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Research Article

Yeast Diversity of Ethnic Rice-Based Alcoholic Starters of the North Bengal Region

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Abstract

North Bengal region of India is highly rich in fermented foods and alcoholic beverages, the ethnic communities of the region prepare, consume, and sell diverse types of fermented foods for their sustainable livelihood. Marcha, dabai, chot, and ranu goti, are traditionally prepared dried amylolytic starters used to produce various local alcoholic beverages in this region. In the present study the phenotypic characterization gives the metabolic fingerprints of yeasts present in all alcoholic starters of North Bengal samples which showed major dominance of (yeasts) *Saccharomyces cerevisiae, Wickerhamomyces anomalus* and *Candida glabrata, Kluyveromyces marxianus, Issatchenkia, Saccharomycopsis fibuligera, Pichia guilliermondii, Candida glabrata, Pichia kudriavzevii, Pichia anomala, Pichia terricola, Hyphopichia burtonii*. Scanning electron microscope (SEM) has been performed to reveal the surface structures of yeast isolates. The alcohol content of the fermented product ranges between 6-6.5 %, and the alcohol tolerance is up to 17 % (v/v), pH ranges between 3.9 to 4.0 and titratable acidity (g/100 ml) ranges between 0.17 to 0.48. No pathogenic contaminants are observed in all eight alcoholic starers of North Bengal. The present study reveals the presence of yeasts with amylolytic, ethanol tolerance, and ethanol-producing ability from amylolytic starters of North Bengal which could be a good source for future bioprospection. The yeasts community constitutes a large and heterogeneous group of microorganisms that is currently a major attraction for food scientists and the biotechnological industries worldwide.

Keywords: Alcoholic Starters; North Bengal; Sustainable Production; Yeast Diversity; SEM; Fermentation; Livelihood

Introduction

Fermented foods and alcoholic beverages are produced by the action of different microorganisms themselves (bacteria, yeast, and mycelia fungi) and their enzymes as well [1]. Fermentation is defined as the enzyme-catalyzed, energy-yielding pathway in the cells which involves the breakdown of molecules such as glucose anaerobically. When it comes to food preservation, fermentation is one the oldest and most economical methods used in food preservation. Apart from preservation, fermentation adds up to some more benefits to certain foods and beverages which include, the prevention of food spoilage by microorganisms, enhancing the nutritional value of the foods and beverages through the synthesis of essential amino acids and vitamins during the process of fermentation, enhancing the digestibility of food that is often difficult to assimilate from a nutritional perspective [2] and fermentation also makes the products better in terms of taste, aroma texture, ethanol content and so on, detoxification and destruction of certain undesirable substances present in the raw foods such as phytates, tannins, and polyphenols [3] is also carried out during fermentation.

Microorganisms carrying out fermentation may be the indigenous microflora present on the substrate or may be added externally as a starter culture. A starter culture is a microbial preparation containing many cells of one or more microbial species that are added to raw material to produce fermented food by accelerating and directing fermentation.

These starters are mainly prepared by the tribal and rural people or communities of different regions using their indigenous knowledge. The North Bengal region of West Bengal (state) is one such region where such starters are prepared by the local rural and tribal people. Marcha is one of the starters that is prepared and used for producing different fermented alcoholic beverages by the people of North Bengal. Not only in India but marcha is used as a starter and is known by different names in different countries say marcha in Nepal and Bhutan, ragi in Indonesia, loogpang in Thailand, bubod in the Philippines, Chinese yeast in Taiwan [4], and nuruk in Korea [5]. The raw material used for the preparation of marcha is glutinous rice.

Marcha can be either round and small solid balls or large like a flattened cake ranging from 2 cm - 13 cm in diameter and 10 gm - 95 gm in weight. It can be either whitish or creamy in colour sometimes with a slight yellowish texture on the surface. Marcha in some places has a sweet odour itself. In the North-Bengal region, this starter is used for the preparation of various amylolytic/alcoholic beverages such as Tongba, Bhaati jaanr, Makai ko jaanr, Gahoon ko jaanr of Darjeeling, Haria, and Handia of Matigara, Siliguri and other nearby areas as well. These amylolytic/alcoholic beverages or drinks are prepared by the different tribal communities including Adivasi, Rava, Toto, Santhal, Saibo, and Uraon which have strong ritualistic importance among these communities. These drinks are consumed by the tribal people on different occasions including marriages, birth ceremonies, annaprashana, and death ceremonies and some people consume it regularly as well. The microflora of Marcha consists of filamentous molds such as *Mucor circinelloides* and *Rhizopus chinensis*, yeasts such as *Saccharomycopsis fibuligera* and *Pichia anomala*, and bacteria such as *Pediococcus pentosaceus* [6]. Generally, marcha are yeast and mold dominant but in a few marcha samples, lactic acid bacteria (LAB) were also found to be present.

The present study is focused on revealing the yeast diversity of Marcha of North Bengal.

Materials and Methods

Survey

Extended surveys were conducted in all seven districts of North Bengal visiting the tribal areas to gather information about different fermented foods, alcoholic beverages, and starters used for their preparation. However, the main objective of the survey was to gather information about the starter called Marcha, the raw materials used for its preparation, and the process of preparation.

