Chapter 4: Identification of fungal and lactic acid bacteria isolates from rice beer and starter cultures and evaluation of their functional properties

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4.1 Introduction

The starters for rice beer allows the growth of various natural fungi, yeast and lactic acid bacteria (LAB). The final produce is obtained through a rough filtration of the fermented mash, and hence contains high count of the microbes involved in the fermentation process [1]. The moulds present in the starters produce a-amylase and amyloglucosidase that results in liquefaction and saccharification of rice starch thereby producing dextrins and maltose, mainly glucose [2]. Here parallel fermentation occurs i.e. progressive saccharification of starch and alcoholic fermentation of the liberated glucose liberated by yeasts, thereby avoiding the exposure of yeast cells to high concentrations of sugar, and contributing to the high ethanol production [3].

The moulds present in the starters produce the starch degrading enzymes viz., α-amylase and amyloglucosidase (glucoamylase) which result in liquefaction and saccharification of rice starch into dextrins and maltose, but mainly into glucose [2]. α- amylase catalyzes the hydrolysis of internal α-1,4-O-glycosidic bonds in polysaccharides and the products retain the α-anomeric configuration. Their activity, structural integrity and stability is dependent on the presence of calcium ions. They are classified as endoamylases (cleave internal α-1,4 bonds), exoamylases (cleave α- 1,4 or α-1,6 bonds of the external glucose residues) debranching enzymes (hydrolyze α-1,6 bonds exclusively) and transferases (cleave α-1,4 glycosidic bond and transfers part of the donor to a glycosidic acceptor) [4]. Glucoamylase on the other hand is capable of hydrolyzing a-1,4 glycosidic linkages in oligosaccharides with the inversion of the anomeric configuration to produce D-glucose. It successively removes glucose units from the non-reducing end of amylose or amylopectin molecules. It also slowly hydrolyzes a-1,6 glycosidic linkages of starch [5,6].

Fermentation of the cereal extracts by yeasts is the most important step involved in brewing. Brewing strains of yeast are polyploid or aneuploid, having 3 or 4 copies of each chromosome. This multiplicity of gene copies results in a more stable organism, leading to a boost of enzyme production and hence rapid metabolism of wort components [7]. *Saccharomyces cerevisiae* is a diplontic and homothallic yeast. Therefore it has high clonal reproduction ability and confers the possibility of regenerating a diploid cell from a haploid. The genetic diversity of *S. cerevisiae* strains is revealed by the numerous works carried out on

the microflora diversity of wine and other fermented foods. A high rate of homozygote strains found are found in yeasts and this could also be a way to adapt to the various environments by modifying the dosage of certain important genes [8]. *Saccharomyces cerevisiae* has the ability to convert sugars rapidly to ethanol and carbon dioxide, under both anaerobic and aerobic conditions. They are also categorized as Crabtree-positive yeasts, which under aerobic conditions, carries out respiration with oxygen as the final electron acceptor, and Crabtree-negative yeasts, which under aerobic conditions produces biomass and carbon dioxide are the sole fermentative products [9]. Each type of beer has its own prevailing aroma due to the formation of higher alcohols and esters triggered by the yeast strain responsible for fermentation and this involves complex enzymatic and regulatory pathways [10]. Most yeast strains can tolerate alcohol concentration of 10–15 %, however, certain strains can tolerate higher amounts of alcohols resulting in fermented beverages ranging from 5–21 % alcohol content [11]. The desirable characteristics in yeasts to be used for alcoholic fermentation are good fermentation speed, low production of volatile acidity, high alcohol tolarance, production of ethanol according to the quantity of sugar in the must and limited production of higher alcohols, complete fermentation of sugars present, growth at high temperature, good glycerol production, proper settling after fermentation, resistance to SO_2, low SO_2 and H_2S production, low foaming and low acetaldehyde production [12]. The use of selected yeast strains in beer making results in better quality product. The local selected strains are more effective as compared to the commercial yeasts as they are better acclimated to the environmental conditions and dominate the fermentation. It also assures the maintenance of typical region wise sensory properties [12].

The LAB represent a group of microorganisms that are functionally related by their ability to produce lactic acid during fermentation [13]. They are aero tolerant anaerobes i.e. they can tolerate oxygen but do not use it [14]. The lactic acid bacteria (LAB) have been used traditionally for the preservation of food materials through fermentation by producing organic acids to control putrefactive microorganisms and pathogens. They are responsible for the taste, texture, color, flavor, and the nutritional properties of fermented foods [15]. LAB selected to carry out fermentation need to possess broad substrate utilization ability [16]. The complete utilization of sugars by LAB during primary fermentation is desired since any sugars remaining after the lactic fermentation can serve as substrates for other fermentative bacteria and yeasts to grow [17]. The LAB have been categorized into two groups viz. the homolactics that produce lactic acid as its only or major end product using the glycolitic pathway and the heterolactics which produce only one molecule of lactic acid along with carbon dioxide and ethanol or

acetate as its major products. The homolactics produce twice as much energy then heterolactics as they produce two lactic acid molecules from one glucose molecule. Thus, the LAB can further be categorized into the obligate homofermentative (which only use the glycolitic pathway), the facultative heterofermentative (which use the glycolitic pathway but are able to use the heterolactic process) and the obligate heterofermentative (which only use heterolactic process) [18,19]. Even though simultaneous carbohydrate utilization has been demonstrated in few species of LAB, they are well known to consume citrate with glucose, lactose or maltose [16]. Lactic acid (CH3–CH (OH)– COOH) which is a nonvolatile, odorless organic acid and is classified as GRAS (generally recognized as safe) is widely used as a preservative chemical in food industry, acidulant in pharmaceutical, cosmetic and chemical industry [20].

Although certain characteristics of the rice beer prepared in Assam have been reported earlier, taxonomical identification of microbes associated is missing. In the present study, the amylolytic fungi, yeasts and LAB associated with some starter cakes and rice beer produced in Assam have been isolated, identified and their physiological and functional properties were studied.

4.2 Materials and Methods

4.2.1 Materials

The starter cakes and rice beer samples were collected from the households of different regions of Assam, India. All the samples were collected in three replicates in sterile bottles, marked according to the place of collection, brought to the laboratory under refrigerated condition on the same day and stored at 4 ^{o}C. The chemicals were obtained from HiMedia (India) and Sigma-Aldrich Corporation (USA). The organisms *Aspergillus oryzae* ATCC 10124, *Fusarium oxysporum* MTCC 1755, *Aspergillus niger* MTCC 281 and *Escherichia coli* MTCC 40, *Staphylococcus aureus* MTCC were obtained from the Department of Food Engineering and Technology, Assam, India.

4.2.2 Identification and studies on amylolytic properties of moulds isolated from rice beer starter cakes

4.2.2.1 Isolation of moulds from starter cakes

The isolation was done within 24 h of storage. Rose Bengal chloramphenicol agar was used for the enumeration and isolation of moulds. The plates were maintained at 27 ºC for 48 h. Counting of the colonies was done and the results obtained were expressed as log of colony forming units (CFU) per gram of sample. The individual colonies were then removed from the plated and sub- cultured on potato dextrose agar plates and slants.

4.2.2.2 Microscopic observation

The fungal mycelia were separated with the help of needles and stained with lactophenol blue (aqueous). The slides were observed in a trinocular microscope (DM 3000, Leica Microsystems, Germany) using 40x magnification.

4.2.2.3 Identification of the isolates

For DNA extraction 0.5g of lyophilized mycellar pad was grinded and 10 ml of CTAB (containing proteinase K to a final concentration of 0.3 mg/mL prior to use) extraction buffer was added. It was gently mixed and kept for 30 min at 65 °C. Then cooled and added an equal volume of chloroform: isoamly alcohol (24:1), mixed and centrifuged at 2000xg for 10 min. To the aqueous supernatant an equal volume of isopropanol was added. The DNA which precipitates upon mixing was spooled out with a glass rod, rinsed with 70% ethanol, air dried, added 5 ml of TE and resuspended overnight at 4 ºC [21].

For PCR amplification, 40 µl of master mix and 10 µl of DNA solution were taken in a PCR tube. The master mix consisted of Taq polymerase 5 U/µl-0.1 µl, PCR buffer 10x-5 µl, $MgCl_2$ 1.5 mM-3 µl, each NTP 10 mM-1 µl, forward primer 10 mM-1 µl (1.0 mM), reverse primer 10 mM-1 µl (1.0 mM), dH_2O-15.4 µl. The primer sets used for ITS region were ITS1 forward primer (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 reverse primer (5'-TCCTCCGCTTATTGATATGC-3'). Amplification was carried out in a thermocycler (Mastercycler nexus gradient, Eppendorf, USA). The conditions used for 18S region were initial denaturation at 95 °C for 15 min, followed by 40 cycles at 95 °C (30 s), 55 °C (30 s), 72

°C (30 s), linked to 72 °C (5 min) and then to 5 °C. The conditions used for ITS-I region were initial denaturation at 96 °C for 10 min, followed by 30 cycles at 95 °C (1 min), 60 °C (1 min), 72 °C (1 min), linked to 72 °C (10 min) and then to 5 °C. The conditions used for ITS-II region were initial denaturation at 96 °C for 10min, followed by 30 cycles at 95 °C (1 min), 56 °C (1 min), 72 °C (1 min), linked to 72 °C (10 min) and then to 5 °C.

The amplified DNA was purified using a PCR product purification kit (GeneJET® K0701, Fermantas, EU) consisting of purification column and elution buffers. All centrifugations were carried out for 30-60 s in a bench top centrifuge at room temperature. The purified DNA obtained was stored at -20°C. The electrophoresis of the purified PCR products were performed on 0.6 % agarose gel and PCR-amplified bands were observed under UV illumination in a Gel-Doc system (MiniBIS Pro, DNR Bio-Imaging Systems, Israel) using the software Gel Quant Express®. The PCR products were sequenced by Sanger method in an automated sequencer at the SciGenom Labs, Cochin India. The organisms were identified by matching the sequences with a sequence with the highest maximum identity score from the GenBank database by using the Basic Local Alignment Search Tool (BLAST). The sequences were then submitted by using the BankIt tool to NCBI for obtaining the accession numbers.

4.2.2.4 Construction of phylogenetic tree

The sequences of identified phylogenetic neighbours were aligned and phylogenetic tree was constructed by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) 7.0.14 software after aligning the sequences using the integrated ClustalW algorithm [22]. The arbitrarily rooted tree was created from evolutionary distances using a maximum-likelihood approach and there were a total of 551 positions in the final dataset. The Maximum Likelihood method based on the Tamura-Nei model [23] was used to infer the evolutionary history. The branch lengths were measured in the number of substitutions per site and 1st, 2nd, 3rd and noncoding codon positions were included after eliminating all positions with gaps and missing data. The tree with the highest log likelihood (-2701.2462) was selected.

4.2.2.5 Test for starch degradation

From 72 h old cultures of the fungi in PDA plates, mycelia was dug out with a 8mm diameter cork borer and placed on the centre of a of starch agar medium (HiMedia, India) plate.

These were incubated for 48 h at 30 °C and visualization of starch degradation was done by flooding with a 0.25 % iodine solution. Clearing of the typically blue coloration of the starch with iodine indicated starch degradation [2].

To test for degradation of rice starch, 20 g of glutinous rice powder was soaked in 100mL of mineral media [24] in a 250 mL conical flask and soaked for 4 h at 22 °C. The mixture was then autoclaved for 1 h at 100 °C, cooled to 35 °C and inoculated with all the fungi dissolved in sterile physiological salt solution to obtain a final level of 10^6 spores g^{-1}. After incubation for 4 days at 30 °C, the saccharified mass was centrifuged, the supernatant was collected and saccharification ratio was calculated according to Eq. 4.1.

$$Saccharification\ Ratio(\%) = \frac{PS}{TS \times 100} \quad \text{Eq. (4.1)}$$

Where, TS= Initial content of total sugars in the medium (total soluble sugars - reducing sugars) and PS= Final content of reducing sugars generated from the saccharification reaction in the medium.

4.2.2.6 Assay for glucoamylase and α-amylase activity

The enzymes were produced by cultivating the mould in 50 ml of the basal medium [1 % $Fe(NH_4)_2(SO_4)_3$; 1 % $ZnSO_4$; 0.5 % $MnSO_4$; 0.08 % $CuSO_4$; 0.1 % $CoSO_4$, and 0.1 % H_3BO_4] in 250 ml Erlenmeyer flasks. A 2% spore suspension prepared from 4-day-old culture of the moulds was used as inoculum. After fermentation, the contents of the flasks were filtered through Whatman No. 1 filter paper, and the culture filtrates were used as the source of extracellular enzymes [25].

For measuring glucoamylase activity, 5 ml of 1 % soluble starch, 1 ml of 0.2 M acetate buffer at pH 4.5, 1 ml of distilled water and 1 ml enzyme solution were incubated at 40 °C in water bath for 10 min and the amount of reducing sugar was estimated. One unit of glucoamylase was defined as 1μ mol reducing sugar liberated per minute under assay condition [26].

The assay for α-amylase activity was done by modification of the method of Fuwa [27]. The filtrate and 1.5% starch in 100 mM Tris-HCl buffer, pH 7.0 were pre-incubated separately at 37 °C for 5 min. The reaction was started by adding 1.0 ml of starch solution to 0.5 ml of the filtrate followed by incubation at 37 °C for 10 min and stopped by adding 2.5 ml of 0.1 N HCl. Then 1 ml of the reaction mixture was added to 10 ml of 0.05 % potassium iodide solution, left at room temperature for 20 min and absorbance was taken at 660 nm. One unit of α-amylase

activity (liquefying activity) was defined as the amount of α-amylase which produced 10 % fall in the intensity of blue colour at the above conditions.

4.2.2.7 Purification of glucoamylase enzyme and its molecular size determination and study of their enzymatic activity

Actively growing fungal mycelia in PDA plates were transferred to 50 ml liquid medium in 250 ml Erlenmeyer flasks. The media contained soluble starch (15.0 g), yeast extract (4.0 g), $K_2HPO_4 \bullet 3H_2O$ (1.0 g) and $MgSO_4 \bullet 7H_2O$ (0.5 g) dissolved in 1 L of distilled and tap water (3: 1) and pH was adjusted to 6.5. The incubation was carried out at 30 °C for 7 days and 120 rpm in a rotary incubator shaker after followed by centrifugation of the fermentation broth at 10,000×g for 30 min at 4 °C. Then the supernatant was brought to 40% ammonium sulphate saturation, precipitated by centrifugation (12,000×g, 30 min) and again supernatant made to 60% ammonium sulphate saturation and precipitated. The precipitate was dissolved in 50 mM phosphate buffer (pH 6.0), and dialysed overnight against the same buffer. This solution was applied to a Sepharose CL-6B (CL6B200-100ML, Sigma Aldrich, USA) column (1.6 cm × 100 cm) previously equilibrated with the same buffer. The active fractions were than eluted, pooled, and applied to a DEAE-Sepharose CL-6B (DCL6B100-50ML, Sigma Aldrich, USA) column (2.6 cm × 10 cm) previously equilibrated with the same buffer. The inactive proteins were eluted with the same buffer and the amylase was eluted with a linear gradient of NaCl (0.0 to 0.5 M) in the same buffer. The eluted enzymatically active fractions were pooled and used for molecular size determination. The active portions were tested by spotting onto starch agar plates and flooding with iodine [28].

SDS PAGE (10 % polyacrylamide) was carried out in a vertical gel electrophoresis unit (PerfectBlue Dual Gel System Twin M, Peqlab, Germany) at a constant voltage of 150 v. Following electrophoresis the gel was stained with a 0.1% coomassie blue solution and destained with a solution of 5% acetic acid and 10% methanol. A midrange protein marker of 14-95 kDa (BLM003, Sisco Research Laboratories, India) was used. For detecting the amylase activity of the bands separated by SDS-PAGE, the gel was immersed in a 1% soluble starch solution prepared in 0.1 M acetate buffer of pH5 for 30 min at 50 ºC, followed by immersing the gel in the same buffer for 10 min. The gel was then dipped in 0.05% iodine solution for 10 min and observed for clear zones [29]. The images were observed and captured in a gel documentation system.

4.2.2.8 Test for the production of mycotoxins

This was done by modification of the method of Richard et al. [30] followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) detection. *Amylomyces rouxii* TU460, *Rhizopus oryzae* TU465 and *Fusarium oxysporum* MTCC 1755 (positive control) were cultivated for 72 h in potato dextrose broth and the media was filtered. To 50 mL of liquid culture 10 mL of saturated NaCl solution and 120 mL of $CHCl_3$was added. The partitioning was done for10 min after which the chloroform fraction was collected and filtered. This was loaded onto a silica gel column (1.0 cm i.d. x 30 cm glass) containing 1 % H_2O slurried in $CHCl_3$. The column was first eluted with 25 ml hexane, followed by 25 ml diethyl ether and then finally with 60 mL of acetonitrile: $CHCI_3$ (1:4) which was collected as the portion containing the mycotoxin. This was further concentrated under reduced pressure and filtered through 0.22 µm membrane filter and used for LC-MS/MS analysis. The instrument used was Agilent 6410 Triple Quad MS-MS (Agilent Technologies, USA). The compounds separated by the LC run were split by mass spectroscopy after identified after comparison of their m/z values with NIST Standard Reference Database, USA.

4.2.3 Identification and studies on physiological properties of yeast strains isolated from rice beer and starter cakes

4.2.3.1 Isolation of yeasts strains from starter cakes

The isolation was done within 24 h of storage. Potato dextrose agar supplemented with 0.1 % tartaric acid was used for the enumeration and isolation of yeasts. The plates were maintained at 27 ºC for 24 h. Counting of the colonies formed was done and the results obtained were expressed as log of colony forming units (CFU) per gram of sample. The individual colonies were then removed from the plated and sub- cultured on potato dextrose agar plates and slants.

4.2.3.2 Microscopic observation

The yeast cells were heat fixed, stained with methylene blue (aqueous) and observed in a trinocular microscope (DM 3000, Leica Microsystems, Germany) using 100x oil immersion lens.

4.2.3.3 Biochemical characterization of the isolates

4.2.3.3.1 Diazonium blue B test - Yeast cultures grown on yeast malt agar for 10 days were held at 55 °C for 5 hr and then flooded with ice cold DBB reagent. A positive test was indicated by the culture turning red within 2 min at room temperature [31].

