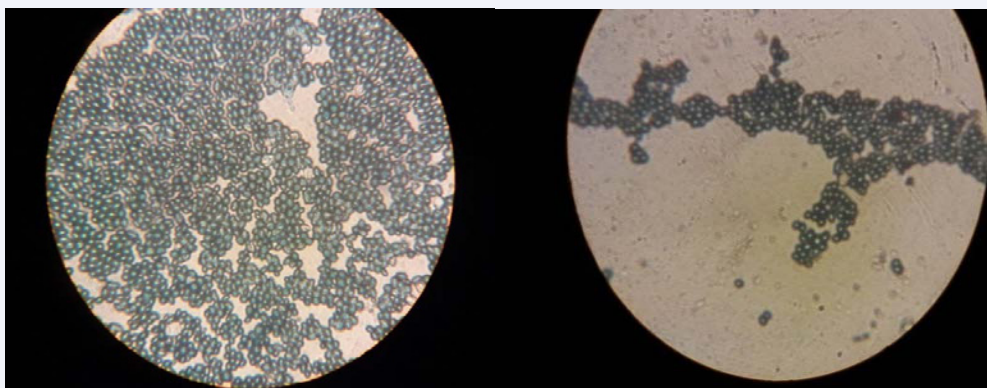


Figure 2 Ascus containing many ascospores (A), Ascus containing one to four ascospores (B).



Figure 3 Observation of H₂S gas production on BSA.

yeast isolates had anti-bacterial capability against the test organism. Table 2 shown *Sorghum* (Sr3) isolate gives the best antimicrobial activity (22 mm clear zone diameter) against *Salmonella* and 12 mm clear zone diameter against *Escherichia coli*. Moreover, all the isolates had shown antagonistic effect against the test organism except B3 and Fm2 isolates which do not have any effect on *Salmonella* and *E.coli* respectively (Table 2).

Sugar fermentation test

Results of the sugar fermentation ability of the yeast isolates indicated that almost all isolates have fermented the sugars except lactose. The abilities to ferment sugar sources were indicated by a color change from blue to orange (Table 3).

Growth characteristic at 37°C

The selected yeast isolates grew favorably at 37°C (body temperature) when compared to a conventional growth temperature at 30°C. Yeast isolates growth was monitored by measuring optical density (turbidity or concentration) of the culture at (OD_{600nm}) using

A spectrophotometer as a measure of growth. The growth rate of each of the yeast isolates at 30 and 37°C for 72 hrs is presented in Figure 4. The result indicated that all the isolated yeast strains exhibited a markedly higher growth rate at 37°C than 30°C.

Survival at low pH (acidic environment)

Yeast isolates growth and survival at low pH was monitored by colony counting at three different pH values ranging from 2, 3, and 5.5. The result indicated that almost all the isolated yeast

strains exhibited a markedly higher growth at pH 3 and 5.5 (Figure 5). Good growth was also recorded at pH 2 except B3 which shows result below conventional standard count value.

DISCUSSION

In this study, 6 yeast isolates from local fermented Cereals were selected. The isolates had, white and creamy color, oval, circular, spherical, and round microscopic shape, one up to many ascospores in ascus and budding. These results were consistent with the previous findings that indicated yeasts with similar features were grossly identified as *Saccharomyces* [20, 21]. The selected six yeast isolates fermented the given carbohydrate and classified as sugar fermenters. Sugar fermenter yeast produces organic acids thereby reducing the pH of their environment into an acidic condition which creates an unsuitable environment for pathogenic organisms [16, 22-23].

In the current study, some of the yeast isolates produced H₂S in BSA media. But, the three yeast isolates (Tf5, Sr3, and Mz5) were found not producing H₂S on the BSA medium. H₂S production is associated with an off-flavor, unpleasant taste. Moreover, Gastrointestinal disorders of animals have been attributed to the consumption of foods spoiled by yeasts that produced H₂S [24].

The probiotic and health benefit potential of yeast has also been reported in recent times by Fijan [25]. *S. cerevisiae* and *S. boulardii* are clinically proven yeasts being used as an animal probiotic and has shown to positively influence host’s health by antimicrobial effect, nutritional effect, inactivation of bacterial toxins, quorum sensing, trophic effects, immunomodulatory effects, anti-inflammatory effects, cell restitution and maintenance of epithelial barrier integrity [26]. The current finding shows that potential probiotic yeasts were isolated from cereal sourdough and identified and characterized as yeasts based on morphological, biochemical characteristics, and antimicrobial activity. Many other studies reported probiotic yeast isolated from different samples [19, 27]. AlZubaidy and Khidhr [28] also identified *Saccharomyces cerevisiae* from fruits with probiotic properties such as antimicrobial activity and gastric acid tolerance.

To be a successful probiotic, any microorganisms must have the capability to be tolerant of stresses that prevail inside the animal body. The optimal temperature for most yeast growth is 28-30°C but potential probiotic yeast must retain viability and metabolic functions active at body temperature, 37°C [29,30]. In this study, the isolated yeasts showed growth at 37°C. A similar

Table 2: Average zone of inhibition of growth of bacteria in diameter (mm).