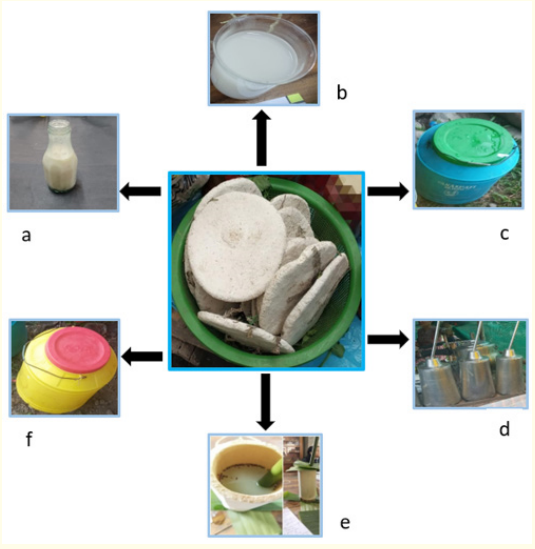


Figure 1: The starter marcha used in the preparation of different alcoholic beverages such as, (a) Bhaati jaanr (b) Haria (c) and (f) Gahoon ko jaanr (d) Makai ko jaanr and (e) Tongba.

Sample collection

The traditionally prepared marcha (alcoholic/amylolytic starter) samples were collected from all seven districts of North Bengal which include Darjeeling, Coochbehar, Alipurduar, Kalimpong, Jalpaiguri, Uttar Dinajpur, and Dakshin Dinajpur. A total of 21 samples were collected 3 from each district. These samples were then packed in gamma-irradiated sterile polybags and transported to the laboratory in a sterile condition for further analysis.

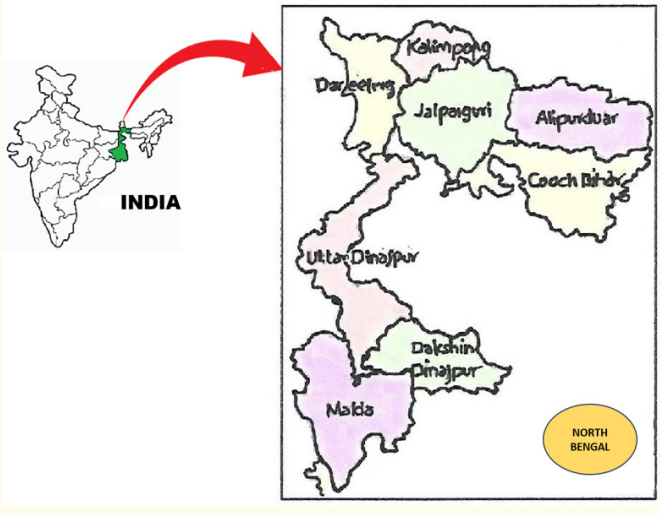


Figure 2: Map of North Bengal.

Isolation of microorganisms

One gram of the sample was homogenized with 9 ml of sterile distilled water and was kept for 1 minute. Thereafter, serial dilution was carried out in the same diluent. The same method was followed for all the samples collected respectively. Now, yeast isolation was done on yeast-malt agar (YM agar) (M424, HiMedia, India) supplemented with 10 IU ml^{-1} benzylpenicillin and 12 mg ml^{-1} streptomycin sulfate following the pour plate method. The plates were incubated at 28°C for 3 days in aerobic conditions and were observed for the appearance of yeast colonies. The purity of the isolates was checked by streaking again on YM agar plates followed by microscopic examination. Isolation of yeast strains was typically based on morphotypes and criteria including size, color, shape, and appearance of fully grown culture on growth media. Colonies were counted as colony forming units (cfu)/g sample. Identified strains of yeasts were preserved in 20% glycerol at −20 ◦ C [7].

Phenotypic and Biochemical Characterization

Growth at 37oC

Yeast-malt extract agar plates were inoculated with cells of actively grown yeast isolates, incubated at 37°C for 4 days, and observed for growth [8].

Growth at 45oC

Yeast-malt extract agar plates were inoculated with cells of actively grown yeast isolates, incubated at 45°C for 4 days, and observed for growth [8].

Sugar fermentation

Yeast isolates were grown at 28°C on yeast-malt (YM) broth for 3 days. Tubes of 10 ml of fermentation basal medium (Wickerham 1951) were supplemented with 2% w/v sterile sugars inoculated with the above yeast culture, incubated at 28°C, and shaken with the help of hands to observe [8].

Sugar assimilation

The yeast isolates were grown at 28°C on yeast-malt (YM) broth for 3 days. Tubes containing a 5 ml mixture of yeast nitrogen base and different carbon sources were inoculated with cultures and incubated at 28°C for 3 days. The control test tube was made by adding 0.5 ml of yeast nitrogen base in 4.5 ml of sterilized distilled water (devoid of any carbon source). The assimilation of carbon sources will be observed by comparing it with the control (Yarrow, 1998). Yeast isolates will be identified at the genus level according to the criteria laid down by [9] and Yarrow [8].