4.2.3.3.2 Urea hydrolysis - Urea broth medium 18 (HiMedia, India) in aliquots of 0.5 ml was taken aseptically in tubes and a loopful of yeast cells for 2 day old culture was suspended in the broth. Then incubated at 37 °C. The tubes were examined every half hour up to 2 hours for change of colour to red, which indicates urease activity [31].

4.2.3.3.3 Production of extracellular starch like compounds - After a positive growth test in liquid media with a sugar, one drop of Lugol's iodine solution was shaken with the medium. A blue, purple or green colour indicated the presence of starch [31].

4.2.3.3.4 Growth in presence of cycloheximide - Sterilized bromo cresol purple broth base (BCPBB) (HiMedia, India) containing D-glucose in test tubes were supplemented with filter sterilized cycloheximide to final concentrations of 0.1 % and 0.01 %. These were then inocluled with 100 µl of a fresh yeast suspension (approx. 10^7 cells/ml) and incubated at 25 °C for up to 7 days, regularly checking for presence of growth [31].

4.2.3.4 Genetic identification of the isolates

For DNA extraction the yeast DNA extraction kit (78870, Thermo Fisher Scientific, USA) was used. Overnight, grown cultures of yeasts were pelleted by centrifugation at 5000×g for 5 minutes at room. The cells (100 mg) were suspended in Y-PER Reagent (8 µL/1 mg pellet) and mixed by gentle vortexing. It was then incubated at 65 °C for 10 minutes followed by centrifugation at 13,000×g for 5 minutes. The pellet was mixed 400 µL of DNA Releasing Reagent A and 400 µL of DNA Releasing Reagent B and incubated at 65 °C for 10 minutes. Following this, 200 µL of protein removal reagent was added, mixed well and centrifuged at 13,000 × g for 5 minutes and the supernatant was collected and to it 600 µL of isopropyl alcohol was added. After mixing gently, the precipitated genomic DNA was pelleted by centrifuging the mixture at 13,000×g for 10 minutes. Then 1.5 mL of 70 % ethanol was added to the pellet,

invert several times and centrifuged at 13,000×g for 1 minute, and the pelleted DNA was resuspend in 50 µL TE buffer.

For PCR amplification, 40 µl of master mix and 10 µl of DNA solution were taken in a PCR tube. The master mix consisted of Taq polymerase 5 U/ µl-0.1 µl, PCR buffer 10x-5 µl, MgCl2 1.5 mM-3 µl, each NTP 10 mM-1 µl, forward primer 10 mM-1 µl (1.0 mM), reverse primer 10 mM-1 µl (1.0 mM), dH2O-15.4 µl. The primer sets used were ITS1 forward primer (5'-TCCGTAGGTGAACCTGCGG -3') and ITS4 reverse primer (5'-TCCTCCGCTTATTGATATGC -3'). Amplification was carried out in a thermocycler (Mastercycler Nexus Gradient, Eppendorf, USA). The conditions used were initial denaturation at 95°C for 10 min, followed by 30 cycles of 95°C (1 min), 55°C (1 min), 72°C (90 sec), linked to 72 °C (10 min) and then to 5°C.

The amplified DNA was purified using a PCR product purification kit (GeneJET® K0701, Fermantas, EU) consisting of purification column and elution buffers and rest of the steps were carried out according to section **4.2.2.3.**

4.2.3.5 Construction of phylogenetic tree

This was carried according to section **4.2.2.4.**

4.2.3.6 Determination of ethanol tolerance

This was done according to the method of Gupta et al. [32]. Potato dextrose broth (5 ml) was adjusted with varying concentration of ethanol 3-15%, (v/v) and inoculated with 100 µl active yeast cultures (~10^7 CFU/ml) individually and incubated for 48 h at 27 ºC. Cultures were then evaluated for cell viability by plating on PDA plates and measuring the optical density at 600 nm. The specific growth rate constant (k) for all the tested strains was calculated according to Eq. 4.2.

$$Log_{10}N - Log_{10}N_0 = \frac{k}{2.303} \times (t - t_0) \qquad \text{Eq. (4.2)}$$

Where, N = CFU/ml at specific time t; N_0 = CFU/ml at 0 h; t_0 = time (0 h) and t = time (48 h)

4.2.3.7 Ability to grow at different temperatures

Fresh cultures of the yeasts were inoculated onto 5 mL of potato dextrose broth in test tubes and grown at different temperatures ranging from °C to 45 °C and grown for 24 h. The tubes were then observed for the presence of growth [31].

4.2.3.8 Growth at high osmotic pressure

Slants of yeast malt agar containing 50 or 60 % D-glucose and 5, 10 or 16% NaCl in test tubes were prepared. They were inoculated with the respective strains by streaking and the cotton plugs were sealed with paraffin tape. They were incubated at 25 and examined for growth for up to 4 weeks [31].

4.2.3.9 Ability to use sugars anaerobically

Durham tubes were inserted in test tubes containing 10 ml of BCPBP containing 50 mM of each test sugar and then autoclaved. Each tube was inoculated with 100 µl of a fresh yeast suspension of approx. 10^7 cells/ml of yeast extract broth. The tubes were incubated at 25 °C for 7 days, shaking then each day to sediment the yeasts and checked for the presence of CO_2 gas bubble inside the Durham tube, which indicated the ability to ferment the sugar anaerobically [31].

4.2.3.10 Ability to use sugars as sole source of carbon for aerobic growth

Test tubes with 5 ml of BCPBP containing 50 mM of each test sugar and then autoclaved. Each tube was inoculated with loopful of yeast cells from an actively growing culture on yeast malt agar (HiMedia, India) to a final concentration of approx. $25x10^6$ cells/ml. The tubes were incubated in tilted position at 25 °C for 7 days with hand shaking each day. The growth was assessed by the change in colour of the media relative to un-inoculated tube as negative control [31].

4.2.3.11 Determination of alcohol dehydrogenase (ADH) activity

The yeasts were subcultures in yeast malt broth for 48 h and then harvested by centrifugation at 8000 rpm for 10 min and cell pellets washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2mM EDTA. They were then resuspended in 10 mM potassium phosphate buffer containing 2 mM $MgCl_2$ and 2mM dithiothreitol and disrupted using a sonicating probe (UW2070, Bandelin, Germany) for 2 min at 30 s interval (133 v, 0.5 repeating cycles per s) by keeping in ice. Crude enzyme extract was obtained by centrifugation at 12000xg for 20 min at 4 ºC and collecting the supernatant [32]. Protein content in the extract was estimated was then estimated.

The reaction velocity for ADH activity was determined by adapting the method of Longhurst et al. [33]. To 0.8 ml of the enzyme extract, 1.5 ml of 0.1 M sodium pyrophosphate buffer (pH-9.6), 0.5 ml of 2.0 M ethanol and 1.0 ml of 0.025 M NAD were added. The increase in absorbance (at 340 nm) of the reaction mixture at 25 °C was recorded for 3-4 min. The enzyme activity was calculated according to Eq. 4.3 by considering the absorbance (340 nm/min) from the initial linear portion of the curve. Here one enzyme unit is the amount of enzyme that reduces one micromole of NAD^+ per min under the specified conditions.

$$\text{ADH unit/mg protein} = \frac{\text{A340/min}}{6.22 \times \text{mg protein/ml reaction mixture}} \qquad \text{Eq. (4.3)}$$

4.2.3.12 Determination of alcohol producing capacity of the yeast strains

The yeast strains (8 nos) showing higher ethanol tolerance and ADH activity were tested for their alcohol producing capacities. This was done by modification of the method of Okunowo et al. [34]. The rice grains were milled in a laboratory grain mill (Pulverisette14, Fritsch, Germany) fitted with a 0.5 mm sieve. The resulting grist was mixed with distilled water (50g/200 ml) and stirred in an incubator shaker at 100 rpm at 45 °C for 10 min, 50 °C for 60 min, 63 °C for 40 min and finally at 95 °C for 60 min. At concentrations of 0.5g/100g protease was added in the first step followed by α-amylase in the subsequent step. The resulting wort was filtering through Whatman filter paper No. 1 and adjusted to 12 °Bx with distilled water. To the wort, diammonium hydrogen phosphate (0.5 g/l), magnesium sulphate (0.2 g/l) and urea (0.5 g/l) was added and the pH was adjusted to 6.8. It was then sterilized by autoclaving.

The yeasts were subcultured on yeast malt agar at 27 °C for 24 h aerobically. The cells were then pelleted by centrifugation at 8000 rpm for 10 mins and washed twice with sterile 0.89 % NaCl solution. They were adjusted to a concentration of approximately 5×10^7/ml in sterile distilled water and 2 ml of this was added to 200 ml of the wort in conical flasks. Fermentation was allowed to proceed at 25 °C for five days in an incubator shaker at 120 rpm. The resulting alcoholic solution was analyzed every 24 h after clarification with 0.1 % bentonite. The pH and total sugars were analyzed and the alcohol content was calculated by using the alcohol by volume (ABV) calculator according to Eq. 4.4.

$$ABV = \frac{1.05}{0.79} \times \left(\frac{Starting\ SG - Final\ SG}{Final\ SG}\right) \times 100 \qquad \text{Eq. (4.4)}$$

Where, SG is the specific gravity 1.05 is number of grams of ethanol produced for every gram of CO_2 produced and 0.79 is the density of ethanol.

4.2.3.13 GC-MS analysis for the analysis of volatile organic compounds (VOCs)

The yeast strains (8 nos) showing higher ethanol tolerance and ADH activity were tested for the production of VOCs. The VOCs present in the yeast culture media were extracted using a slight modification of the method of Patakova-Juzlova et al. [35]. Samples (100 mL) were first filtered through Whatman no. 4 filter paper and then distilled in a rotary evaporator (8763.RV0.000 Roteva, Equitron, India) until 20 % of the total volume had been collected as distillate. This distillate was then extracted with 10 % dichloromethane (DCM) in a separating funnel by shaking for 10 min. The DCM extract was filtered through a 0.2 μm pore size filter driven syringe and stored at 4 °C until analysis.

For carrying out the analysis, a gas chromatograph (7890A, Agilent Technologies, USA and mass spectrometer (240 Ion Trap MS, Agilent Technologies, USA) was used. The carrier gas was helium at a flow rate of 2.2557 mL min^{-1}. A split type injector at 20:1 ration was used and the injection volume was 1 μL. The column used was HP -5 (5 % phenyl methyl siloxane) and was 30 m in length and 0.320 m in diameter, and the film thickness was 0.25 μm. The injection temperature was 250 °C. The oven temperature was at 35 °C for 2 min and then programmed to 200 °C at a rate of 10 °C min^{-1}, and held for 5 min. The mass range selected was from m/z 50 to 500.

4.2.4 Identification and studies on functional properties of lactic acid bacteria isolated from rice beer and starter cakes

4.2.4.1 Isolation of lactic acid bacteria

The microbial isolation of the samples was started immediately after the samples were brought to the laboratory. The plating was done according to the method described by Brown [36]. All the samples were serial diluted on 0.86 % NaCl solution and plated on MRS Agar (Himedia, India) supplemented with $CaCO_3$ and bromocresol purple indicator using double layer technique. These plates were incubated in an anaerobic gas pack system (LE012, Himedia, India) at 37 °C for 48 h. After the appearance of growth, the colonies were isolated with the help of toothpicks and subcultured on MRS broth. The colonies were further purified using the continuous streaking technique. The individual colonies were then picked, subcultured and grown on MRS broth. A Gram's staining kit (K001-1KT, HiMedia, India) was used for staining the cells (48 h old culture). The slides were observed in a trinocular microscope (DM 3000, Leica Microsystems, Germany) using oil immersion lens at 100x magnification. The isolates were maintained in 30 % glycerol stocks (1:1) at -80 °C for further investigation.

4.2.4.2 Genetic identification of the isolates based on 16s rRNA sequencing

For isolation and purification bacterial genomic DNA, a genomic DNA purification kit (K0512, Thermo Scientific, EU) was used. Bacterial culture (18h old) was centrifuged for 10 min at 5000xg. Then 20 mg of bacterial cells was resuspended in 200 µl of TE buffer, mixed with 400 µl of lysis solution and incubated at 65 °C for 5 min. Immediately 600 µl of chloroform was added, gently emulsified by inversion (3-5 times) and centrifuged at 10000 rpm for 2 min. The upper aqueous phase was transferred to a new tube and 800 µl of freshly prepared precipitation solution was added. It was mixed gently by several inversions at room temperature for 1-2 min and centrifuged at 10000 rpm for 2 min. Supernatant was completely removed and DNA pellet was dissolved in 100 µl of NaCl solution. Then 300 µl of cold ethanol was added, kept for 10 min at -20 °C and centrifuged (10000 rpm, 3-4 min). The pellet was washed once with 70 % cold ethanol and redissolved in 100 µl of sterile deionized water. The concentration of DNA was measured spectrophotometrically.

For PCR amplification, 40 µl of master mix and 10 µl of DNA solution were taken in a PCR tube. The master mix consisted of Taq polymerase 5 U/ µl-0.1 µl, PCR buffer 10x-5 µl, MgCl2 1.5 mM-3 µl, each NTP 10 mM-1 µl, forward primer 10 mM-1 µl (1.0 mM), reverse primer 10 mM-1 µl (1.0 mM), dH2O-15.4 µl. The 16S rRNA universal primers set consisted of "27f" forward primer (5'-GAGAGTTTGATCCTGGCTCAG-3') and "1495r" reverse primer (5'-CTACGGCTACCTTGTTACGA-3').Amplification was carried out in a thermocycler (Mastercycler nexus gradient, Eppendorf, USA). The conditions used were initial denaturation at 95 °C for 3 min, followed by 30 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (1 min), linked to 72 °C (10 min) and then to 5 °C.

The amplified DNA was purified using a PCR product purification kit (GeneJET® K0701, Fermantas, EU) consisting of purification column and elution buffers and rest of the steps were carried out according to section **4.2.2.3.**

4.2.4.3 Construction of phylogenetic tree

This was carried according to section **4.2.2.4**

4.2.4.4 General characteristics of the strains

For catalase test the strains were inoculated onto MRS agar slants by means of streak inoculation and were incubated at 37 °C for 24 h under anaerobic conditions. After this, few drops of 3 % H_2O_2 was pipetted over the growth on the slant and observed for the appearance of gas bubbles. The appearance of bubbles indicated a positive test. To test the production of gas from glucose, phenol red broth was supplemented with glucose (500 mg/100ml) and Durham`s tubes were placed in inverted position inside the tubes inoculates with the strains. A positive test was shown by the deposition of a gas bubble inside the Durham`s tube [37]. To test for the production of ammonia from arginine, ammonia production broth tubes were inoculated with microbial culture (10 µL) and then incubated at 37 °C for 48 hours. Then add few drops Nesslers reagent and observed for the appearance of yellow or brown colouration to confirm the presence of ammonia [38]. Gelatinase activity was tested by growth on nutrient gelatin agar plates followed by treating the plates with saturated ammonium sulfate solution. A clear zone indicates a positive reaction [39]. Haemolysis activity was investigated as described by Gerhardt et al. [40]. For this, 2 µl of a 6 h old culture broth was spot inoculated

into sterile blood agar. After growth the plates were observed for clear zones surrounding colonies (positive reaction for ß-haemolysis).

4.2.4.5 Growth characteristics of the strains at different pH, salt concentration and temperature

For pH test, pH of 3.9, 9.6 and 7.0 were adjusted in MRS broth by using 1N HCl and 10 % NaOH and then autoclaved. Tubes will be inoculated with microbial culture (10 µL) and then incubated at 35 °C for 48 hours after which growth was observed. For salt tolerance test, NaCl concentrations of 6.5, 10 and 18 % were adjusted in MRS broth by using NaCl. Tubes were inoculated with microbial culture (10 µL) and then incubated at 35 °C for 48 hours after which growth was observed. To test the influence of temperature, MRS broth tubes were inoculated with microbial culture (10 µL) and then incubated at 10, 15 and 45 ºC after which growth will be observed [37].

4.2.4.6 Carbohydrate fermentation tests

Tubes of phenol red broth were supplemented with different carbohydrates (500 mg/100 ml) and inoculated with microbial culture (10 µL). Incubation was done at 37 °C for 48 hours. Tubes were examined between 2, 4, 8, 18 hours in order to avoid false negatives. All carbohydrate broth cultures were examined for evidence of acid production, which is detected by the medium turning yellow [41].

4.2.4.7 Antibiotic susceptibility test

The strains were tested for their resistance against a selection of twelve antibiotics using a slightly modified version of the agar disc diffusion method. Strains were grown in MRS broth for 48 h at 37 °C. Following the preparation of a 10-fold dilution in PPS, freshly poured MRS agar plates were equally inoculated with this dilution. Antibiotic discs were placed on the inoculated plates using the disc dispenser. Following 24 h incubation at 37 °C, inhibition zones around the discs were measured using an antibiotic zone scale [42].

4.2.4.8 Antibiosis activity tests

The antibacterial activity of the strains was determined by agar spot-on-lawn test introduced by Schillinger and Lucke [43] with some modification. The indicator bacteria used were *Escherichia coli* and *Staphylococcus aureus*. Overnight culture (1 µl each) of selected isolates were spotted on MRS agar plates and incubated under anaerobic conditions for 48 h to develop colonies. A portion of 0.25 ml of 1:10 dilution of an overnight culture of the indicator bacteria was inoculated in 9 ml of antibiotic susceptibility test media (Himedia, India) soft agar (0.7 % agar) after autoclaving and cooled to 50 °C. The medium was immediately poured over the agar plate on which the tested LAB was grown. The plates were incubated aerobically at 37 °C for 24 h in inverted position. The antibacterial activity will be related to the inhibition clear zone which will be calculated as the difference between the total of inhibition zone and the diameter of growth spot of selected strains. For antifungal activity, the LAB strains were inoculated as 2 cm long lines on MRS agar plates and incubated at 37 °C for 48 h on anaerobic condition. The plates were then overlaid with 10 ml of malt extract soft agar (0.7 %) containing 10^4 fungal (*Aspergillus niger*) spores/ml. The plates were again incubated aerobically at 30 ºC in inverted position for 48 h after which they were observed for clear zones of inhibition surrounding the bacterial colonies [44].

4.2.4.9 Test for antioxidant activity

The tests for accessing antioxidant activity were performed according to Li et al. [45]. To test the resistance to hydrogen peroxide, overnight cultures of the strains were inoculated at 1% (v/v) into MRS broth and MRS broth containing 0.4, 0.7 or 1.0 mM hydrogen peroxide and incubated at 37 C for 8 h. The cell growth was measured spectrophotometrically at 600 nm and results were given as optical density (OD).