Yeast isolates	<i>Salmonella</i>	<i>E. coli</i>
Cy	19.4	13
Sr3	22	12
Fm2	11.5	0
Tf5	21.8	12.8
B3	0	13.2
Mz5	15.7	5
D1	20.4	9
AVR	15.83	9.29

Table 3: Sugar fermentation test.

Yeast isolates	Types of sugars						
	Glucose	Fructose	Galactose	Maltose	Sucrose	Sorbitol	Lactose
Cy	+	+	+	+	+	+	-
Fm2	+	+	±	+	±	+	-
B3	+	+	+	+	±	+	-
Sr3	+	+	+	+	+	+	-
Mz5	+	+	+	+	+	+	-
Tf5	+	+	+	+	+	+	-
D1	+	+	+	+	±	±	-
-ve control	-	-	-	-	-	-	-

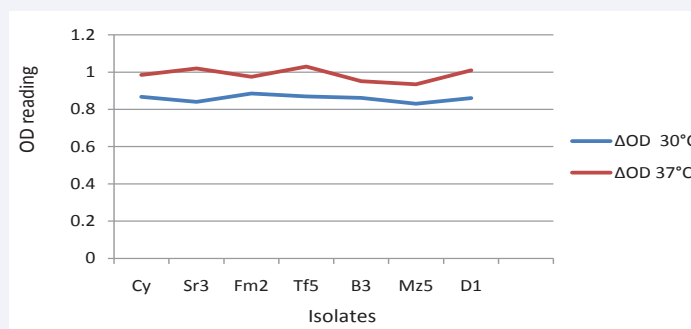


Figure 4 Absorption rates of growth of yeast isolates after 72 hours at A₆₀₀

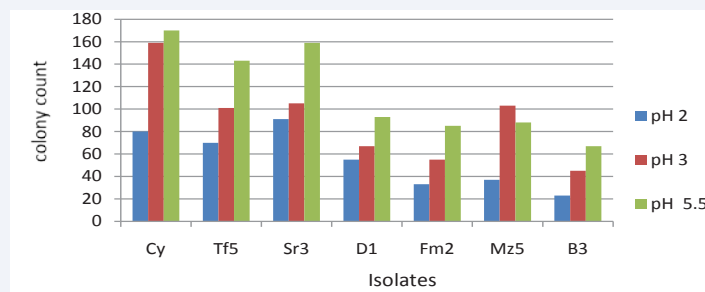


Figure 5 Survival of yeast isolates at low pH.

observation was reported by Psomas et al. [31], where all yeast isolates from the infant’s faces grew at 37°C. The current finding indicates that the selected yeast isolates grew favorably at 37°C when compared to a conventional growth temperature of 30°C.

The pH in the stomach, ranging from 2.5 to 3.5 for a fed individual due to the secretion of gastric juice is lethal to most microbes [32]. Yeast can survive over a wide pH range such that little yeast species isolates have been reported from highly acidic environments [22, 32]. The current finding reveals that the selected yeast strains survived at acidic pH 2.0. The acid tolerance demonstrated by the test yeast strains was in agreement with reports from previous studies assessing the probiotic potentials of different yeast species from various animal and food sources, including spontaneously fermented olive brines, avian and infant feces, feta cheese, and *fura* [31, 33-34].

Antimicrobial activities in yeasts are desirable properties of probiotic. It is also important for the probiotic strain to have a competitive advantage and prevent the colonization of the intestine by pathogens [35]. Most yeast that is investigated for probiotic potentials do not produce antibacterial metabolites or have antagonistic activity against pathogens [36,37]. In this experiment, yeast isolates have shown a great zone of inhibition against *Salmonella* and *Escherichia coli*. This observation was supported by a report that established that certain pathogenic bacteria possess binding molecules on their surfaces that can bind to yeasts due to mannan and polysaccharides on the outer layer of their cell wall [38].

CONCLUSION AND RECOMMENDATIONS

The present study was isolated probiotic yeasts that can contribute to the improvement of animal production.

Experimentally, 50 yeast isolates were collected from mixed cereal flour dough. The cultural characteristics indicated that the isolates were either creamy or white creamy in color, rough or smooth margin, and either spread or raised elevation. Morphologically they were spherical, oval, circular, produced ascospore, and buds that are similar to the genus *Saccharomyces*. Six isolates with the best antimicrobial activity were selected and further characterized. The selected yeast isolates were also utilized seven sugars except for lactose. The yeast isolates; Sr3, Tf5, and Mz5 with the best features of Sugar fermentation, not producing H₂S, growth at 37°C, tolerant for acidic pH (2 and 3), and with antimicrobial activity have considerable probiotic features. These isolates showed promising probiotic activities and possessed comparable attributes with other reported probiotic yeasts. Supplementation of foods with these yeasts to improve the quality of animal life is profitable, but before therapeutic application, further research should be done to ensure the safety and efficacy of the potential of this probiotic yeast. In addition to the further identification of the growth factors of the probiotic, the identification of the yeast at the species level and further investigation to clarify more antimicrobial and antagonistic activity of these yeast isolates and evaluate their capacity to adhere to intestinal epithelium are recommended.

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