Ethanol tolerance

24-hour-old yeast cultures of different yeast isolates were grown on liquid YEPD-medium. Ethanol tolerance of the strains was observed after 48-72 hrs of incubation at 37°C [10] for different ethanol concentrations including 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, and 20%.

Estimation of alcohol produced

The ethanol yield of yeast isolates was determined after the growth at 28°C for 3, 4, 5, and 6 days in YM-broth containing 10% glucose [11]. The percent of ethanol produced was estimated using an alcohol meter.

Cell Morphology and Budding Type

The yeast isolates were grown at 28°C on yeast-malt (YM) broth for 3 days. Thereafter, one drop of the broth was taken on grease-free slides and then the slides were observed under the microscope(100X) to determine the cell morphology and budding type of the live yeast cells, respectively. Yeast identification was carried out using the methods of [12,13].

Determination of amylolytic activity of the yeast

Active yeast cultures were streaked in the center of the surface of soluble starch agar plates (4% soluble starch, 5% yeast extract, and 1.5% agar) and were allowed to grow. After incubation for 72 hours at 37°C. The Petri plates were flooded with Lugols Iodine solution (2gm iodine, 2gm ammonium sulfate, and 300ml distilled water) for 1 minute and the diameter of the clear zone and colony was measured. The amylolytic activity was expressed as the ratio of clear zone diameter to colony diameter [14].

Detection of pathogenic contaminants

The enumeration of the pathogenic contaminants of the samples was carried out on specific selective media such as *Bacillus cereus* agar base for *Bacillus cereus*, Baird Parker agar base for *Staphylococcus aureus*, *Salmonella-Shigella* agar for Salmonella and Shigella, and Eosin Methylene blue (EMB) agar for *E. coli* respectively [15].

Bacillus cereus

Sample dilution was carried out in sterilized distilled water. The pour plate method was followed using *Bacillus cereus* agar base medium. The plates were then incubated at 30°C for 24-48 hours. Characteristic turquoise to peacock blue colonies surrounded by a zone of precipitate of the same color were regarded as presumptive *Bacillus cereus*.

Staphylococcus aureus

After serial dilution of the sample enumeration of *Staphylococcus aureus* was carried out using Baird Parker agar base following the pour plate method. The plates were incubated at 30°C for 24-48 hours. The black colonies surrounded by a clear zone extending 2-5 mm into the opaque medium appeared will be regarded as presumptive *Staphylococcus aureus*.

Salmonella and Shigella

Salmonella-Shigella (SS) agar was used for the detection of *Salmonella* and *Shigella* in the sample. After serial dilution, the SS-agar plates were incubated at 37°C for 48 hours and were observed in a dark background for presumptive colonies. *Salmonella* colonies appear dark-centred while colourless colonies were regarded as presumptive *Shigella*.

Escherichia coli

To detect *Escherichia coli (E. coli),* one type of coliform bacteria in the sample Eosin methylene blue (EMB) agar was used. After serial dilution, the plates were incubated at 37°C for 18-24 hours and observed for the presumptive colonies. Blue-black colonies with a green metallic sheen were regarded as presumptive *Escherichia coli.*

Scanning electron microscopy

Scanning electron microscopy has been used to study colony structures of 5 yeast isolates associated with alcoholic starters. Preparation of the yeast cultures for scanning electron microscopy (SEM) was done following the protocol for cultured microorganisms by Das Murtey and Ramasamy (2018) [16]. From the YPD agar plate, single colonies were taken and grown in YPD broth for 24 hrs at 25°C. One milliliter of the sample was centrifuged at 900 g for 2 min for pellet formation and resuspended in 5% glutaraldehyde solution prepared in 0.1 M phosphate buffer (pH 7.2) for fixation. 30 minutes later the sample was centrifuged, the supernatant was discarded and the pellet was washed twice in 0.1M phosphate buffer. The pellet was resuspended in 1% osmium tetroxide prepared in 0.1M phosphate buffer. Sample dehydration was then carried out using ethanol series of 35, 50, 75, and 95%, absolute ethanol, and hexamethyldisilazane (HDMS) for 30 minutes per step, centrifuging and discarding the supernatant in each case. Lastly, the second HDMS was discarded and the sample was left to dry overnight in a desiccator.

Now, the dehydrated yeast sample was mounted on plain aluminum stubs using carbon double surface adhesive and coated with a 5 nm gold-palladium (80:20) layer using a Gold Sputter Coater (BIO-RAD Polaron Division, SEM coating system, United Kingdom) and observed under a constant accelerating voltage of 5 kV under a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan).