To test the hydroxyl radical scavenging activity, the strains were grown in MRS broth at 37 °C for 18 h and harvested by centrifugation at 6000xg for 10 min. They were then washed twice with deionised water and resuspended in deionised water to a count of 10^8 CFU/ ml. Then were incubated with 1 mg/ml lysozyme at 37 °C for 30 min followed by ultrasonic disruption (UW2070, Bandelin Electronic, Berlin, Germany) for five 1-min intervals in an ice bath. The cell debris were removed by centrifugation at 8000xg for 10 min and resulting supernatant was obtained as the intracellular cell-free extract. To assay was conducted by incubating 1.0 ml of intracellular cell-free extract with 1.0 ml of brilliant green (0.435 mM), 2.0 ml of $FeSO_4$ (0.5

mM), 1.5 ml of H_2O_2 (3.0 %, w/v) at room temperature for 20 min and then the absorbance was measured at 624 nm. The hydroxyl radical scavenging activity was expressed according to Eq. 4.5.

$$Scavenging\ activity\ (\%) = [(A_S - A_o) \div (A - A_O)] \times 100 \quad \text{Eq. (4.5)}$$

Where, A_S is the absorbance in the presence of the sample, A_0 is the absorbance of the control in the absence of the sample, and A is the absorbance without the sample.

To test the DPPH free radical scavenging activity, the strains were grown in MRS broth (30 ml) 37 °C for 24 h on anaerobic condition. The cells were then harvested by centrifugation (6000 g, 10 min, 4 ºC), washed thrice with deionised water and resuspended in deionised water (10 ml). Then 2 ml of the cell suspension was added to 4 ml of ethanolic DPPH solution (0.05 mM). The mixture was then mixed vigorously and incubated at room temperature in the dark for 30 min. The controls include deionised water and DPPH solution. The blank contained only ethanol and the cells. The absorbance of the resulting solution was measured in triplicate at 517 nm after centrifugation at 8000g for 10 mins. The DPPH free radical scavenging activity was expressed according to Eq. 4.6.

$$Scavenging\ activity(\%) = \left[1 - \left(A_{Sample} - A_{Blank}\right) \div A_{Blank}\right] \times 100 \quad \text{Eq. (4.6)}$$

4.2.4.10 Test for acid tolerance of the isolates LAB strains

This was done by modification of the method of Jacobsen et al. [46]. This was MRS broth was used to simulate acidic conditions of gut after adjusting to different pH values namely 1.5 and 2.0 with 1.0 N HCl. Another set of broth was adjusted to neutral pH (7.0) to serve as a control. The broth tubes adjusted at different pH values were inoculated (at 10^8 CFU/ml) with overnight grown cultures of isolates and incubated at 37 °C. One ml of culture was taken from each tube after an interval of 0, 1, 2, 3, 5, 6, 7, 8, 24 and 48 h and 10-fold serial dilutions were prepared in 0.1% peptone water and plated on MRS agar. The plates were incubated at 37 °C for 48 h and the CFU were counted.

4.2.4.11 Test for bile tolerance of the isolated LAB strains

This was done by modification of the method of Jacobsen et al. [46]. Fresh cultures were inoculated in MRS broth supplemented with bile salts (1 % to 14 %) followed by incubation at 37 °C. Aliquots were withdrawn after 24 h interval and plated on MRS agar at 37

°C for 24 hours. Bile tolerance was assessed in terms of viable colony counts after the aforesaid incubation at 37 °C.

4.2.4.12 Test for cellular aggregation of the isolated LAB strains

Autoaggregation assay was performed according to Del Re et al. [47] with certain modifications. The strains were grown for 18 h at 37 °C in MRS broth. The cells were harvested by centrifugation at 5000xg for 15 min, washed twice and resuspended in phosphate buffered saline (PBS) to give viable counts of approximately 10^8 CFU ml^{-1} (absorbance of approx. 0.5 at 600 nm). The cell suspensions were mixed by vortexing for 10 s and autoaggregation was determined during 5 h of incubation at room temperature. Every hour 0.1 ml of the upper suspension was transferred to another tube with 3.9 ml of PBS and the absorbance was measured at 600 nm. The autoaggregation percentage was expressed according to Eq. 4.7.

$$Autoaggregation\ (\%) = 100 \times (A_O - A_t) \div A_O \qquad \text{Eq. (4.7)}$$

Here, A_t represents the absorbance at time t = 1, 2, 3, 4 or 5 h and A_0 the absorbance at t = 0.

4.2.4.13 Test for microbial adhesion to solvents (MATS) of the isolated LAB strains

The bacterial adhesion to hydrocarbons was determined by following the method of Rosenberg et al. [48] with slight modification to measure the cell surface hydrophobicity. The bacterial cells grown in MRS broth at 37 °C for 18 h were centrifuged and the cell pellet was washed twice with phosphate urea magnesium (PUM) buffer. The washed pellet was resuspended in PUM buffer and the absorbance was adjusted to approx. 0.7 OD at 600 nm. Microbial cell suspension (3.0 ml) and xylene (1.0 ml) were mixed by vortexing and incubated at 37 °C for 10 min for temperature equilibration. The mixture was again briefly vortexed and incubated at 37 °C for 1 h for phase separations. The aqueous phase was gently taken out to measure its absorbance at 600 nm. The surface hydrophobicity or MATS was calculated as percent decrease in the absorbance of the aqueous phase after mixing (A_f) and phase separations relative to that of original suspension (A_i) as Eq. 4.8.

$$MATS\ (\%) = 100 \times (A_f - A_i) \div A_i \qquad \text{Eq. (4.8)}$$

Similarly two other solvents (chloroform and ethyl acetate) were assayed in the same manner.

The values of MATS obtained with the two other solvents, chloroform and ethyl acetate, were regarded as a measure of electron donor (basic) and electron acceptor (acidic) characteristics of bacteria, respectively [49].

4.2.4.14 Surface associated adhesion proteins of two *Lactobacillus casei* strains

Two of the *Lactobacillus casei* strains, viz., *L. casei* TEZU309 and *L. casei* TEZU374 showing good adhesion properties were studied for their surface associated proteins

4.2.4.14.1 Preparation of cellular extracts

Cultures from 24-h growth were harvested by centrifugation (5000xg, 15 min) and cells were washed twice with phosphate buffered saline and pelleted by centrifugation (5000xg, 15 min). This was termed as the untreated cell pellets (UCP). One set was again extracted with 10 ml of 5 mol l^{-1} LiCl (30 min at 37 °C) and pelleted by centrifugation (5000xg, 15 min). This was termed as the treated cell pellets (TCP). Subsequently, for the isolation of surface proteins the CPE and TCP were resuspended in 1 % SDS for 30 min at 37 °C [50]. After centrifugation at 9000 g for 5 min, the supernatant was transferred to a 6000–8000 kDa cutoff dialysis membrane and dialysed against cold distilled water for 24 h, changing the water every 2 h for the first 8 h. The dialysed precipitate was harvested via centrifugation at 20000 g for 30 min and pellets were resuspended in 10 % SDS solution to obtain the surface protein extract (SPE) [51]. The protein content was quantified spectrophotometrically.

4.2.4.14.2 Adhesion properties with and without the surface proteins

For determining the cellular autoaggregation the UCP and TCP were washed twice and resuspended in phosphate buffered saline (PBS) to give viable counts of approximately 10^8 CFU ml^{-1} (absorbance of approx. 0.5 at 600 nm). The cell suspensions were mixed by vortexing for 10 s and autoaggregation was calculated according to Eq. 4.7

For determining the bacterial adhesion to solvents the UCP and TCP were washed twice with phosphate urea magnesium (PUM) buffer and resuspended in PUM buffer and the absorbance was adjusted to approx. 0.7 OD at 600 nm. MATS was calculated according to Eq. 4.8.

4.2.4.14.3 Adherence to Caco-2 and HT-29 intestinal epithelial cells

This was done according to Johnson et al. [51]. The Caco-2 and HT-29 intestinal epithelial cell line (National Centre for Cell Science, Pune, India) was used and growth media was obtained from Himedia, India. Caco-2 cultures were grown at 37 °C in a 95 % air 5 % CO_2 atmosphere (Galaxy CO-170S CO_2 incubator, New Burnswick Scientific, USA). The cells were grown on a minimal essential medium (MEM) supplemented with 1 mM sodium pyruvate, 20 % (v/v) heat-inactivated FBS, 0.1 mM non-essential amino acids, penicillin G (100 mg ml^{-1}), streptomycin sulfate (100 mg ml^{-1}) and amphotericin (0.25 mg ml^{-1}). Cells were used for adherence assay at post confluence. Concentration of cells was determined by trypsinizing the cells for 10 min at 37 °C and counting them using a heamocytometer. Monolayers for the adhesion assay were prepared in 12-well tissue culture plates (Eppendorf, USA) by seeding approximately $6.5x10^4$ cells per well in 2 ml cell culture medium. The culture medium was replaced every 2 days, while the monolayers were used for the assay 2 weeks post confluence.

The UCP and TCP were resuspended in PBS to a final concentration of approx. $1x10^8$ CFU ml^{-1}. The monolayers were washed twice with 1 ml PBS before adding 1 ml MEM without antibiotics and incubating at 37 °C in a 5 % CO_2 atmosphere. Then, 1 ml of the bacterial suspension was added to each well of the cell monolayer in triplicate and incubated at 37 °C in a 5 % CO_2 atmosphere for 1 h. Following incubation, monolayers were washed five times with 1 ml PBS and treated with 1 ml 0.05 % (v/v) Triton X-100. After 10 min at 37 °C, cell monolayers were disrupted via pipetting and transferred to microcentrifuge tubes. Finally, microbial cells from the monolayer were diluted and enumerated (A_1 CFU/ml) after plating onto MRS agar plates. The bacterial cells initially added to each culture were also counted (B0 cfu/ml). The adhesion percentage was calculated according to Eq. 4.9.

$$\% \, Adhesion = \frac{A_1}{A_0} \times 100 \qquad \text{Eq. (4.9)}$$

4.2.4.14.4 Imaging of the cell surfaces by transmission electron microscopy (TEM)

TEM analysis on the *L. casei* strains was done following the method of Vellaiswamy et al. [52]. The UCP and TCP were washed twice with PBS and fixed in 2 % glutaraldehyde and cacodylate buffer (0.1 M) overnight at 4 °C. They were then washed with cacodylate buffer

(0.1 M) and further fixed for 1 h at room temperature with 2 % osmium tetroxide (0.1 M), followed by dehydration in an ascending series of ethanol (30 % to 100 %). The cells were embedded in epoxy resin and cut into ultrathin sections (70 nm). There were transferred on to Ultrathin C Type-A 400 mesh Cu grid (270215, Ted Pella Inc., USA) which were pre-treated twice with 50 mM NH_4Cl in PBS for 5mins. The grids were then allowed to dry at room temperature before examined on a Tecnai G2 20 S-Twin (200 KV) transmission electron microscope (FEI Company, USA) with a resolution of 2.4 A° and the images were processed using the TIA imaging software.

4.2.4.14.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the surfaces associated proteins

The SPE was used for running SDS-PAGE. Before loading, proteins were precipitated by mixing 20 % TCA with protein samples (1:1, 10 % TCA final concentration). Then kept in ice for 20 min and centrifuged for 10 min at 16000xg. The protein pellet was washed with ethanol/ethyl acetate (1:1) and centrifuged for 10 min at 16000xg three times. The final protein pellets was dissolved in 2X loading buffer at 100 °C for 15 min. SDS PAGE (12.5 % polyacrylamide) was carried out in a vertical gel electrophoresis unit (PerfectBlue Dual Gel System Twin M, Peqlab, Germany) at a constant voltage of 150 v. Following electrophoresis the gel was stained (0.1 % coomassie blue, 45 % methanol, 10 % acetic acid,) and destained with a solution of 5 % acetic acid and 10 % methanol. A midrange protein marker of 14-95 kDa (BLM003, Sisco Research Laboratories, India) was used. The images were observed and captured in a gel documentation system (MiniBIS Pro, DNr Bio-Imaging Systems, Israel), and processed using the GelQuant Express software.

4.2.4.15 Utilization of different carbohydrates for production of organic acids by the LAB strains

Few of the isolated LAB strains were tested for their ability to produce various organic acids by utilizing D-glucose, maltose and lactose.

4.2.4.15.1 Growth of the LAB strains in formulated media

The basal media used for the growth of the LAB strains was adapted from de Man [53] and consisted of peptone (1.0 %), beef extract (0.8 %), yeast extract (0.4 %), glucose (2.0 %), sodium acetate trihydrate (0.5 %), polysorbate 80 (0.1 %), dipotassium hydrogen phosphate (0.2 %), triammonium citrate (0.2 %), magnesium sulfate heptahydrate (0.02 %), manganese sulfate tetrahydrate (0.005 %). This was supplemented with 2.0% of D-glucose, maltose or lactose. The pH in all the three media was adjusted to 6.2. The media (10 ml) was inoculated with 100 µl of 24 h old culture of the LAB strains and incubated under anaerobic conditions for 48 h.

4.2.4.15.2 HPLC analysis for the production of various organic acids

Following the incubation period the cultures were centrifuged for 10 min at 10,000 rpm. The supernatant diluted 1:20 times with deionized water, filtered through Whatman no. 4 filter paper and washed twice with n-hexane. This was then passed through 0.25µm pore size syringe driven filter and then subjected to solid-phase extraction using a Sep-Pak® C18 cartridge which was previously activated with methanol. This extract was used for analysis of the organic acids. The analysis was carried out in an HPLC system (Ultimate 3000, Dionex, Germany) equipped with an autosampler. The injection volume was 20 µL and the detector used was an Ultimate 3000 Variable Wavelength detector at 210 nm (UV range). The column used was Acclaim OA® (5 µm bead size, 4.0 × 250 mm, Thermo Scientific). The mobile phase was 0.2 M sodium sulphate solution (pH adjusted to 2.68 with methane sulphonic acid). An isocratic run was used with a constant flow rate of 0.6 mL min^{-1} at a temperature of 30 °C.

The organic acid standards which were used in the HPLC analysis along with their retention times were: gluconic acid (3.70 min), oxalic acid (3.85 min), tartaric acid (4.10 min), pyruvic acid (4.25 min),formic acid (4.40 min), maelic acid (4.51 min), lactic acid (4.80 min), acetic acid (5.00 min),citric acid (5.38 min),succinic acid (6.01 min), propionic acid (7.63 min) and butyric acid (15.25 min).

4.3 Results and discussions

4.3.1 Identification and studies on amylolytic properties of moulds isolated from rice beer starter cakes

4.3.1.1 Identification of the isolates

The initial count of moulds in both the starters i.e *amou* and *perok-kushi* (Fig 4.1) was within 8 log CFU g^{-1} (Table 4.1). Based on the sequencing of the 18S ribosomal RNA gene's internal transcribed spacer region, the fungus from *amou* and *perok-kushi* was identified as *Amylomyces rouxii* TU460 and *Rhizopus oryzae* TU465 with NCBI accession numbers KU500802 and KU535588, respectively.

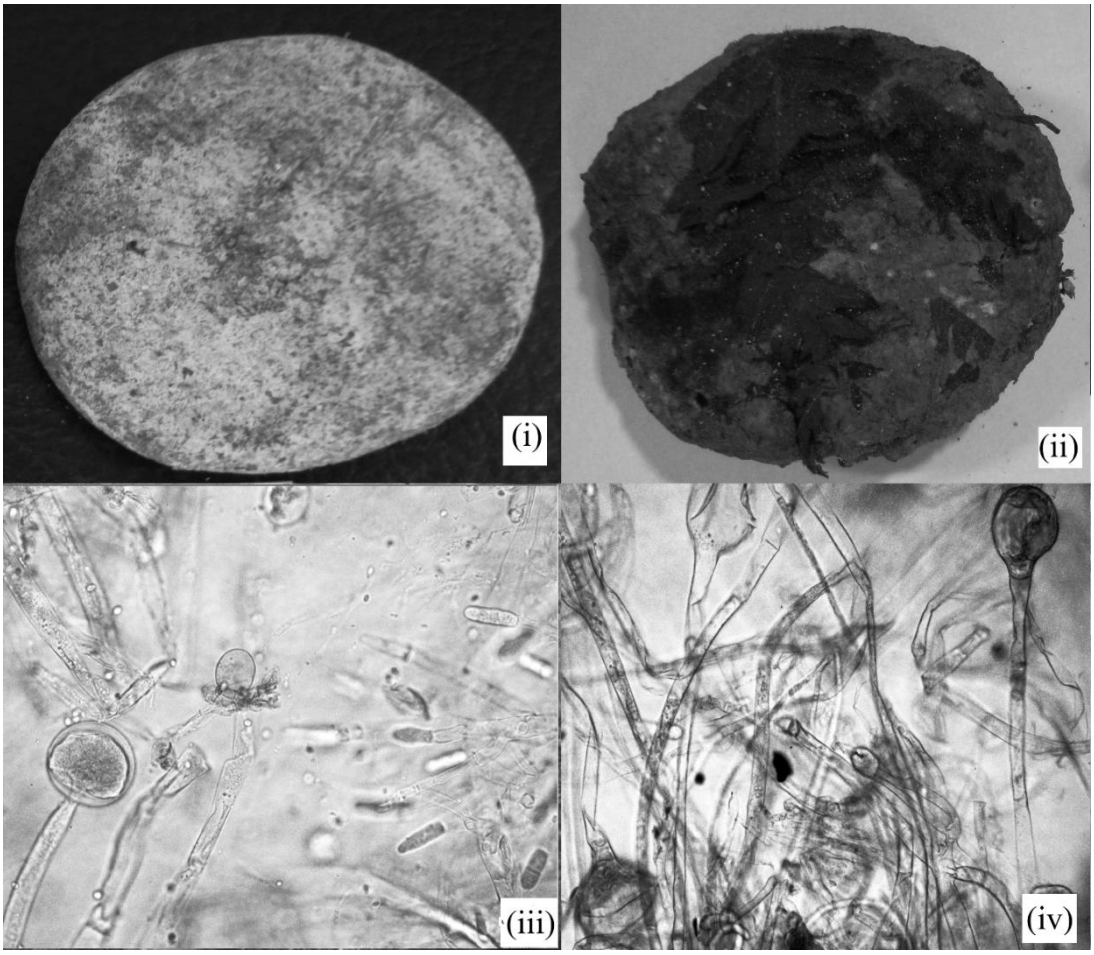

Fig 4.1 The starter cake samples (i) *Amou* (ii) *Perok-kushi* and isolates (iii) *Amylomyces rouxii* TU460 (iv) *Rhizopus oryzae* TU465

Table 4.1 The starter cakes collected for study and the count of moulds in them

Starter name/ Community	Initial count of moulds (log CFU g^{-1})	Identified fungus	NCBI accession number
Amou, Bodo	7.67±0.26	*Amylomyces rouxii* TU460	KU500802
Perok-kushi, Deori	7.58±0.55	*Rhizopus oryzae* TU465	KU535588

The mucoromycotina fungus *Rhizopus* and *Amylomyces* are indispensable microorganisms for the production of fermented foods in Asia [54]. *A. rouxii* belongs to the monotypic genus Amylomyces. Its strains vary from white to light gray-brown colonies containing many abortive sporangia. All strains produce abundant chlamydospores [55]. *A. rouxii* have also been studied for the degradation of pentachlorophenol (PCP), which is a dangerous halogenated pollutant with persistence in the environment [56,57]. The distinctive morphological characteristics of *A. rouxii* are the enormous number of chlamydospores produced in the aerial and substrate mycelium [58] which is seen in Fig 4.1. *R. oryzae*, a fast growing filamentous fungus is a member of the order Mucorales and has high prevalence in tropical and subtropical regions. It is a primary or secondary colonizer which readily invades easily accessible and digestible substrates [59]. *R. oryzae* is also considered as an alternative microorganism for lactic acid fermentation as it synthesizes L-lactic acid from starchy materials by the action of external amylases in the presence of ammonium salts as sole nitrogen source [58]. In their study related to phylogenetic analysis, Kito et al. [54] took twenty-one strains of *A. rouxii* isolated from starters of Asian fermented foods and concluded that the species *A. rouxii* is composed of two distinct types, derived from *R. oryzae* or *R. delemar* via domestication in the starters. *A. rouxii* shows by the formation of rhizoids, stolons, and black pigmented sporangia and thereby suggesting its closeness to *R. oryzae* [58]. Also, studies have indicated similarity of *A. rouxii* and *R. oryzae* revealed by DNA-DNA complementation. However, the distinction between these two genera is supported by the pattern of utilization of sucrose, maltose, and glycerol by strains of *A. rouxii* and *R. oryzae* [55]. However, in the present study, the sequencing of the ITS region brought about a distinction in between the two species.

Phylogenetic tree showing the evolutionary relationship between *A. rouxii* TU460 and *R. oryzae* TU465, and three other related species (*A. rouxii* CBS 438.76, *R. oryzae* 8-3M and *A. oryzae* YI-A6) based on 16S rRNA gene sequences is shown in Fig 4.2. Based on 500 samplings, the bootstrap values are shown next to the branches as the percentage of trees in

which the associated taxa clustered together. The Neighbor-Join and BioNJ algorithms were applied to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then the topology with superior log likelihood value was selected to obtain the initial trees for the heuristic search. It was seen that *A. rouxii* TU460 was very closely related to *A. rouxii* CBS 438.76. Whereas, less similarity was observed in between *R. oryzae* TU465 and the outgroup organisms *R. oryzae* 8-3M and *A. oryzae* YI-A6, thereby suggesting a genetic drift.

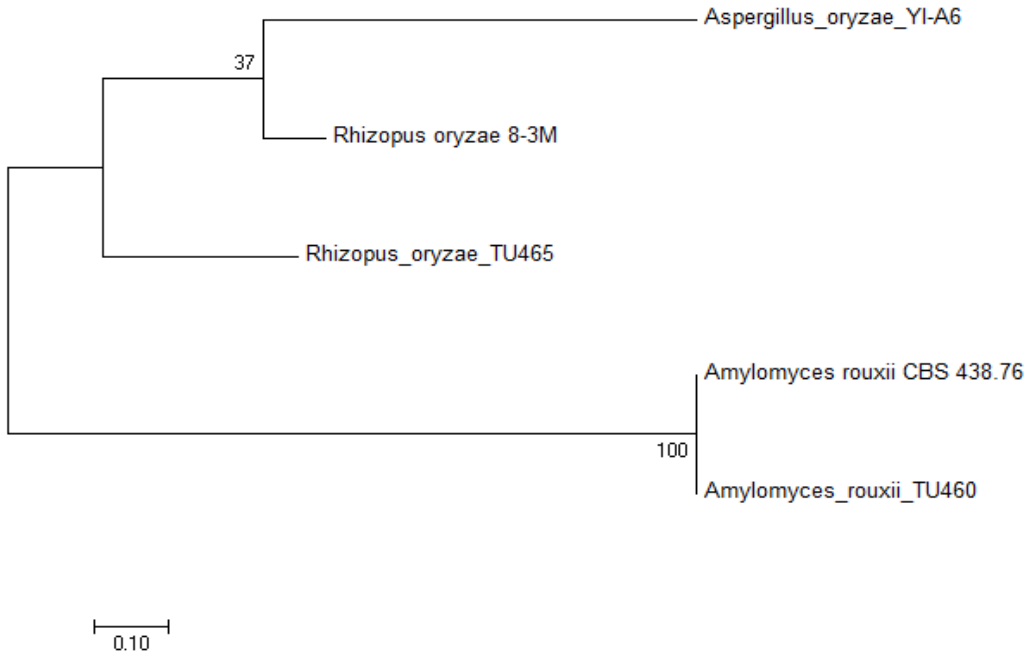

Fig 4.2 Phylogenetic tree based on the ITS region gene sequence showing the evolutionary relationship between *Amylomyces rouxii* TU460 and *Rhizopus oryzae* TU465, and three other related species (*Amylomyces rouxii* CBS 438.76, *Rhizopus oryzae* 8-3M and *Aspergillus oryzae* YI-A6) whose ITS gene sequences were obtained from the NCBI database. The bar at the bottom of the figure represents the length of branch that represents an amount genetic change of 0.10

4.3.1.2 Starch hydrolysis activity

The starch saccharifying enzymes viz., amylase and amylogluosidase produced by fungus can attack native starch granules thereby leading to pores which penetrate into the inner portions of the granules [60]. They can also act directly on raw starch granules below the gelatinization temperature of starch [61]. Zone of starch degradation and saccharification ratios shown by the fungal strains are given in Table 4.2. The zones on the starch agar plates shown by *A. rouxii* TU460 was 39.67 mm, which was close to the zone shown by the positive control

A. oryzae ATCC 10124. The saccharification ratio was also higher in *A. rouxii* TU460 (64.25 %), which was even higher than the positive control. *R. oryzae* TU465 on the other hand exhibited comparatively smaller degradation zones and lower sacccharification ratio. The negative control *F. oxysporum* MTCC 1755 did not show any such zone or scarification activity.

Table 4.2 Starch degradation, saccharification ratio and enzymatic activity of the fungal strains

Fungal strain	Zone of starch degradation (mm)	Saccharification ratio (%)	Glucoamylase activity (µmol/min)	α-amylase (unit mL^{-1})
A. rouxii TU460	39.67±1.53	64.25±3.50	14.92±1.58	7.02±0.98
R. oryzae TU465	27.67±1.53	5.26±0.11	1.41±0.26	6.09±0.74
A. oryzae ATCC 10124	40±0.00	30.00±0.26	2.74±0.78	7.36±0.56
F. oxysporum MTCC 1755	0.00	0.00	0.00	1.61±0.18

Note: The results are means of three replications followed by standard deviation

4.3.1.3 Enzymatic activity of the strains

Glucoamylase, which is best known to be produced by species of *Aspergillus*, is an enzyme of considerable industrial importance as it has the capability of converting starch quantitatively to glucose [62]. As shown in Table 4.2, the glucoamylase activity of *A. rouxii* TU460 (14.92 µmol/min) was considerably higher than that of *R. oryzae* TU465 and *A. oryzae* ATCC 10124 which was used as a positive control. No production was shown by the negative control *F. oxysporum* MTCC 1755. Wang et al. [62] reported that *A. rouxii* glucoamylase is a glycoprotein and has an optimum pH around 4.5, optimum temperature 60 °C, a molecular weight of 55.6 kDa, and Km values of 15.8 for soluble starch and exists in only one form. However, Nahar et al. [63] reported that the strain *Rhizopus* RFF isolated from Bangladesh was a potential producer of glucoamylase with highest activity at 45 °C and pH 4.5. Also, Fujio and Morita [64] reported the production of glucoamylase to a high concentration by *Rhizopus* sp. A-11 when the basal liquid medium was supplemented with zinc and calcium ions.

The activities α-amylase in *A. rouxii* TU460 and *R. oryzae* TU465 were 7.02 and 6.09 unit mL^{-1} and these values were close to the positive control *A. oryzae* ATCC 10124 (Table 4.2). The negative control *F. oxysporum* MTCC 1755 on the other hand showed comparatively

very little production of α-amylase. The production of α-amylase *R. oryzae* has also been reported by other authors like Yu and Hang [65] who reported production of high amylase activity by *R. oryzae* NRRL 395 when grown at 30 °C on substrates such as rice, barley, corn etc. Ray (2004) reported the production of extracellular amylase (3.8 units mL^{-1}) by *R. oryzae* in liquid medium in which the sole carbon source was 2 % soluble starch or cassava starch. Ghosh and Ray [66] also reported the highest production of extra-cellular isoamylase by *R. oryzae* PR7 in solid-state fermentations of millet, oat, tapioca, and arum after 72 h of growth at 28 °C and pH of 8.0. In another study, Watanabe and Oda [58] studied the properties of two sucrose-hydrolyzing enzymes produced by *R. oryzae* NBRC 4785 and *A. rouxii* CBS 438.76 and found that the enzymes could be classified as glucoamylase and invertase, respectively. They also found that the enzyme from the *R. oryzae* strain was more unstable than that from the *A. rouxii* strain under conditions of lower pH and higher temperature.

4.3.1.4 Molecular weight and enzymatic activity of the extracellular enzymes

The SDS PAGE revealed that *A. rouxii* TU460 produced two distinctive enzymes of 59 kDa and 31 kDa and *R. oryzae* TU465 produced only one enzyme of 72 kDa (Fig 4.3). In similar studies, Kanlayakrit et al. [67] reported the production of two types of glucoamylases by *Amylomyces* sp. 4-2 isolated from *loogpang kaomag*, a starter used for making sweet fermented rice in Thailand. One enzyme with MW 68 kDa had the ability to digest starch and be adsorbed onto it, while the other with MW 50 kDa lacked such activities. In contrast, two *R. oryzae* 99-880 glucoamylase enzymes, AmyC and AmyD were purified by Mertens et al. [68] and found them to have molecular mass of 52 kD and 47 kD respectively on the SDS-PAGE gel. Kareem et al. [69] also purified thermostable glucoamylase from *R. oligosporus* SK5 mutant and found that the enzyme showed two major bands with corresponding molecular weights of 36 kDa and 50 kDa. Moreover, in the Fig 4.4, it can be seen that after iodine staining, clear zones of were formed around the bands corresponding to the separated proteins, thereby indicating starch degrading amylase activity of the separated protein fractions in the gel.

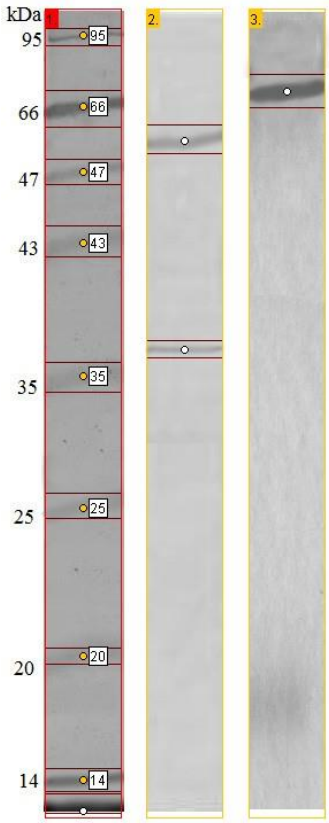

Fig 4.3 SDS- PAGE of the two purified extracellular enzymes produced. Lane 1: 14-95 kDa protein marker, Lane 2: *Amylomyces rouxii* TU460 and Lane 3: *Rhizopus oryzae* TU465

Fig 4.4 Iodine stained SDS-PAGE gels (with 1% starch) of the two enzymes after electrophoresis; (a) *Amylomyces rouxii* TU460 and (b) *Rhizopus oryzae* TU465

4.3.1.5 LC-MS/MS analysis to test the production of mycotoxin by the fungal strains

Mycotoxins are the low molecular weight secondary metabolites produced by certain molds. A complex biosynthetic pathway involving sixteen steps, mediated by individual major genes involved in both the regulatory and biosynthetic pathways are implicated in the production of mycotoxins [70]. Their structures may include single heterocyclic rings with molecular weights of 50 Da to irregularly arranged 6 or 8 membered rings with molecular weights greater than 500 Da. They have acute, chronic, mutagenic and teratogenic toxicity and impose various acute and chronic effects on human health, include including deterioration of liver or kidney function. Certain examples are aflatoxins, ochratoxins, ergot alkaloids etc. [71].

In order to analyze the high number of mycotoxins occurring in food materials, reliable, sensitive, robust and fast methods are necessary. These help to properly assess the relevant risk of exposure and the toxicological risk for humans and animals, and also help to ensure that regulatory levels fixed by international organisations like the EU are met. LC-MS/MS is a promising technique for multi-mycotoxins analysis as it provides simultaneous analysis of mycotoxins with good sensitivity, confirmation and without derivatization [72]. These methods provide low levels of detection with visual fragmentation of sample constituents and elimination of impurities based on mass [73].

The chromatograms obtained after liquid chromatography of the growth media extracts of *A. rouxii* TU460, *R. oryzae* TU465 and *Fusarium oxysporum* MTCC 1755 (positive control) are shown in Fig 4.5 and the *m/z* values detected in the EI-mass spectra by MS/MS analysis of all the peaks are shown in Table 4.3. The compounds detected by NIST library search of the m/z values of the separated compounds are shown in Table 4.3. The results revealed that almost same category of compounds were present in both *Amylomyces rouxii* TU460 and *Rhizopus oryzae* TU465, and none of those compounds belonged to the mycotoxin category. Hence these two wild fungal strains can be considered safe in terms of health effects and can be used in human foods. However, in case of the positive control *Fusarium oxysporum* MTCC 1755, which is a known producer of mycotoxins, two potent toxins viz. penitrem A and azaspiracid were detected. Penitrem A is a mycotoxin usually on ryegrass and produced by certain species of *Aspergillus, Claviceps*, and *Penicillium*. It is neurotoxic and acts by inhibiting potassium channels in smooth muscles [74]. Whereas, azaspiracids are a group of toxins causing AZA poisoning characterized by symptoms such as nausea, vomiting, diarrhoea and stomach cramps and are generally produced by shellfish [75].

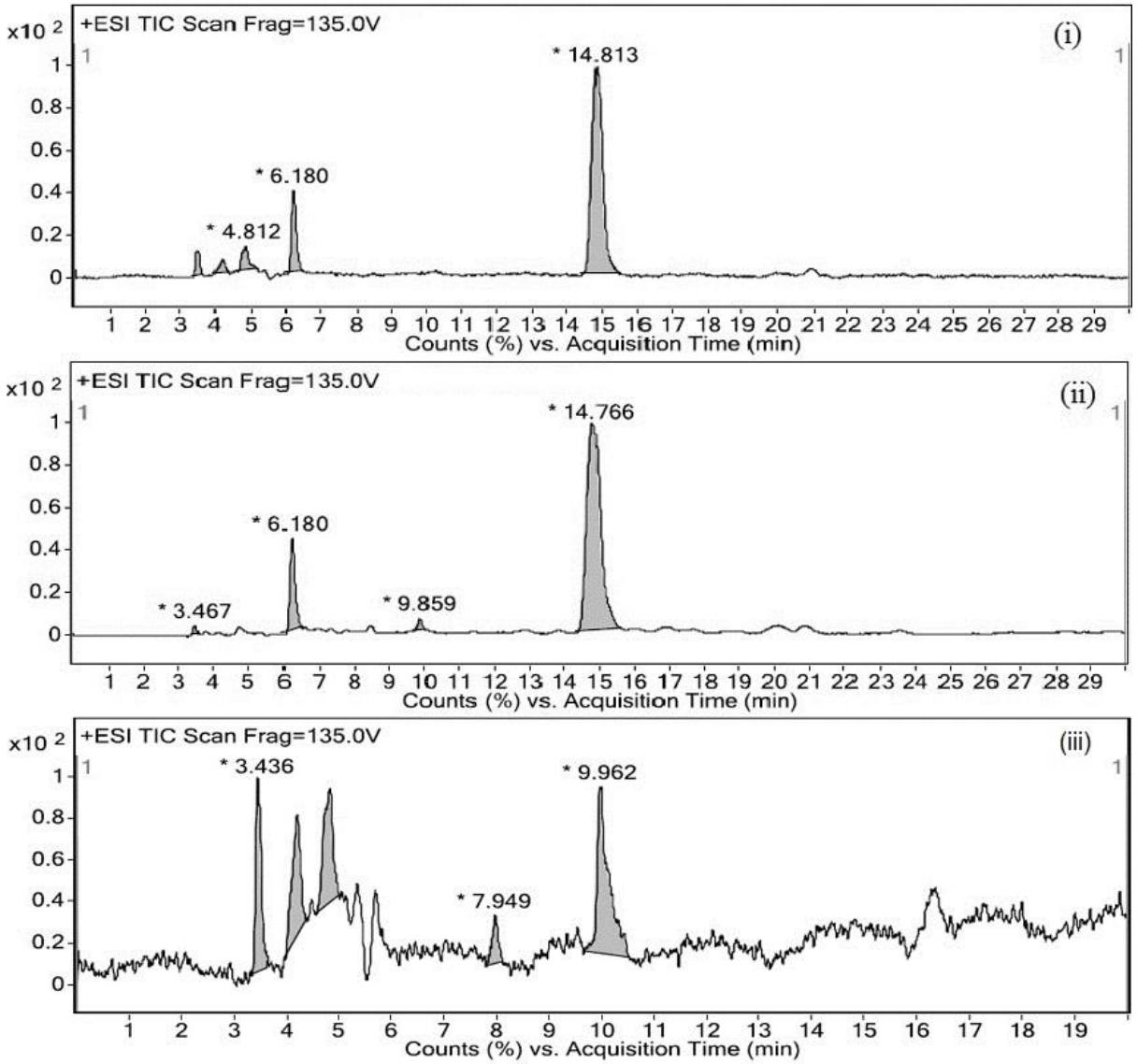

Fig 4.5 LC chromatograms of the culture supernatants for the detection of mycotoxins (i) *Amylomyces rouxii* TU460 (ii) *Rhizopus oryzae* TU465 (iii) *Fusarium oxysporum* MTCC 1755