Results and Discussion

Survey

From the survey conducted it was found that Marcha is generally prepared by the tribal people for selling so that they can earn for their living. They sold the Marcha in the nearby markets, haats, and local shops. The cost varied depending on the size of the Marcha, ranging from Rs 20-50. The preparation of Marcha is a very lengthy process and is prepared traditionally without using any artificial means. The entire traditional process of Marcha preparation is discussed in the form of a flowchart below:

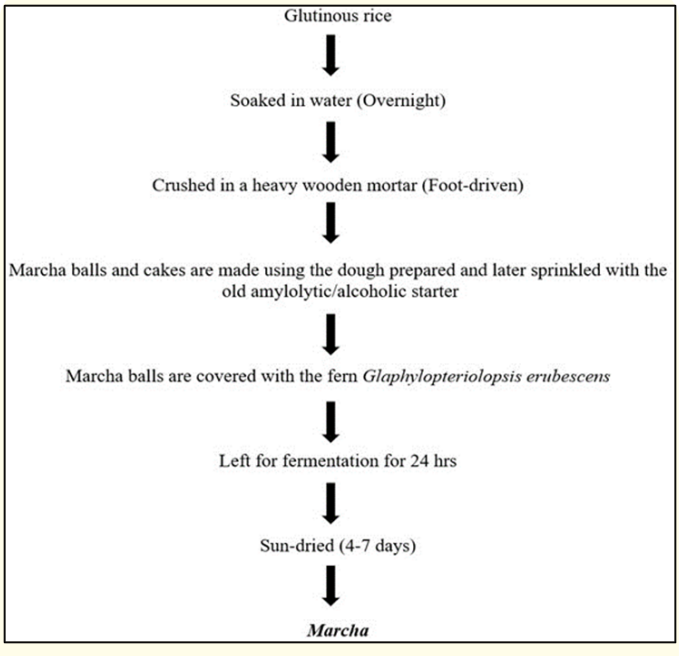


Figure 3: Traditional method of Marcha preparation.

Sample collection

21 samples were collected from different markets, haats, and shops of all seven districts of North Bengal and were used for further analysis. The detailed information on the samples collected is discussed below in a tabular form:

The samples collected (Marcha) have different shapes and sizes, from round and small to large like a flattened cake. Some images of the samples that were clicked are depicted below in Figure 4.

Number ofsamples collected (n)	Place of collection (Districts)	Common/LocalName of the sample	cfu/g x 10^6	pH	Titrable acidity
3	Darjeeling	Marcha	7.3 (7.2-7.4)	3.4	0.19
3	Coochbehar	Chot, Dabai	6.9 (6.8-7.1)	3.6	0.17
3	Alipurduar	Dabai	6.8 (6.5-7.1)	3.7	0.43
3	Kalimpong	Marcha	7.3 (7.2-7.4)	3.6	0.25
3	Jalpaiguri	Dabai, Ranu goti	6.9 (6.8-7.1)	3.9	0.48
3	Uttar Dinajpur	Ranu goti, dabai,	7.1 (7.0-7.2)	3.5	0.26
3	Dakshin Dinajpur	Ranu goti	7.1 (7.0-7.2)	4.0	0.43

Table 1: Representation of samples.

Figure 4: Amylolytic/alcoholic starters (Marcha).

Isolation of microorganisms

A total of 21 yeasts were isolated from the samples. Here, 4 yeasts were isolated from the Alipurduar sample, 3 from the Coochbehar sample, 2 from the Jalpaiguri sample, 3 from the Kalimpong sample, 4 from the Darjeeling sample, 3 from the Uttar Dinajpur sample, and 2 from the Dakshin Dinajpur sample and these yeast isolates were represented by the codes AY, CY, JY, KY, DY, UDY, and DDY respectively. After isolation, the isolates were preserved in 30% glycerol at −20 ◦ C for further analysis.

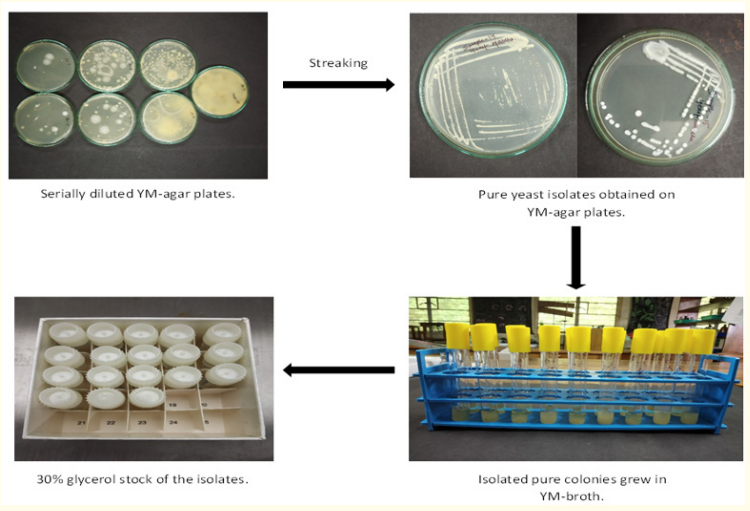


Figure 5: Isolation and preservation of yeast isolates.

Growth at 37°C

All 21 isolates when grown on YM-agar at 37°C showed a positive result i.e., all isolates were able to grow at 37°C. The presence of colonies on the YM-agar plates was observed for confirmation.

Growth at 45°C

The presence of colonies on YM-agar plates showed the growth of all 21 isolates at 45°C thereby showing a positive result.