Table 4.3 LC-MS/MS analysis to test the presence of mycotoxins in the culture media of the identified fungi *Amylomyces rouxii* TU460 and *Rhizopus oryzae* TU465 and that of known mycotoxin producer *Fusarium oxysporum* MTCC 1755

Fungal isolate	Retention time (mins)	m/z values detected in the EI-mass spectra (abundance in bracket)	Suggested compound
Amylomyces rouxii TU460	3.49	311.3(14721.6), 338.5(17287.8), 352.4(285140.7), 353.4(80171.1), 354.4(22354.5), 369.4(26325.2), 639.6(16871.4)	N-(9-Anthrylmethylene)-2-((2-(4-morpholinyl)-2-oxoethyl)thio)-1,3-benzothiazol-6-amine
	4.18	104.1(4892.3), 127(6103.7), 203.1(94472.3), 204.1(6593.1), 338.4(8828.4), 352.4(5709.14), 353.2(13141.5), 365.1 (12112.2), 369.4 (7561.4), 639.6 (8712.2)	2-(3-Chlorophenyl)-4-nitro-1H-isoindole-1,3(2H)-dione
	4.81	155.2(20740.3), 169(27536.3), 171.1(10195.6), 181.1(13229.9), 227.2(137457.6), 228.2 (16987.2), 249.2(21017.4), 261.2(123510.2), 262.1(26074.3), 283.1(49228.5)	3-Hydroxy-3-(2-(2-hydroxyphenyl)-2-oxoethyl)-1,3-dihydro-2H-indol-2-one
	6.18	77.1(50400.9), 105.1(402086.2), 149.1(481518.8), 150.1(46046.5), 211.1(42161.7), 233.1(702090.8), 234.1(83838)	2-(1-Methylhydrazino)-4,6-di(1-pyrrolidinyl)-1,3,5-triazine
	14.81	105.1(241741.1), 149.1(1999804.5), 150.1(185779.8), 315.2(170300.7), 337.2 (1155669.4), 338.1(21660.8), 651.2(355119.5), 652.2(146063.3)	5-(3,5-Dimethoxybenzylidene)-2-thioxo-1,3-thiazolidin-4-one
Rhizopus oryzae TU465	3.47	352.4(311637.1), 353.4 (97643.3), 354.4 (26428.2), 369.2(20517.4)	N-(9-Anthrylmethylene)-2-((2-(4-morpholinyl)-2-oxoethyl)thio)-1,3-benzothiazol-6-amine
	6.18	77.1 (240167), 105.1 (2020924), 106.1 (156030.2), 149.1(2098115.8), 150.1(203172.7), 211.1 (152604.9), 233.1 (2152190.3), 234.1 (243191.3)	2-(1-Methylhydrazino)-4,6-di(1-pyrrolidinyl)-1,3,5-triazine
	9.86	116.1(60748), 135(74342.1), 349.1(41602.8), 233.1(25151.8), 242.2(18065.2), 275.1 (105825.6), 277(16849.2), 289.1 (57170.6), 353.2 (20620.6), 415.2(16886.3)	5-(4-Ethoxybenzylidene)-2-(4-morpholinyl)-1,3-thiazol-4(5H)-one
	14.77	105.1(1027730.3), 149.1(7810172), 150.1(778311.1), 315.2(502561.8), 337.2 (2812214.3), 338.2 (540621.3), 651.2(1062496), 652.3(423360.5)	5-(3,5-Dimethoxybenzylidene)-2-thioxo-1,3-thiazolidin-4-one
Fusarium oxysporum MTCC 1755	3.436	338.4(46381.1), 339.4(14102.5), 352.4(241207.6), 353.3(74940.2),354.4(17838.6), 369.5(17202.5)	N-(9-Anthrylmethylene)-2-((2-(4-morpholinyl)-2-oxoethyl)thio)-1,3-benzothiazol-6-amine
	4.183	203.1(50151.3), 205.1(5546), 338.2(28229.4), 339.3(7664.2), 352.4(57739.3), 353.2(17301.9), 354.3(5643.9), 365.2(18588.8), 639.4(13006.1), 640.4(7653.7)	Azaspiracid
	4.804	155.1(12559.9), 169.9(32528.5), 181.1(19637.3), 203.1(15968.7), 227.1(55180.2), 2282.3(7922.1), 249.1(12128.4), 259.1(10593.5), 261.1(48189.9), 283.1(22751.8)	3-Hydroxy-3-(2-(2-hydroxyphenyl)-2-oxoethyl)-1,3-dihydro-2H-indol-2-one
	7.949	100.1(4319.1), 155.3(3846.9), 229.2(4019.7), 320.3(29500.4),321.2(5202.1), 325.2(51458.9), 326.3(7739.3), 352.6(5931.3), 639.3(14206.8), 640.3(8276.5)	1-(4-Nitro)-3-((2-(3-methylphenoxy)ethyl)amino)phenyl)pyrrolidine
	9.962	352.5(46616),353.2(13521.3), 412.2(16182.5), 415.2(15837.30), 417.2(12920.3), 432.3(7985), 437.3(7792.7),445.2(7143.7), 639.3(39563.5), 640.3(17052.9)	Penitrem A

When LC–MS/MS was used by Lattanzio et al. [76], for the simultaneous detection of 11 mycotoxins in maize belonging to aflatoxins, ochratoxin A, fumonisins, DON, zearalenone, and T-2 and HT-2, the limits of detection were found to range from 0.3 to 4.2 µg/kg. The limit of quantification for the aflatoxins and ochratoxin A was found to be 1 mg/kg and in the range of 10 to 200 mg/ kg for other mycotoxins by Spanjer et al. [77], when HPLC-ESI-MS/MS was developed for simultaneous determination of 33 mycotoxins in various plant products. Silva et al. [78] also reported that LC–MS/MS provides higher sensitivity for fumonisins B1 and B2 (12 µg/kg) in corn-based food when compared to mass spectrometry (40 µg/kg) or fluorescence detection (20 µg/kg for fumonisin B1; 15 µg/kg for B2). In the LC-MS/MS method developed by Elbert et al. [79], nine different mycotoxins deoxynivalenol (DON), zearalenon (ZON), ochratoxin A (OTA), 3- cetyldeoxynivalenol (3- AcDON), , HT-2, T-2, fusarenon X (FUS X), 15- acetyldeoxynivalenol (15-AcDON), nivalenol (NIV) and diacetoxyscirpenol (DAS) produced by *Fusarium* sp. were detected in one single run. The limits of quantification were found to be 0.3 µg/kg for OTA, 5 µg/kg for HT-2, T-2 and ZON and at 10 µg/kg for AcDON, DON, FUS X, DAS and NIV and were found to meet the National and European law required detection limits.

In order to set a limit for the minimum amount (threshold limit) of mycotixins capable of causing toxic effects on human health, the maximum permitted levels of mycotoxins in foods for human consumption have also been established by the European Union (EU) Commission Regulations. The threshold limits of the mycotoxins have been set as follows: 200 to 1750 µg/Kg deoxynivalenol, 20 to 400 µg/Kg zearalenone and 200 to 4000 µg/Kg fumonisins according to EC No 1126/2007 for fusarium toxins; 40 to 150 µg/Kg for sum of the aflatoxins B1, B2, G1 and G2 according to EU No 165/2010; 10 µg/Kg in dried fruits to be subjected to sorting, 4 µg/Kg in dried fruits for direct human consumption and 10 µg/Kg in dried figs for aflatoxins according to EU No 1058/2012; 30 µg/Kg in cereal products, 15 µg/Kg in spices and 80 µg/Kg in wheat gluten for ochratoxin A according to EU No 594/2012; 2000 µg/Kg citrinin in food supplements based on fermented rice according to EU No 212/2014 [80].

4.3.2 Identification and studies on physiological properties of yeast strains isolated from rice beer and starter cakes

4.3.2.1 General biochemical characteristics and identification of the yeast isolates

The DBB test helps in differentiating yeasts with ascomycetous affinity (no colour formation) from yeasts with basidiomycetous affinity (colour formation) [81]. The color formation is a result of intermediates of tryptophane degradation and ketones associated with the cell wall [82], and is therefore a reflection of different chemical phenomena in different yeasts [83]. Urea hydrolysis serves as a confirmatory test for the recognition of anascosporogenous species [84] as urease-positive strains generally belong to nonfermenting genera like *Cryptococcus*, *Sporobolomyces*, *Pullularia*, *Rhodotorula*, *Candida* and *Trichosporon*. It thus helps in eliminating saprophytes and in recognition of possible pathogens [85]. Certain non-fermenting capsulated yeasts cells produce extracellular starch like glycoproteins as a response to hyper-osmotic pressure or acidic conditions and the starch produced consists of two different polysaccharides, one corresponding to amylose and the other a pentosan [86,87]. This phenomenon is invariably associated with the presence of a capsule or less well organized extracellular slimes and occurs sporadically throughout the genera of *Cryptococcus*, *Lipomyces*, *Candida*, *Rhodotorula*, *Trichosporon* and *Bullera* (88)[106]. *Cycloheximide* causes a significant inhibition of fermentation by large number of yeast strains in concentrations of 0.5 to 5 ug per ml [89]. It is a potent inhibitor of protein synthesis which blocks the translocation of peptidyl-tRNA from the A site to the P site of the 60S eukaryotic ribosomal subunit. Low level resistance is conferred by an amino acid substitution in the ribosomal protein L29 [90].

These characteristics of the isolated strains are shown in Table 4.4. In the present study, it was seen that all the strains were negative for DBB test, urea hydrolysis and starch production. Except for the yeast isolates from *thap* (*W. anomalus* TU122) and *sujen* (*P. membranifaciens*) all the other isolates belong to *Saccharomyces crevisae*. The microscopic images for the yeast strains are shoe in Fig 4.6. The NCBI accession numbers were obtained for all the strains after identification and sequence submission. The strains of yeast identified after sequencing of their ITS region are shown in Table 4.4. *S. cerevissae* TU7, *S. cerevissae* TU21 and *P. membranifaciens* TU77 did not grow in presence of 0.01 % cycloheximide and were joined by eight other strains when the concentration was raised to 0.1 %. All the other strains exhibited weak growth under the same conditions.

Table 4.4 The samples of starter cake and rice beer, biochemical characteristics of the respective strains and their identification

Origin	Isolate code	Diazonium blue B test	Urea hydrolysis	Starch production	Growth in presence of cycloheximide		Identified strain	NCBI accession number
					0.01 %	0.1 %		
Umhu, Dimasa	TU96	-	-	-	**w**	**w**	*Saccharomyces cerevisiae* TU96	KU535600
Judima, Dimasa	TU4	-	-	-	**w**	-	*Saccharomyces cerevisiae* TU4	KU535589
	TU7	-	-	-	-	-	*Saccharomyces cerevisiae* TU7	KU535606
Thap, Karbi	TU118	-	-	-	**w**	**w**	*Saccharomyces cerevisiae* TU118	KU535601
	TU122	-	-	-	**w**	-	*Wickerhamomyces anomalus* TU122	KU535604
Hor alank, Karbi	TU121	-	-	-	**w**	-	*Saccharomyces cerevisiae* TU121	KU535602
Perok kushi, Deori	TU74	-	-	-	**w**	**w**	*Saccharomyces cerevisiae* TU74	KU535598
Sujen, Deori	TU77	-	-	-	-	-	*Pichia membranifaciens* TU77	KU535599
Mod pitha, Ahom	TU46	-	-	-	**w**	-	*Saccharomyces cerevisiae* TU46	KU535607
	TU63	-	-	-	**w**	-	*Saccharomyces cerevisiae* TU63	KU535596
Xaj pani, Ahom	TU45	-	-	-	**w**	-	*Saccharomyces cerevisiae* TU45	KU535595
Aopo pitha, Mising	TU11	-	-	-	**w**	**w**	*Saccharomyces cerevisiae* TU11	KU535591
Aopo pitha, Mising	TU123	-	-	-	**w**	**w**	*Saccharomyces cerevisiae* TU123	KU535605
Apong, Mising	TU71	-	-	-	**w**	-	*Saccharomyces cerevisiae* TU71	KU535597
Amou, Bodo	TU14	-	-	-	**w**	-	*Saccharomyces cerevisiae* TU14	KU535592
	TU21	-	-	-	-	-	*Saccharomyces cerevisiae* TU21	KU535593
Jou bishi, Bodo	TU9	-	-	-	**w**	**w**	*Saccharomyces cerevisiae* TU9	KU535590

Note: “w” – Weak positive response; “-” – Negative

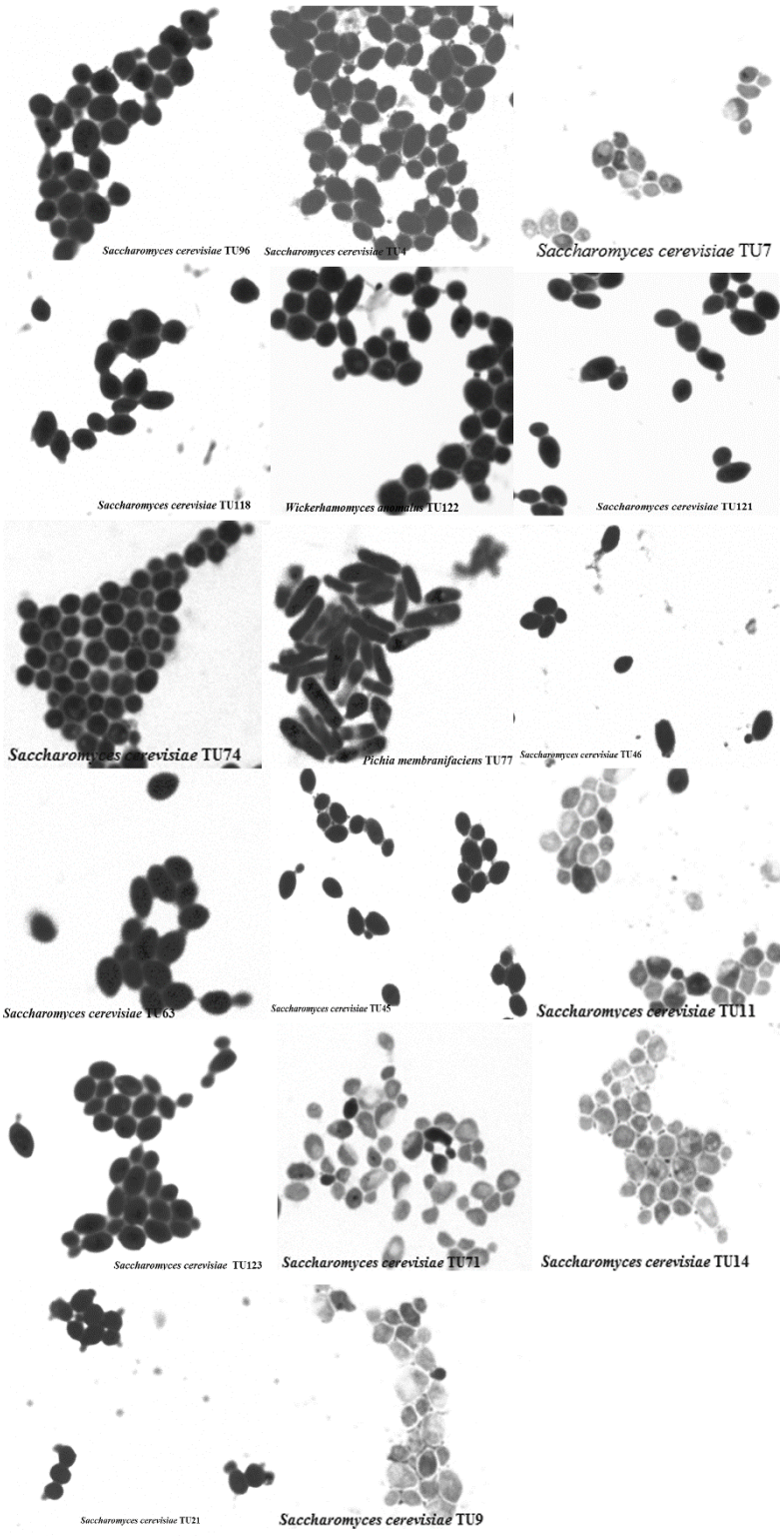

Fig 4.6 Microscopic view (100x) of the yeast strains

Since prehistoric times, *Saccharomyces cerevisiae* has been exploited to convert sugars into ethanol and desirable flavor compounds and to obtain foods and beverages with extended shelf-life, and to improve sensory properties and digestibility [91]. The name *Saccharomyces*

cerevisiae originated from the ancient words “Saccharomyces” meaning sugar mold or fungus and “cerevisiae” origination from Gaelic word “kerevigia” and the French word “cervoise”, both meaning beer. It is an essential component of many important human activities including baking, brewing, distilling, wine making and laboratory models. It has also been proposed that *S. cerevisiae* cells are found only in association with human activities [92] and this has led to the idea that it is a domesticated species i.e. wild species selected and bred by humans to obtain cultivated variants that thrive in man-made environments, but behave suboptimally in nature [91]. It is proposed to be specialized for the fermentation of alcoholic beverages, with other isolates of the species from other sources simply migrating from these fermentations [93].

Pichia membranaefaciens is a type species of the genus *Pichia*, which produces spherical, hemispherical or globular ascospores, ferments glucose, grows in presence of alcohol and has had film-forming ability. Its GC content ranges from 41.5–46.0 mol % and the optimum growth temperature is between 35–41 ^{o}C, thereby suggesting that it might be a heterogeneous complex species [94]. While studying the enzymatic activity of non-*Saccharomyces* yeasts for potential in wine-making by Fernández et al. [95], it was seen that *P. membranifaciens* possessed proteolytic activity, and also a possibility of intraspecific differences among the species. The strain *P. membranifaciens* CYC 1086 also secretes a killer toxin named PMKT2 that is inhibitory to a variety of spoilage yeasts and fungi. It has been found to inhibit *Brettanomyces bruxellensis*, while being completely ineffective against *Saccharomyces cerevisiae*. Thus it may be used in fermentation processes as a killer yeast which will have no deleterious effects on the fermentative strain, but at the same time will avoid the development of spoilage yeasts [96]. *P. membranifaciens have also been identified* from the inside of a cask involved in lambic beer fermentation [97].