Sugar fermentation

To determine the yeast isolates sugar fermenting ability, a total of 8 different sugars were used such as glucose, rhamnose, galactose, sucrose, maltose, lactose, trehalose, and arabinose. It was found that out of the 21 isolates, all were able to ferment all sugars except AD-1 which couldn't ferment lactose and trehalose, AD-2 which couldn't ferment maltose, lactose, and arabinose, AD-3 was unable to ferment rhamnose, lactose, and arabinose, AD-4 which couldn't ferment lactose and, trehalose, the isolates JY-1 and JY-2 were unable to ferment lactose, KY-2 lacked the ability to ferment lactose and arabinose, KY-3 couldn't ferment lactose, trehalose, and arabinose, DY-1 couldn't ferment arabinose, UDY-1 was unable to ferment trehalose and arabinose, UDY-2 which couldn't ferment rhamnose and sucrose, DDY-1 which couldn't ferment trehalose and lastly DDY-2 which was unable to ferment the sugars such as glucose, galactose, sucrose, and arabinose.

Straincode	Sugars Fermented								Tentative Identification
	Glucose	Rhamnose	Galactose	Sucrose	Maltose	Lactose	Trehalose	Arabinose	
AD-1	+	+	+	+	+	-	-	+	*Saccharomyces cerevisiae*
AD-2	+	+	+	+	-	-	+	-	*Candida glabrata*
AD-3	+	-	+	+	+	-	+	-	*Kluyveromyces marxianus*
AD-4	+	+	+	+	+	-	-	+	*Saccharomycopsis fibuligera*
CY-1	+	+	+	+	+	+	+	+	*Pichia guilliermondii*
CY-2	+	+	+	+	+	+	+	+	*Candida glabrata*
CY-3	+	+	+	+	+	+	+	+	*Pichia tropicalis*
JY-1	+	+	+	+	+	-	+	+	*Pichia anomala*
JY-2	+	+	+	+	+	-	+	+	*Issatchenkia*
KY-1	+	+	+	+	+	+	+	+	*Wickerhamomyces anomalus*
KY-2	+	+	+	+	+	-	+	-	*Issatchenkia*
KY-3	+	+	+	+	+	-	-	-	*Pichia terricola*
DY-1	+	+	+	+	+	+	+	-	*Saccharomyces cerevisiae*
DY-2	+	+	+	+	+	+	+	+	*Issatchenkia*
DY-3	+	+	+	+	+	+	+	+	*Pichia anomala*
DY-4	+	+	+	+	+	+	+	+	*Candida glabrata*
UDY-1	+	+	+	+	+	+	-	-	*Issatchenkia*
UDY-2	+	-	+	-	+	+	+	+	*Saccharomyces cerevisiae*
UDY-3	+	+	+	+	-	-	-	+	*Pichia anomala*
DDY-1	+	+	+	+	+	+	-	+	*Saccharomycopsis fibuligera*
DDY-2	-	+	-	-	+	+	+	-	*Saccharomycopsis fibuligera*

Table 2: Sugar fermentation test.

(+) = Sugars fermented

(-) = Sugars not fermented.

Sugar assimilation

To determine the sugar assimilating ability of the yeast isolates a total of 8 different sugars were used such as glucose, rhamnose, galactose, sucrose, maltose, lactose, trehalose, and arabinose. It was found that out of the 21 isolates, all isolates were able to assimilate the different sugars except AD-1 and UDY-2 which couldn't assimilate any of the sugars, and DY-4 which only assimilated maltose.

Strain Code	Sugar Assimilated								Tentative Identification
	Glucose	Rhamnose	Galactose	Sucrose	Maltose	Lactose	Trehalose	Arabinose	
AD-1	-	-	-	-	-	-	-	-	*Saccharomyces cere*
AD-2	+	+	+	+	+	+	+	+	*Candida glabrata*
AD-3	+	+	+	+	+	+	+	+	*Kluyveromyces mar*
AD-4	+	+	+	+	+	+	+	+	*Saccharomycopsis*
CY-1	+	+	+	+	+	+	+	+	*Pichia guilliermond*
CY-2	+	+	+	+	+	+	+	+	*Candida glabrata*
CY-3	+	+	+	+	+	+	+	+	*Pichia tropicalis*
JY-1	+	+	+	+	+	+	+	+	*Pichia anomala*
JY-2	+	+	+	+	+	+	+	+	*Issatchenkia*
KY-1	+	+	+	+	+	+	+	+	*Wickerhamomyces*
KY-2	+	+	+	+	+	+	+	+	*Issatchenkia*
KY-3	+	+	+	+	+	+	+	+	*Pichia terricola*
DY-1	+	+	+	+	+	+	+	+	*Saccharomyces cere*
DY-2	+	+	+	+	+	+	+	+	*Issatchenkia*
DY-3	+	+	+	+	+	+	+	+	*Pichia anomala*
DY-4	-	-	-	-	+	-	-	-	*Candida glabrata*
UDY-1	+	+	+	+	+	+	+	+	*Issatchenkia*
UDY-2	-	-	-	-	-	-	-	-	*Saccharomyces cere*
UDY-3	+	+	+	+	+	+	+	+	*Pichia anomala*
DDY-1	+	+	+	+	+	+	+	+	*Saccharomycopsis*
DDY-2	+	+	+	+	+	+	+	+	*Saccharomycopsis*

Table 3: Sugar assimilation test.

(+) = Sugars assimilated

(-) = Sugars not assimilated.

Ethanol tolerance

Ethanol tolerance for different concentrations of ethanol including 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, and 20% was observed and it was found that all the isolated yeast strains were able to survive at ethanol concentration 7-20% respectively.

Estimation of alcohol production

The percent of ethanol produced was estimated using an alcohol meter, it was found that all the isolates were able to produce alcohol on the 3rd day except DY-4, UDY-1, and UDY-2, with a minimum of 1% and a maximum of 4% alcohol was estimated on the 3rd day. On the 4th day, a maximum of 5% alcohol was estimated. On the 5th day maximum of 6% alcohol was produced. Lastly, on the 6th day, 7% alcohol was estimated to be the maximum. The isolate AD-4 produced the maximum i.e., 7% of ethanol at the end of the 6th day.

Cell morphology and budding type

The cell morphology and the budding type of all 21 isolates were observed individually under the microscope and it was found that the shape was ellipsoidal, spheroid, and cylindrical, and the budding types bipolar, tripolar, and multipolar as depicted in the table below.

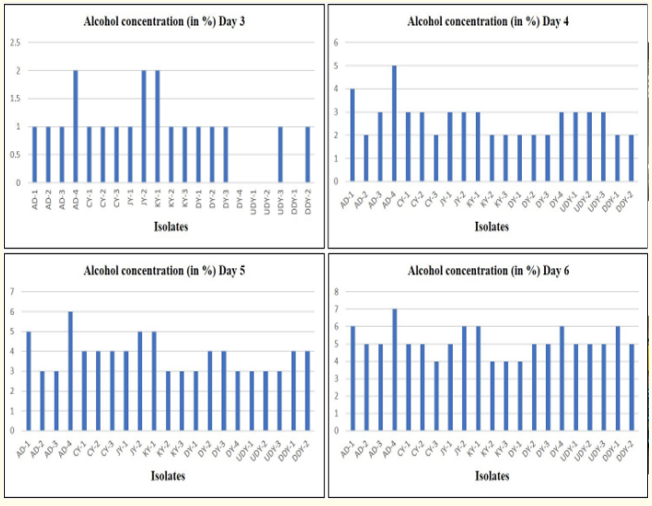


Figure 6: Ethanol production by the isolates at days 3,4,5, and 6.

Strain code	Cell morphology	Budding type
AD-1	Ellipsoidal	Bipolar
AD-2	Spheroid	Bipolar
AD-3	Spheroid	Bipolar
AD-4	Ellipsoidal	Bipolar
CY-1	Spheroid	Bipolar
CY-2	Spheroid	Bipolar
CY-3	Spheroid	Tripolar
JY-1	Ellipsoidal	Bipolar
JY-2	Ellipsoidal	Bipolar
KY-1	Spheroid	Multipolar
KY-2	Spheroid	Bipolar
KY-3	Spheroid	Multipolar
DY-1	Spheroid	Tripolar
DY-2	Spheroid	Tripolar
DY-3	Cylindrical	Tripolar
DY-4	Spheroid	Bipolar
UDY-1	Ellipsoidal	Bipolar
UDY-2	Ellipsoidal	Bipolar
UDY-3	Spheroid	Bipolar
DDY-1	Spheroid	Tripolar
DDY-2	Spheroid	Bipolar

Table 4: Yeast cell morphology and budding type.

The cell morphology and the budding type of the yeast isolates as observed under the microscope are shown in figure 7.

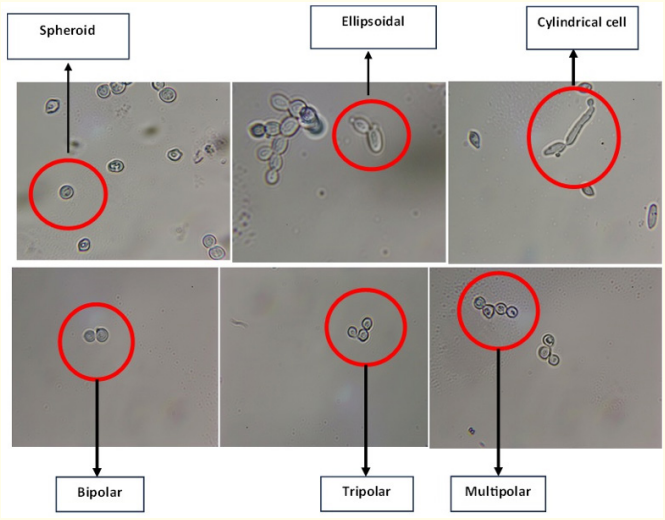


Figure 7: Cell morphology and budding type seen in yeasts (100X).