Wickerhamomyces anomalus (formerly *Pichia anomala*) is a yeast of the *Wickerhamomyces* clade is frequently found in natural environments (plants, soil, fruits, animals) and in various fermentations. Its beneficial effects include flavor enhancement, phytase production, food and feed processing, dairy fermentation, vaccine development, waste water treatment and agent for biopreservation and folate biofortification [98,99]. *W. anomalus have also been identified* from the outside of a cask involved in lambic beer fermentation [97]. It has the ability to tolerate extreme environmental conditions like pH, osmotic stress, oxidative, salt and temperature shocks. It also displays broad-range antimicrobial activity against a variety of fungal and bacterial species, which is due to the biosynthesis of volatile compounds such as ethyl propionate, ethyl acetate, isoamyl acetate and/or production of killer proteins like glucanases [98]. The antifungal activity of *Wickerhamomyces anomalus* in

sourdough fermentation against *Penicillium roqueforti* have also been obtained Coda et al. [110]. The wine yeast *Wickerhamomyces anomalus* AS1 have been found to hydrolyse a number of synthetic and natural glycosides under oenological conditions, thereby releasing volatile aroma compounds which can have a major impact on aroma development and sensory profile of alcoholic beverages [101]. *W. anomalus* has been use to ferment *Saraca asoca* bark for preparing polyherbal formulation as it causes deglycosylation during fermentation [102].

4.3.2.2 Construction of phylogenetic tree

The tree with the highest log likelihood (-2309.9782) was chosen and shown in Fig 4.7. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 275 positions in the final dataset. It was observed that *S. cerevisiae* TU4, TU11, U118, TU123, TU121, TU9, TU74 and TU14 were closely related, while *S. cerevisiae* TU7,TU46, TU96 and TU45 were closely related to the reference strain *S. cerevisiae* SB1. Close relation was observed between *S. cerevisiae* TU71 and TU21. The later groups were to some extent related to reference strain *P. anomala* MTCC237. However, difference was observed between *P. membranifaciens* TU77 and the reference strain *P. membranifaciens* TU77 NCL53. The genetic diversity among 651 strains of *Saccharomyces cerevisiae* from 56 different geographical origins were analysed by Legras et al. [8]. Their genotyping revealed 575 distinct genotypes organized in subgroups of bread, beer, wine and sake yeast types. They observed that strains used for rice wine and sake were most closely related to beer and bread strains. It was also observed that up to 28 % of genetic diversity between those groups was associated with geographical differences, thereby suggesting local domestications.

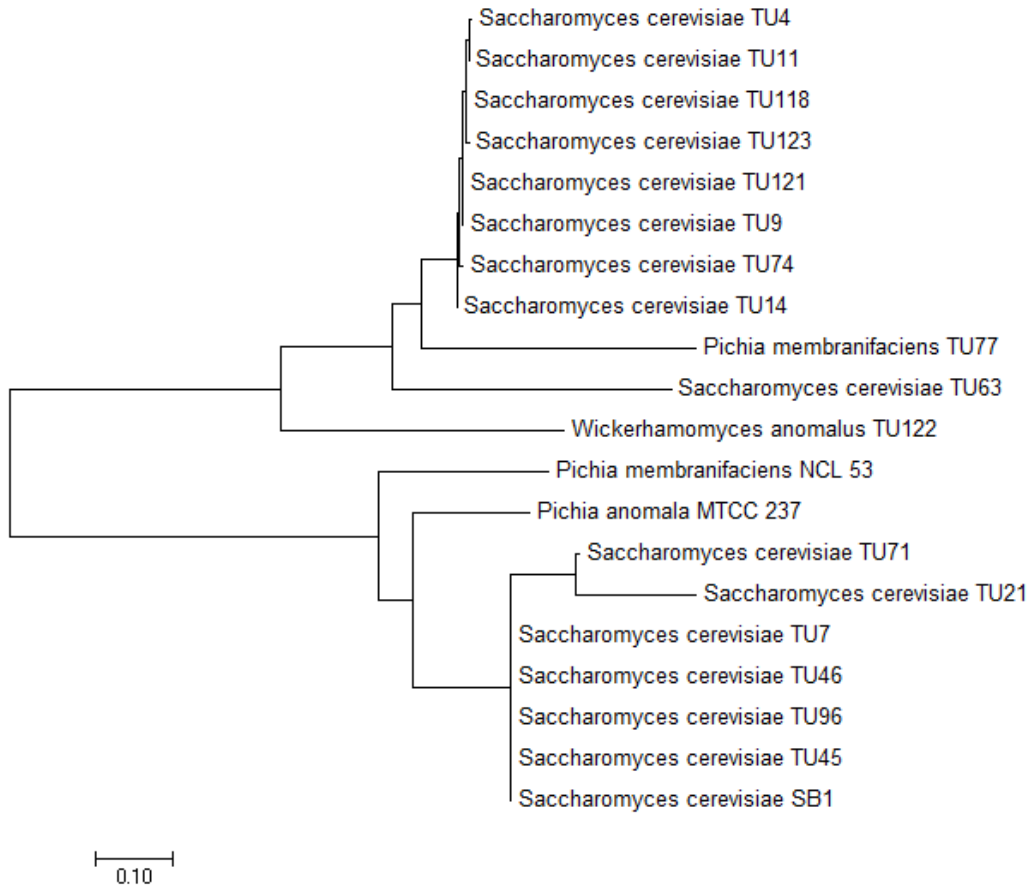

Fig 4.7 Molecular Phylogenetic analysis of the isolated and reference strains based on the sequence of ITS region by maximum likelihood method

4.3.2.3 Ethanol tolerance of the yeast strains

Even though ethanol is the major product from yeast sugar fermentation, yet increased ethanol concentration has different and separable effects on the viability of yeast cells and its specific rate of growth and fermentation the target sites on the yeast cell for ethanol includes the plasma membrane and membrane of cellular organelles, thereby leading to the inhibition and denaturation of intracellular proteins, glycolytic enzymes, glucose, maltose, ammonium, amino acid transport, glucose induced proton accelerated passive re-entry of protons and depression of the optimum growth temperature and causing lipid solubilisation. The ethanol tolerance in yeasts is defined as the threshold concentration of ethanol above which the cells are no longer able to maintain a proton gradient across their plasma membrane. The higher the ethanol production by a particular yeast strain, the more ethanol tolerant it is considered to be. The ethanol tolerance is also influenced by the plasma membrane composition, media composition, osmotic pressure, temperature, mode of substrate feeding, intracellular ethanol

accumulation and by-product formation. Tolerance is mediated by an increase in length of fatty-acyl chain and increase in proportion of unsaturated fatty acids and sterols present in the membrane, thereby resulting in tightening of the membrane [103].

The ethanol tolerance of the yeast isolates major in terms of their optical density, log of colony forming units and specific growth rate constants are shown in Table 4.5. At 0 % ethanol concentration the highest cunt was observed in *S. cerevissae* TU14, *S. cerevissae* TU71, *W. anomalus* TU122. Their specific growth rate constants were also highest at this point, which continued till the concentration was 3%. However at 6 % ethanol concentration high growth rate was shown by *S. cerevisae* TU71 and *S. cerevisae* TU14. At 9 % ethanol concentration tolerance was also shown by *S .cerivissae* along with the other tolerant strains. Finally at the highest concentration of 15 % ethanol *S. cerivissae* TU46, *S. cerevisae* TU63 and *S. cerevisae* TU45 proved to be the most tolerant strains.

Table 4.5 Test for ethanol tolerance of the yeast strains

Strain	Ethanol concentration																	
	0 %			3 %			6 %			9 %			12 %			15 %		
	OD	C	k	OD	C	k	OD	C	k	OD	C	k	OD	C	k	OD	C	k
S. cerevisiae TU96	1.54	7.77	0.366	1.16	7.53	0.355	1.21	7.63	0.359	1.07	7.18	0.338	0.86	7.06	0.332	0.43	6.08	0.285
S. cerevisiae TU4	1.57	8.00	0.377	1.50	7.71	0.363	1.46	7.52	0.354	1.41	7.46	0.351	1.38	7.35	0.346	1.33	7.20	0.339
S. cerevisiae TU7	0.87	7.90	0.372	0.72	7.82	0.368	0.56	7.73	0.364	0.21	7.65	0.360	0.20	7.26	0.342	0.54	6.96	0.298
S. cerevisiae TU118	1.62	7.72	0.364	1.47	7.31	0.344	1.42	7.51	0.354	1.03	7.08	0.333	0.96	7.04	0.331	0.37	5.00	0.233
W. anomalus TU122	1.73	8.64	0.408	1.56	8.49	0.401	1.43	7.75	0.365	1.12	7.32	0.344	0.61	7.60	0.358	0.84	6.95	0.327
S. cerevisiae TU121	1.59	7.59	0.357	1.20	7.27	0.342	1.45	7.56	0.356	1.29	7.30	0.344	0.74	7.08	0.333	0.42	5.60	0.262
S. cerevisiae TU74	1.46	8.45	0.399	1.29	8.28	0.391	1.19	7.92	0.373	1.06	7.43	0.350	0.95	7.32	0.344	0.59	6.90	0.324
P. membranifaciens TU77	0.75	7.34	0.345	0.63	7.20	0.339	0.29	7.02	0.330	0.18	6.89	0.324	0.17	6.67	0.313	0.16	6.43	0.302
S. cerevisiae TU46	1.60	7.95	0.375	1.54	7.75	0.365	1.43	7.63	0.359	1.35	7.48	0.352	1.32	7.48	0.352	1.30	7.40	0.348
S. cerevisiae TU63	1.56	8.07	0.380	1.47	7.86	0.370	1.28	7.59	0.357	1.16	7.37	0.347	1.13	7.36	0.346	1.10	7.17	0.337
S. cerevisiae TU45	1.59	7.99	0.377	1.52	7.82	0.368	1.45	7.59	0.357	1.40	7.38	0.347	1.38	7.06	0.332	1.36	7.02	0.330
S. cerevisiae TU11	1.71	8.32	0.392	1.60	8.03	0.379	0.34	7.67	0.361	1.06	7.51	0.354	0.89	7.82	0.368	0.42	6.43	0.302
S. cerevisiae TU123	1.69	8.02	0.378	1.66	8.16	0.385	1.22	7.58	0.357	1.24	7.62	0.359	0.98	7.15	0.336	0.37	6.59	0.309
S. cerevisiae TU71	1.64	8.48	0.400	1.27	8.21	0.387	1.24	7.99	0.377	1.02	7.54	0.355	0.40	7.48	0.352	0.47	6.79	0.319
S. cerevisiae TU14	1.67	8.50	0.401	1.44	8.34	0.393	0.99	7.91	0.373	0.81	7.48	0.352	0.47	6.83	0.321	0.28	6.62	0.311
S. cerevisiae TU21	0.71	8.30	0.392	0.65	8.03	0.379	0.45	7.66	0.361	0.19	7.11	0.334	0.18	7.08	0.333	0.14	6.58	0.309
S. cerevisiae TU9	0.86	8.11	0.382	0.73	8.02	0.378	0.43	7.88	0.371	0.29	7.61	0.358	0.24	7.76	0.366	0.33	6.56	0.308

Note: OD- Optical density at 600 nm; C- Log CFU/ml; k- Specific growth rate constant/h

Generally the yeast growth is inhibited at ethanol concentrations of 10 % (v/v), while 20 % (v/v) ethanol is inhibits fermentation capacity [104]. The ethanol tolerance in *S. cerevisiae* is compromised by constraints on energy production, and hence an increased expression of genes associated with glycolysis and mitochondrial function, and decreased expression of genes involved in energy demanding growth related processes takes place [105]. Till date more than 251 genes are found to be involved in influencing ethanol tolerance in *S. cerevisiae* [106]. The tolerance may be a function of decrease in membrane fluidity due to the incorporation of oleic acid into lipid membranes, thereby counteracting the fluidizing effects of ethanol [107]. The maintenance of vacuole function in order to bring about protein turnover and maintenance of ion homoeostasis is also important for ethanol tolerance [105]. In the study of Madrigal et al. [108] with different *Wickerhamomyces* isolates obtained from enological ecosystems in Spain for their enzymatic activity for the winemaking industry, *W. anomalus* and *P. membranifaciens* were found to be the most promising species. Their glycosidase enzymes also showed high degree of tolerance to high concentrations of ethanol and glucose.

4.3.2.4 Growth characteristics of the strains at different temperatures

Temperature has a direct influence on yeast growth and fermentation performance and the preferred temperature for *Saccharomyces* yeasts is between 25 to 35 ^{o}C. However, many types of mesophilic yeast exploited for alcohol production can grow between 0 ^{o}C and 48 ^{o}C. The optimum temperature for yeast growth is a narrow range and analysis of the metabolic response of *S. cerevisiae* to continuous heat stress has demonstrated that when the temperature is increased to 43 ^{o}C, yeast cells began to lose their viability [109]. Yeasts with varied temperature tolerance are desired as temperature control avoids shifts that may result in stuck fermentations above 30 ^{o}C, resulting in more efficient fermentation with better sensory quality. At the same time, fermentations at10–15 ^{o}C prevent the volatilization of primary aromas and increase the synthesis of secondary aromas. In low temperature stress tolerant strains, changes in membrane lipid composition of yeasts occur as an adaptive response. The fluidity of the membrane is maintained due to an increase in the degree of unsaturation of fatty acids and a decrease the chain length of these fatty acids [110]. With increased temperature, the alcohol production in *Saccharomyces* increases with temperature up to 40 ^{o}C, fusel alcohol production increases with increasing temperature with top-fermenting but not with bottom-fermenting

yeasts and acetate production increases. In *S. cerevisiae*, the alcohol dehydrogenase and catalase activity is also affected by change in temperature [111].

The results for growth of the yeast strains at different temperatures are shown in Table 4.6. It was observed that all the strains exihibited positive growth at 25, 30, 35 and 37°C however none of the strains could grow at 40, 42 and 45 °C. In the study of Redón et al. [110], the various adaptation mechanisms of *S. cerevisiae*, *S. bayanus*, *S. uvarum*, and a hybrid *S. cerevisiae/S. uvarum* to low temperatures were compared after culturing at low (13 ^{o}C) and optimal (25 ^{o}C) temperatures. It was observed that at low temperature, the phosphatidic acid content and the phosphatidylcholine/ phosphatidylethanolamine ratio decreased, whereas the medium-chain fatty acid and the triacylglyceride content increased. In order to improve fermentation rate at 13 ^{o}C, except the hybrid strain which showed the best vitality at all preculture temperature, the rest of the species needed a preadaptation at low temperature which involved a change in their lipid composition.

Table 4.6 Growth of the yeast strains at different temperatures

Strain	Different temperatures (°C)						
	25	30	35	37	40	42	45
S. cerevisiae TU96	+	+	+	+	-	-	-
S. cerevisiae TU4	+	+	+	+	-	-	-
S. cerevisiae TU7	+	+	+	+	-	-	-
S. cerevisiae TU118	+	+	+	+	-	-	-
*W. anomalus*TU122	+	+	+	+	-	-	-
S. cerevisiae TU121	+	+	+	+	-	-	-
S. cerevisiae TU74	+	+	+	+	-	-	-
P. membranifaciens TU77	+	+	+	+	-	-	-
S. cerevisiae TU46	+	+	+	+	-	-	-
S. cerevisiae TU63	+	+	+	+	-	-	-
S. cerevisiae TU45	+	+	+	+	-	-	-
S. cerevisiae TU11	+	+	+	+	-	-	-
S. cerevisiae TU123	+	+	+	+	-	-	-
S. cerevisiae TU71	+	+	+	+	-	-	-
S. cerevisiae TU14	+	+	+	+	-	-	-
S. cerevisiae TU21	+	+	+	+	-	-	-
S. cerevisiae TU9	+	+	+	+	-	-	-

Note: "+" – Positive; "-" – Negative

4.3.2.5 Tolerance of the yeast strains to osmotic stress

In the beginning of fermentation process or in order to increase plant capacity operations in the brewing process the yeast cells needs to be able to ferment at high initial concentrations of sugars. However, elevated osmotic pressure of high gravity worts distort yeast metabolism or decrease yeast viability, and thus exerts a negative influence on fermentation performance. Thus the yeast strain selected to carry out fermentation should have the capacity to withstand high osmotic pressures [104,112]. In a hyper osmotic environment, dehydration of the cells takes place and the cytoskeleton collapses leading to growth arrest. The response of yeasts to osmostress involves immediate cellular changes, activation of the primary defence processes and changes in cell homeostasis [113]. Yeast cells decreases their cell volume in response hypertonic stress, mediated by an osmotic water loss resulting in loss of cell turgor pressure and equilibration of the intracellular solute concentration with that of the environment [104]. Also there occurs a transient increases in glycolytic intermediates along with accumulation of cytosolic glycerol, trehalose and glycogen in order to balance the intracellular osmotic pressure with the external environment [113].

NaCl is a commonly used osmolyte to cause hyperosmotic stress and the intracellular concentration of glycerol increases parallel to the external concentration of NaCl [113]. The results for growth of the yeast strains in high osmotic stresses are shown in Table 4.7. *W. anomalus* TU122, *S. cerivissae* TU46, TU 63, TU 45, TU 71 and TU 21 could grow at 50 % dextrose. However at 60 % dextrose *S. cerevissae* TU63 and *S. cerevissae* TU45 could not grow. On the other hand all the strains could grow at 5 % NaCl and only *W. anomalus* TU122, *S. cerivissae* TU46, TU 63 and TU 45 could grow at 10 % NaCl. However none of the strains could grow at 16 % NaCl. In similar studies, Logothetis et al. [113] evaluated the osmotic stress responses of the industrial wine yeast strain *S. cerevisiae* VIN 13 in terms of cell growth and viability at 4 %, 6 % and 10 % w/v NaCl and 20 %, 30 % and 40 % w/v D-glucose. They observed beneficial influences on both cell viability and fermentation performance of the yeast strain due to osmostress induced by mild salt pre-treatments.