Determination of amylolytic activity of yeast cells

All 21 yeasts isolated from the starter called marcha were checked for their amylolytic activity. It was found that all 21 isolates showed negative results, i.e., no clear zone was observed around the colony.

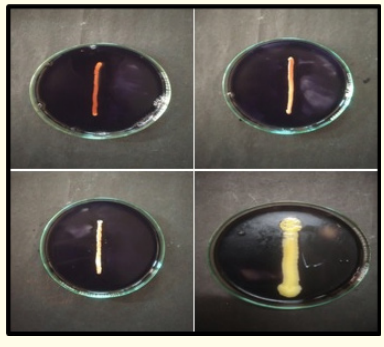


Figure 8: Observed negative results.

Detection of pathogenic contaminants

The pathogenic contaminants test gave negative results for all the collected starter samples. No colonies of *Salmonella* and *Shigella, Staphylococcus aureus*, *Bacillus cereus,* and fecal coliforms were found in their specific culture media. Hence the absence of these microbes makes the drinks fit for consumption that are prepared using the starter marcha. Figure 9 shows the negative results below.

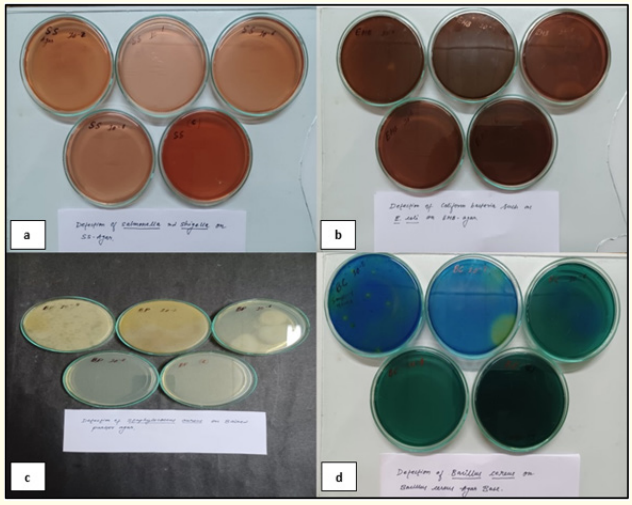


Figure 9: (a) Detection of Salmonella and Shigella on SS-Agar, (b) Detection of Coliform bacteria such as *E. coli* on EMB-Agar, (c) Detection of Staphylococcus aureus on Baird Parker Agar, (d) Detection of *Bacillus cereus* on *Bacillus Cereus* Agar Base.

Scanning electron microscopy of yeast

Scanning electron microscopy (SEM) was performed to analyze different yeast isolates and investigate differences in cell morphology. The SEM images can be seen in Figure 9. SEM was performed for the isolates AD-2, CY-3, JY-1, KY-2, DY-1, and UDY-3 respectively. The shape of the cells varied from spheroid to oval and ellipsoidal. The size of the cell was found to be between 0.761 µm-4.182 µm.

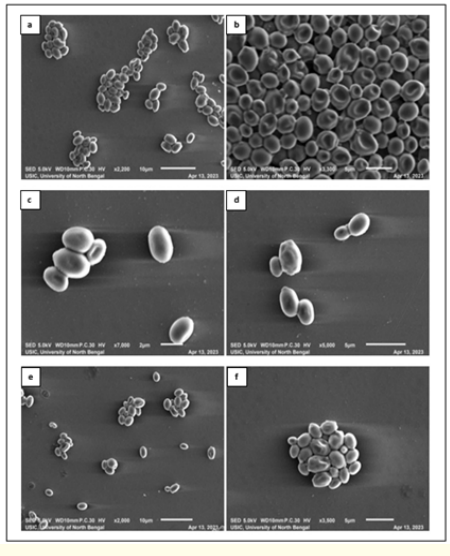


Figure 10: Scanning electron microscopy (a) AD-2, (b) CY-3, (c) JY-1, (d) KY-2, (e) DY-1, and (f) UDY-3.