Table 4.7 Growth of the yeast strains in high osmotic stresses

Strain	Media composition				
	50 % dextrose	60 % dextrose	5 % NaCl	10 % NaCl	16 % NaCl
S. cerevisiae TU96	-	-	+	-	-
S. cerevisiae TU4	-	-	+	-	-
S. cerevisiae TU7	-	-	+	-	-
S. cerevisiae TU118	-	-	+	-	-
W. anomalus TU122	+	+	+	+	-
S. cerevisiae TU121	-	-	+	-	-
S. cerevisiae TU74	-	-	+	-	-
P. membranifaciens TU77	-	-	+	-	-
S. cerevisiae TU46	+	+	+	+	-
S. cerevisiae TU63	+	-	+	+	-
S. cerevisiae TU45	+	-	+	+	-
S. cerevisiae TU11	-	-	+	-	-
S. cerevisiae TU123	-	-	+	-	-
S. cerevisiae TU71	+	+	+	-	-
S. cerevisiae TU14	-	-	+	-	-
S. cerevisiae TU21	+	+	+	-	-
S. cerevisiae TU9	-	-	+	-	-

Note: "+" – Positive; "-" – Negative

4.3.2.6 Anaerobic sugar fermentation by the yeast strains

Depending on oxygen requirement yeasts are classified as obligate aerobes (exclusively respiratory metabolism), facultative anaerobes (both respiratory and fermentative metabolism) and obligate anaerobes (exclusively fermentative metabolism) [9]. In industrial fermentation anaerobic growth is desired in order to overcome the problems encountered due to oxygen gradients in voluminous aerobic fermentations. Moreover, transfer of this ability to other organisms which are able to ferment but not grow under anaerobic conditions can have industrial application [114]. Yeasts growth under oxygen-limited conditions can be directly correlated to the ability to perform alcoholic fermentation [9]. Various genes in yeasts are differentially expressed in response to aerobic and anaerobic growth conditions in order to regulate cellular metabolism. The hypoxic genes are induced at low oxygen tension and are repressed by haeme, which is an intermediate in oxygen levels signalling mechanism in yeast cells [112]. This is evident from the transcription of more than 500 genes changes significantly in cultures growing under aerobic and anaerobic conditions [114].

The results for anaerobic sugar fermentation by the yeast strains are shown in Table 4.8. Except *P. membranifaciens* TU77 all the strains fermented D-glucose. D-galactose, maltose, sucrose, melibiose and raffinose were also fermented by most of the strains. Whereas, Me α-D-glucoside and Me α-D-glucoside was fermented only by *W. anomalus* TU122. On the other hand, αα-trehalose, lactose, melezitose, inulin, starch and D-xylose was fermented by none of the strains. For growing under anaerobic conditions, the yeasts need to ferment sugars, whereby the enzymes pyruvate decarboxylase and alcohol dehydrogenase convert pyruvate into ethanol and carbon dioxide. All yeasts are not capable of fermenting all sugars and any given yeast may be able to ferment certain sugars but not others and this is known as the Kluyver effect [114]. This may be a result of oxygen requirement for sugar transport and activity of the pyruvate decarboxylase, disaccharide hydrolysis or sugar-specific regulatory mechanisms and product inhibition [114,115]. Kluyver effect is widespread among facultatively fermentative yeasts, with a strong strain-dependence on the pattern of disaccharide fermentation [115].

4.3.2.7 Utilization of different carbon sources by the yeast strains

The utilization of various carbohydrates by the yeast strains are shown in Table 4.9. Glucose, galactose, sucrose, fructose, mannose, maltose and trehalose were invariably utilized by almost all the strains. Whereas, the utilization of rest of the carbohydrates was scarce. The robustness of industrial yeasts is defined by its ability to promptly utilize various available nutrients with high yield of a desired product. The performance of brewing yeasts, in particular, is dependent on two major nutrient class viz., carbohydrates and nitrogenous compounds [116]. The ratios of different carbohydrate present affect fermentation performance as different yeast strains can utilize different carbohydrates, whereby lager yeasts has the distinctive ability to ferment melibiose [112]. Apart from the conventional carbon sources like glucose and maltose, the ability to utilize additional carbon sources like glycerol relates to other potential feedstocks [116]. The uptake of carbohydrates occurs in order of the monosaccharides first, followed in increasing order of complexity by disaccharides and trisaccharides and various facilitated diffusion process and energy dependent transporters are involved in the transport of different carbohydrate across the cellular membrane [112]. Under aerobic conditions, yeasts can perform respiration with oxygen as the final electron acceptor, however the Crabtree positive yeasts still exhibits alcoholic fermentation until all the sugars are depleted [117].

Table 4.8 Anaerobic sugar fermentation of the yeast strains

Strain	D-Glucose	D-Galactose	Maltose	Me α-D-Glucoside	Sucrose	αα-Trehalose	Melibiose	Lactose	Cellobiose	Melezitose	Raffinose	Inulin	Starch	D-Xylose
S. cerevisiae TU96	+	+	+	-	+	-	+	-	-	-	+	-	-	-
S. cerevisiae TU4	+	+	+	-	+	-	+	-	-	-	+	-	-	-
S. cerevisiae TU7	+	-	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae TU118	+	+	+	-	+	-	+	-	-	-	+	-	-	-
*W. anomalus*TU122	+	+	+	+	+	-	-	-	+	-	+	-	-	-
S. cerevisiae TU121	+	+	+	-	+	-	+	-	-	-	+	-	-	-
S. cerevisiae TU74	+	-	-	-	-	-	-	-	-	-	-	-	-	-
P. membranifaciens TU77	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae TU46	+	+	+	-	+	-	+	-	-	-	+	-	-	-
S. cerevisiae TU63	+	+	+	-	+	-	+	-	-	-	+	-	-	-
S. cerevisiae TU45	+	+	+	-	+	-	+	-	-	-	+	-	-	-
S. cerevisiae TU11	+	-	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae TU123	+	+	+	-	+	-	+	-	-	-	+	-	-	-
S. cerevisiae TU71	+	-	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae TU14	+	-	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae TU21	+	-	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae TU9	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: “+” – Positive; “-”– Negative

Table 4.9 Ability of the yeast strains to use carbon sources

Carbon sources	Yeast strains																
	S. cerevisiae TU96	*S. cerevisiae* TU4	*S. cerevisiae* TU7	*S. cerevisiae* TU118	*W. anomalus* TU122	*S. cerevisiae* TU121	*S. cerevisiae* TU74	*P. membranifaciens* TU77	*S. cerevisiae* TU46	*S. cerevisiae* TU63	*S. cerevisiae* TU45	*S. cerevisiae* TU11	*S. cerevisiae* TU123	*S. cerevisiae* TU71	*S. cerevisiae* TU14	*S. cerevisiae* TU21	*S. cerevisiae* TU9
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	-	+	+	−	+	+	+	+	+	+	+	+	+
Sorbitol	−	−	−	−	-	−	−	+	−	−	−	−	−	−	−	−	−
Xylose	−	−	−	−	-	−	−	+	−	−	−	−	−	−	−	−	−
Arabinose	−	−	−	−	-	−	−	−	−	−	−	−	−	−	−	−	−
Rhamnose	−	−	−	−	-	−	−	−	−	−	−	−	−	−	−	−	−
Sucrose	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	−	+	−	−	+	+	+	+	+	+	−	+	+
Trehalose	+	+	+	+	**D**	+	−	−	+	+	+	+	+	−	+	+	+
Cellobiose	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Salicin	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Melibiose	−	−	−	−	-	−	−	−	−	−	−	−	−	−	−	−	−
Lactose	−	−	−	−	-	−	−	−	−	−	−	−	−	−	−	−	−
Raffinose	+	−	−	−	+	−	+	−	−	−	−	+	−	+	+	+	−
Mannitol	−	−	−	−	+	−	−	−	−	−	−	−	−	−	-	−	−
Inositol	−	−	−	−	-	−	−	−	−	−	−	−	−	−	−	−	−
Adonitol	−	−	−	−	-	−	−	−	−	−	−	−	−	−	−	−	−
Inulin	−	−	−	−	-	−	−	−	−	−	−	−	−	−	−	−	−
Starch	−	−	−	−	-	−	−	−	−	−	−	−	−	−	−	−	−
Glycerol	**w**	**w**	**w**	**w**	+	**w**	**+**	**+**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**
Ethanol	**w**	**w**	**w**	**w**	+	**w**	**w**	**+**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**
Methanol	−	−	−	−	-	−	−	-	−	−	−	−	−	−	−	−	−

Note: "+" – Positive; "w" – Weak positive response; "-"– Negative; "D" – Positive delayed > 7 days

4.3.2.8 ADH activity of the yeast strains

Alcohol dehydrogenases (ADHs) are a large family of enzymes which plays critical role in sugar metabolism. In yeasts converts the pyruvate resulting from glycolysis is converted acetaldehyde and carbon dioxide. In cells grown anaerobically or in the presence of sugars excess, the alcohol dehydrogenase ADH1 reduces the acetaldehyde to ethanol such that NAD^+ is generated and glycolysis is continued. Another alcohol dehydrogenase ADH2, in yeasts converts ethanol accumulated during aerobic growth back into acetaldehyde when the sugar concentration is low, with the concomitant reduction of NAD1. Thus in presence of these enzymes, the yeast can produce alcohol when sugar is plentiful, or otherwise oxidise the alcohol once the sugar depletes [118,119]. The results for ADH activity of the yeast strains are shown in Table 4.10. A wide variation in the ADH activity was observed in all the yeast strains. The highest activity of 61.29 ADH mg $^{-1}$ protein was shown by *S. cerevisiae* TU71, and this was followed by *S. cerevisiae* TU46 with 54.27 ADH mg $^{-1}$ protein. Both *W. anomalus* TU122 and *S. cerevisiae* TU11 had activity in close range and the rest of the strains showed activity below 40 ADH mg $^{-1}$ protein.

Table 4.10 Estimation of ADH enzyme activity of the yeast strains

Identified organism	Unit ADH mg $^{-1}$ protein
S. cerevisiae TU96	15.31
S. cerevisiae TU4	16.34
S. cerevisiae TU7	10.09
S. cerevisiae TU118	11.29
W. anomalus TU122	42.94
S. cerevisiae TU121	19.24
S. cerevisiae TU74	09.59
P. membranifaciens TU77	06.73
S. cerevisiae TU46	54.27
S. cerevisiae TU63	34.57
S. cerevisiae TU45	32.20
S. cerevisiae TU11	46.72
S. cerevisiae TU123	08.76
S. cerevisiae TU71	61.29
S. cerevisiae TU14	02.67
S. cerevisiae TU21	08.75
S. cerevisiae TU9	07.89

4.3.2.9 Change in pH and total sugars of culture media and alcohol production by the yeast strains

Yeasts are acidophilic organisms and grow better under acidic conditions with pH optima of 4 to 6, depending on temperature and the presence of oxygen. This optimum range of pH is required for the functioning of enzymes, membrane-bound and transport proteins. A deviation from the optimal level results in deactivation of the enzymes and the cells are unable to grow and make ethanol efficiently [120]. However, as yeasts grows they acidify their growth media, largely as a result of the activity of the plasma-membrane H+-ATPase [121]. However, a low pH (4.0 or less) of the growth media of yeasts helps in controlling the growth of contaminating bacteria. The change in pH and content of total sugars and alcohol was analysed in the culture media of all the strains up to a period of 5 days. The change in pH of the growth media of the yeast strains with time is shown in Fig 4.8. The pH was initially 6.8 in all the cultures. *S. cerevisiae* TU63 and *S. cerevisiae* TU71 recorded a rapid decline on the first day which ended up in 5.32 and 5.28 respectively on the fifth day. The pH in all the other strains recorded a gradual decline and the lowest was observed in the strain *S. cerevisiae* TU7.

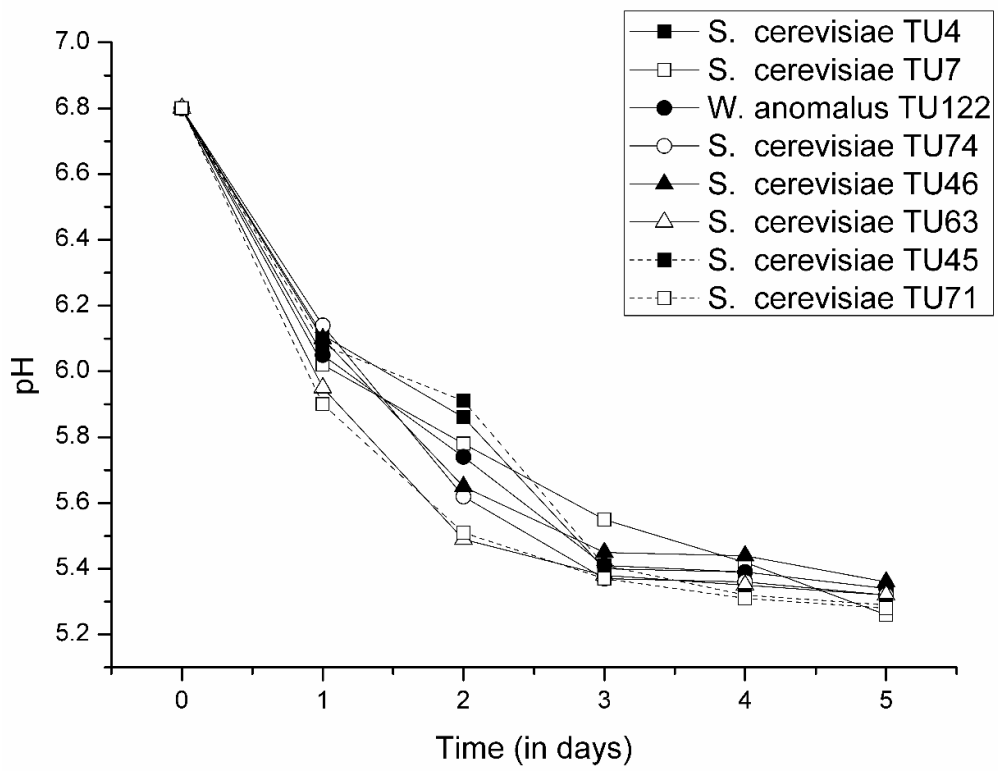

Fig 4.8 Change in pH of the growth media with time

The concentration of fermentable sugars in the culture media of yeasts is a key factor in the regulation of alcoholic fermentation [122]. Since the primary function of yeasts is to rapidly and efficiently catalyze the complete conversion of sugars to alcohol [123], the rate of sugar uptake limits the rate of alcohol production [123]. Both glucose and fructose are utilized simultaneously; however, *S. cerevisiae* has a Km of 1.6 mM for glucose uptake and 20 mM for fructose uptake [124]. The change in total sugars content of the growth media of the yeast strains with time is shown in Fig 4.9. The total sugar started with 13.20 mg/100 mL in all the cultures which gradually decreased with time. The highest utilization was observed in the strains *S. cerevisiae* TU4, TU63 and TU71.

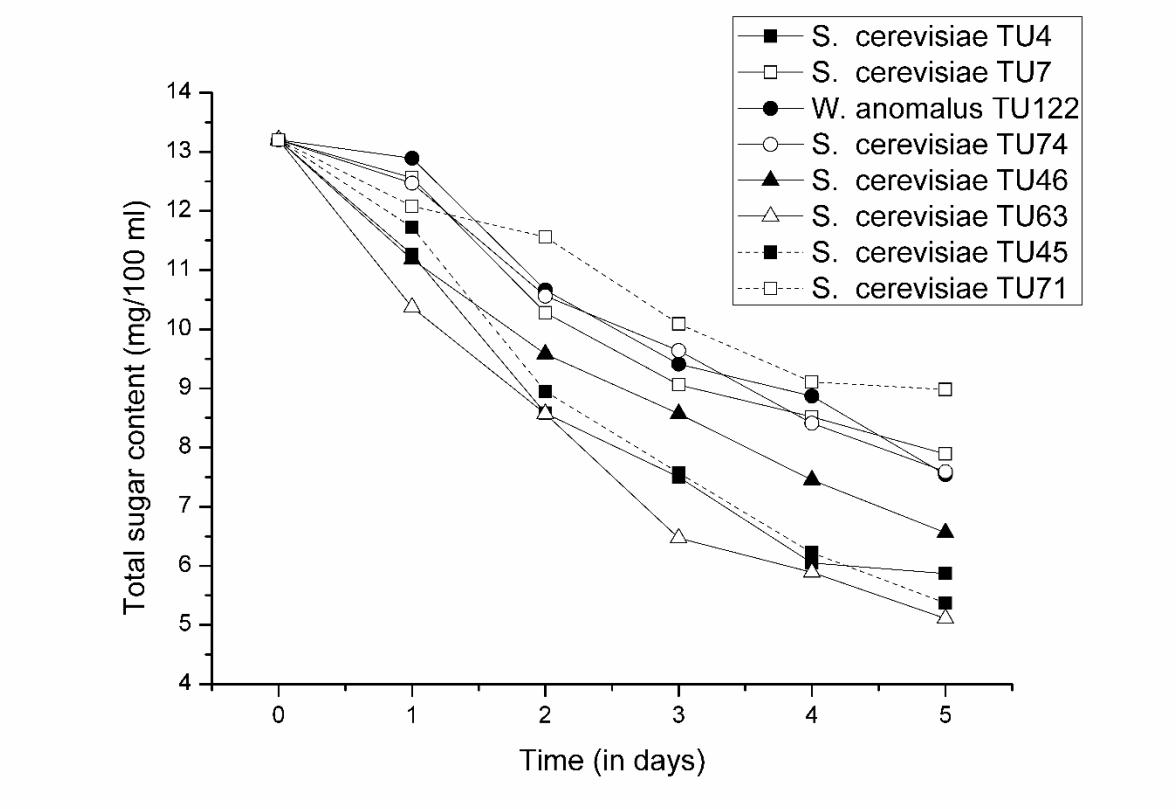

Fig 4.9 Change in total sugars content of the growth media with time

With the proceeding fermentation and depletion of nutrients, ethanol is accumulated and thus the yeast is confronted with the hurdle of its own metabolic activity as ethanol is toxic to most organisms even at low concentrations. As ethanol is a desired product, the robustness of a yeast stress is defined by its ability to accumulate more amount of ethanol. The tolerant yeast overcomes this stress mainly by changing properties of the cellular membranes i.e. increasing membrane permeability and changing in membrane fluidity [125]. The change in

alcohol content of the growth media of the yeast strains with time is shown in Fig 4.10. The alcohol content showed rapid increase in the first day which gradually increased in all the cultures. The highest content of alcohol was exhibited by the strains *S. cerevisiae* TU4, TU63 and TU71.