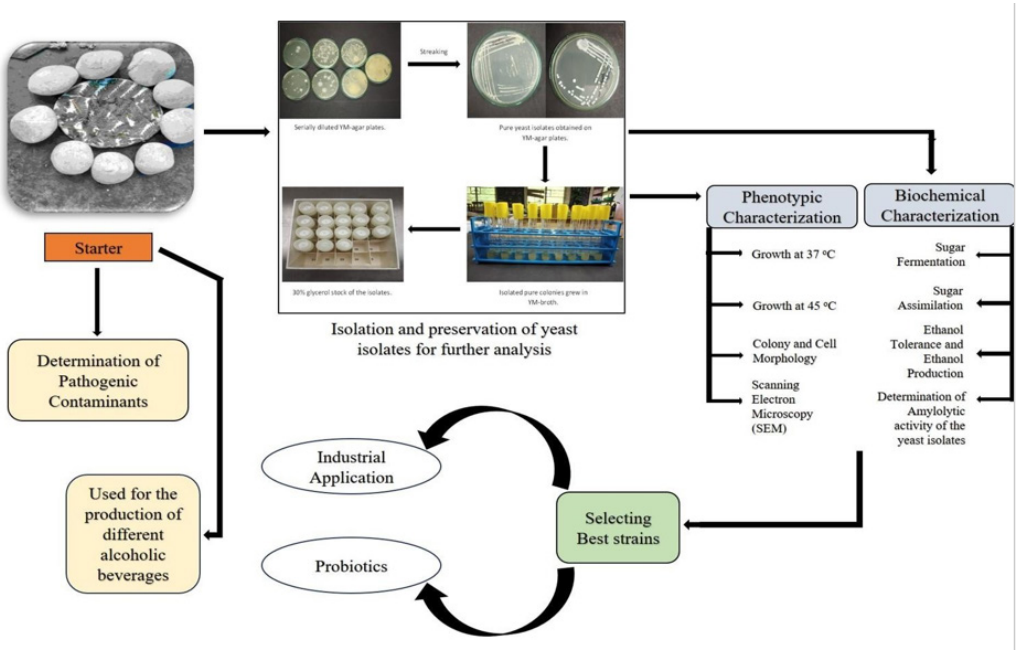
Discussion

The diversity of yeast strains associated with alcoholic and amylolytic starters may be directly related to the raw material or substrate used and the local geographical conditions where they are produced [17]. *Saccharomycopsis fibuligera, Saccharomyces cerevisiae, W. anomala, Pichia guilliermondii*, and *Candida sp.* are the most common yeast isolates observed almost in all geographically located rice-based starters of Asia [18-20]. It is very interesting to investigate that yeast species (*W. anomalus*) could not be detected by conventional media but has been detected in all alcoholic starters, but our studies showed the presence of an abundance of *W. anomalus* after biochemical analysis. *W. anomalus* has been reported in hong-qu and yao-qu, traditional amylolytic starters of China, and banh men, a traditional Vietnamese starter [17,19]. However, we noticed that *Pichia*, *Kluyveromyces, P. anomala, Issatchenkia, S. cerevisiae, Candida musae, S. fibuligera,* and Saccharomycopsis spp., which were detected in *marcha of Sikkim, hamei* of *Manipur, Thiat* of *Meghalaya, Chiowan* of *Tripura and Phut* of *Arunachal Pradesh*. *Saccharomycopsis fibuligera, Saccharomyces cerevisiae, W. anomala, Pichia guilliermondii*, and *Candida sp. were* also observed in most of the North East alcoholic starter samples. The ethanol tolerance and amylolytic activities of alcoholic starters were observed in all the alcoholic starter samples of North Eastern states such as *marcha* of *Sikkim, hamei* of *Manipur, Thiat* of *Meghalaya, Chiowan* of *Tripura, and Phut* of *Arunachal Pradesh* similar to our results. Although the species of *Saccharomyces bayanus* have not been isolated from any other Asian amylolytic starters, the closely related species of *Saccharomyces cerevisiae* was isolated from *ragi* of Indonesia and *banh men* of Vietnam [18-22]. *Saccharomycopsis fibuligera* is the most dominant yeast in *Marcha* which is typically found growing on cereal products. There are no pathogenic contaminants such as *Streptococcus, Staphylococcus, E. coli, B. cereus, Salmonella, or Shigella* were detected in all eight alcoholic starers of North Bengal [28]. Similar results are also shown in the many alcoholic starters of North East India as well as Asian amylolytic or alcoholic starter samples and these amylolytic starters have many yeasts strains with probiotic attributes [23-29].

Conclusion

Amylolytic and alcoholic starter culture-making technology reflects the traditional method of "sub-culturing" desirable inocula or microbial consortia from previous batches to new culture using rice as base substrates by back-sloping method, in the North Bengal region by the local tribes from ancient times. The selection of local alcoholic starters from various geographi-

cal locations with wide diverse microbiomes is gaining the importance of fungal/yeast species diversity as indigenous property. Our ancient tribal communities preserved fungal or yeast or bacterial consortia in the viable form of alcoholic starter for home-based brewing or fermentation with their knowledge of rural biotechnology. These results may enrich our knowledge of indigenous yeasts that may be present in the ethnic alcoholic starters and may be used to promote the development of unique ethnic alcoholic beverages; moreover, data of alcoholic starters of North Bengal. Research on probiotics has been dynamically developing in recent years, including the use of probiotic yeasts, which has been minimized thus far and is gaining more and more interest. This study was carried out for the isolation of pure yeast strains from the starter and their characterization. It was observed that most of the yeast strains showed good biochemical characteristics and can be further used for different aspects such as probiotic potential, the development of pure yeast-starter with improved qualities, and in food industries.



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Contributions

SPS, AS, SPSa were involved in developing the idea of reviewing, drafting the manuscript, and finalizing the manuscript. All authors contributed to the article and approved the submitted version. (SPSah and SPSha share the first authorship) (┼ = First authorship).

Ethical Approvals

This study does not involve experiments on animals or human subjects.

Data Availability

All the raw data of biochemical and phenotypic tests are available with the authors and shall be provided upon request.

Conflicts of Interest

The authors report no financial or any other conflicts of interest in this work.

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