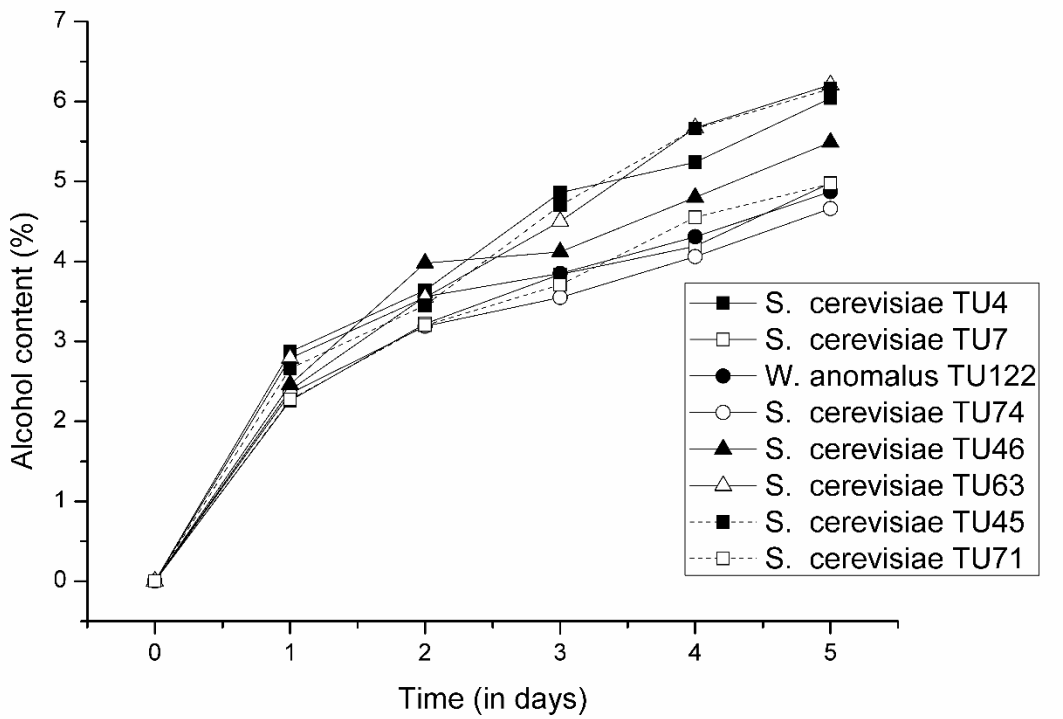

Fig 4.10 Change in alcohol content of the growth media with time

4.3.2.10 Formation of volatile organic compounds (VOCs) by the yeast strains

The volatile organic compounds produced by yeasts are various mixtures of gas-phase, carbon-based compounds and are derived from primary and secondary metabolism pathways. They are small in size and hence can diffuse through the atmosphere and soil. Approximately 250 fungal VOCs have till date been identified where they occur as mixtures of hydrocarbons, alcohols, aldehydes, ketones, phenols, thioalcohols, heterocycles, thioesters and their derivatives [126]. Distinct metabolic conversions are performed by yeasts on different substrates and it is strongly influenced by their capacity of fermenting and assimilating carbohydrates. With higher availability of carbon sources in the medium, the complexity of the composition of the VOCs produced increases [127]. In the natural environment, fungal VOCs play signalling roles between the fungi and plants, arthropods, bacteria, and other fungi. They also find applications as biofuel known as mycodiesel, biocontrol agents to prevent the growth

of plant pathogens prevent post-harvest fungal growth known as mycofumigation and as plant-growth promoters [126].

The volatile organic compounds detected in the culture media of the various yeast strains are listed in Table 4.11. Ethanol, 2-methoxy- was produced by all the strains except *W. anomalus* TU122, which produced ethanol, 2-(4-phenoxyphenoxy)- and isopropyl alcohol. Phenylethyl alcohol (a sweet smelling alcohol) was also produced by all the strains except *S. cerevisiae* TU45. The aromatic dicarboxylic acid, phthalic acid was also found to be produced by all the strains. On the other hand, many distinctive fusel alcohols were found to be varyingly produced by all the strains. The higher or fusel alcohols contribute to the overall beer flavour character. Acetaldehyde imparts a 'grassy' off-flavour to beer if it is present above the flavour threshold of 10–20 mg L^{-1}. The vicinal diketones diacetyl (2,3- butanedione) and 2,3-pentanedione imparts a butterscotch aroma with flavour thresholds of 0.15 mg L^{-1} 0.9 mg L^{-1} respectively. The esters result in the floral/fruity aromas in beer and over 100 different esters have been identified [112]. In the study of Fialho et al. [127], the VOCs produced by *S. cerevisiae* strain CR-1 grown on PDA were found to be high quantities of alcohols as well as esters viz. ethanol, phenylethyl alcohol 3-methyl-1-butanol, 2-methyl-1-butanol, ethyl acetate and ethyl octanoate. Similar results were also obtained by Buzzini et al. [128] who studied the VOCs produced by 98 ascomycetous yeast strains (40 species and 12 genera). Alcohols (amyl alcohol and isoamyl alcohol), esters (ethyl isobutyrate, isoamyl acetate, isobutyl acetate, ethyl isovalerate, 2-methylbutyl acetate, isoamyl propionate and phenylmethyl acetate) and aldehydes (2-isopropyl-5-methyl-2-hexenal and 2-methyl-2-hexenal) were found to be produced, and most of these were well known as flavouring compounds.

Chen and Xu [3] also studied the influence of yeast strains on volatile flavour profiles of Chinese rice wine and found significant differences based on the on the yeast strains used. They found that yeast strains from the Shaoxing region produced higher amounts of 2-phenylethanol and 3-methylthiopropanol, while those from the Shanghai region produced more of the branched-chain higher alcohols. In another study, Mo et al. [129] studied the production of VOCs by two *Pichia* strains *P. farinosa* SKM-1 and *P. anomala* SKM-T. They found that *P. farinose* produced 5 and *P. anomala* produced 12 kinds of VOCs. Phenylethyl alcohol was identified as the most common VOC by them.

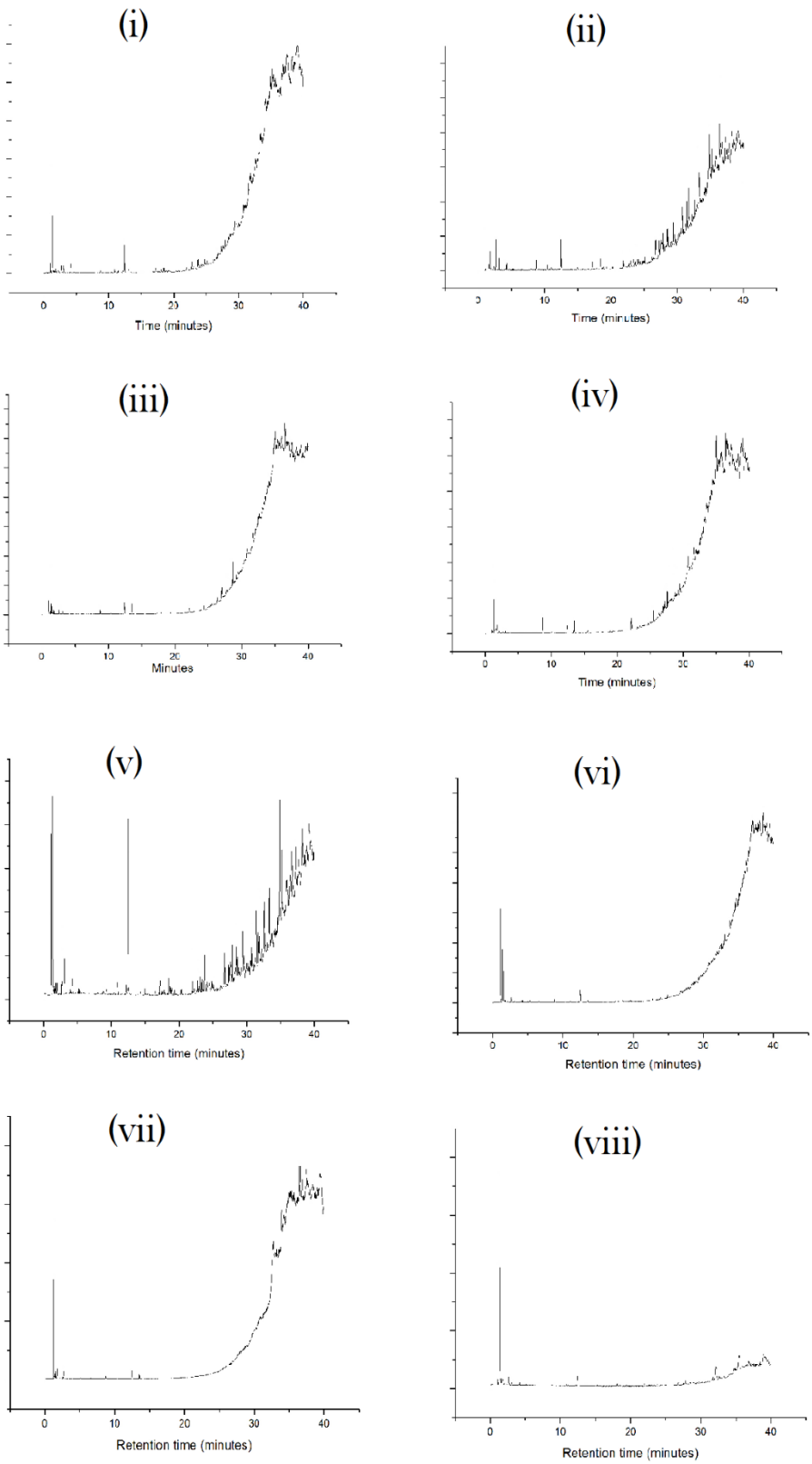

Fig 4.11 GC chromatograms for the detection of volatile organic compounds by the yeast strains. (i) *S. cerevisiae* TU4 (ii) *S. cerevisiae* TU7 (iii) *S. cerevisiae* TU71 (iv) *S. cerevisiae* TU45 (v) *S. cerevisiae* TU46 (vi) *S. cerevisiae* TU63 (vii) *W. anomalus* TU122 (viii) *S. cerevisiae* TU74

Table 4.11 The volatile organic compounds detected in the yeast culture media

Organic compound	Yeast strains							
	S. cerevisiae TU4	*S. cerevisiae* TU7	*S. cerevisiae* TU71	*S. cerevisiae* TU45	*S. cerevisiae* TU46	*S. cerevisiae* TU63	*W. anomalus* TU122	*S. cerevisiae* TU74
Ethanol, 2-methoxy-	√	√	√	√	√	√	-	√
Ethanol, 2-(4-phenoxyphenoxy)-	-	-	-	-	-	-	√	-
Isopropyl alcohol	-	-	-	-	-	-	√	-
Ethylene, 1,2-dichloro-,(Z)-	√	-	-	√	√	√	√	√
3-Hexanol	-	√	-	-	√	-	-	√
3-Oxetanol, 2,2,3-trimethyl-	-	√	-	-	√	-	-	-
3-Penten-2-ol	-	-	-	-	√	-	-	-
2-Propanone, 1-Methoxy-	-	√	-	-	-	-	-	-
1-Pentanol	-	√	√	-	√	√	-	√
Benzeneethanol, alpha.-methyl-	-	-	√	-	-	-	√	-
1-Propanol, 3-phenoxy-	-	√	-	√	-	√	-	-
Phenyl alcohol	-	-	√	-	-	-	-	-
Phenylethyl alcohol	√	√	√	-	√	√	√	√
1-Decanol, 2-ethyl	√	-	-	-	-	-	-	-
1-Ethanone	√	√	-	-	√	-	-	√
Oxalic acid, isobutyl nonyl ester	√	√	-	-	√	-	-	-
1-Octanol, 2-butyl	√	√	-	-	√	-	-	√
Pentadecen-1-ol	-	-	√	-	-	-	-	-
1-Eicosanol	√	-	-	-	-	-	-	-
9H-Fluoren-3-ol, 9,9-dimethyl-	-	-	√	√	-	-	-	-
1-Decanol-2-hexyl-	-	√	-	-	√	-	-	√
Phenol, 2-(1-phenylethyl)-	-	-	√	√	-	-	-	-
Phenol, 2,6-bis(1,1-dimethylethyl)-	√	√	-	-	√	-	-	√
Benzyl Benzoate	-	-	√	√	-	-	-	-
Benzimidazol-4-ol	-	-	-	√	-	-	-	-
1-Heptacosanol	-	-	-	-	-	-	-	√
Phthalic acid	√	√	√	√	√	√	√	√
Oleyl alcohol, trifluoroacetate	-	-	-	-	√	-	-	-
1-Hexadecanol	-	√	-	-	-	-	-	√
1-Pentacontanol	-	-	-	√	-	-	-	-

Note: "√" – Detected; "-" – Not detected

4.3.3 Identification and studies on functional properties of lactic acid bacteria isolated from rice beer and starter cakes

4.3.3.1 The LAB strains identified in the starter cakes

The different identified species of LAB isolated and identified from various rice beer starter cakes used by different communities in Assam are shown in Table 4.12. From the various starters, ten strains of *Lactobacillus casei*, one strain of *Lactobacillus pentosus*, two strain of *Lactobacillus plantarum* and sixteen strains of *Pediococcus pentosaceus* were identified. The *L. casei* group currently consists of three species: *L. casei*, *L. paracasei* and *L. rhamnosus.* The species diversity of *L. casei* in regards to its ecological versatility and genome evolution has also been recently proven by comparative genomic analyses [130]. Several strains of *L. casei*, have already been identified with potential probiotic traits and satisfying stringent technological [131]. *L. casei* has also been used successfully as a probiotic a number of commercial fermented food products ([132]. The species *L. pentosus* is considered to be safe and suitable for the Qualified Presumption of Safety (QPS) approach by EFSA [133]. It is a versatile candidate for fermentation as it can ferment hexoses using the Embden-Meyerhoff-Parnas pathway and pentoses using the phosphoketolase pathway. It has been shown to inhibit the growth of Helicobacter pylori and other pathogens such as *Escherichia coli, Salmonella, Aspergillus niger, Aspergillus oryzae*, *Streptococcus pneumonia* and influenza virus [134]. *L. plantarum* is a versatile organism commonly encountered in dairy, meat and plants products, as well as in GI tract of humans and animals. It has high occurrence in traditional fermented foods from Korea, China, and India. The behaviour of different strains of *L. plantarum* is diverse; some may exhibit resistance to extreme acidic conditions, whereas others can resist high concentration of bile salt. This may be attributed to the presence and absence of different genes encoding production of bacteriocin, exopolysaccharides and genes involved in sugar metabolismwhich in turn affects the adaptability and survivability [135,136]. *P. pentosaceus* is a homofermentative probiotic LAB, and is known to prevent cardiovascular diseases, attack of harmful pathogens in the GI and provoke immune reaction [137]. It has been used as a starter culture in meat, vegetables and dairy fermentation and is responsible for characteristic flavour changes and extending the shelf life. It also produces bacteriocins active against a broad spectrum of Gram positive bacteria like *Listeria monocytogenes, Staphylococcus aureus* etc. [138].

Table 4.12 Identified strains and their NCBI accession numbers

Source	Organism name	NCBI Accession Number
Umhu, Dimasa	*Lactobacillus casei* TEZU309	KR781616
Judima, Dimasa	*Lactobacillus pentosus* TEZU174	KR781611
Thap, Karbi	*Lactobacillus casei* TEZU468	KT273335
Thap, Karbi	*Pediococcus pentosaceus* TEZU469	KT273350
Thap, Karbi	*Pediococcus pentosaceus* TEZU470	KT273334
Thap, Karbi	*Pediococcus pentosaceus* TEZU472	KT273333
Thap, Karbi	*Pediococcus pentosaceus* TEZU473	KT273332
Hor-alank, Karbi	*Pediococcus pentosaceus* TEZU199	KR781612
Perok-kushi, Deori	*Pediococcus pentosaceus* TEZU366	KT273346
Perok-kushi, Deori	*Pediococcus pentosaceus* TEZU451	KT273336
Sujen, Deori	*Pediococcus pentosaceus* TEZU213	KR781617
Sujen, Deori	*Pediococcus pentosaceus* TEZU298	KR781615
Mod-pitha, Ahom	*Pediococcus pentosaceus* TEZU270	KT273348
Mod-pitha, Ahom	*Lactobacillus plantarum* TEZU272	KT273347
Xaj pani, Ahom	*Pediococcus pentosaceus* TEZU427	KT273337
Apop-pitha, Mising	*Pediococcus pentosaceus* TEZU481	KT273331
Apop-pitha, Mising	*Pediococcus pentosaceus* TEZU482	KT273330
Apop-pitha, Mising	*Pediococcus pentosaceus* TEZU486	KT273328
Apong, Mising	*Lactobacillus casei* TEZU262	KR781613
Apong, Mising	*Lactobacillus plantarum* TEZU263	KT273349
Amou, Bodo	*Pediococcus pentosaceus* TEZU293	KR781614
Amou, Bodo	*Pediococcus pentosaceus* TEZU410	KT273338
Jou-bishi, Bodo	*Lactobacillus casei* TEZU368	KT273345
Jou-bishi, Bodo	*Lactobacillus casei* TEZU369	KT273344
Jou-bishi, Bodo	*Lactobacillus paracasei* TEZU370	KT273343
Jou-bishi, Bodo	*Lactobacillus casei* TEZU371	KT273342
Jou-bishi, Bodo	*Lactobacillus paracasei* TEZU372	KT273341
Jou-bishi, Bodo	*Lactobacillus casei* TEZU373	KT273340
Jou-bishi, Bodo	*Lactobacillus casei* TEZU374	KT273339

In similar studies, Sujaya et al. [139] isolated and identified LAB from five different types of *ragi tape*, a type of dry starter for rice wine preparation in Indonesia. On the basis of 16S rDNA sequencing, the isolates were identified as *Pediococcus pentosaceus*, *Enterococcus faecium*, *Lactobacillus curvatus*, *Weissella confusa* and *W. paramesenteroides*. The diversity of LAB in Korean *makegolli*, a type of rice wine wasstudies by Min et al. [140] and they found that *L. ingluviei*, *L. fermentum* and *L. harbinensis* were the most frequent. The molecular identification of LAB in Chinese rice wine was carried out using species specific multiplex PCR by Ke et al. (15)[34]. The strains identified belonged to *Lactobacillus curvatus*, *L. brevis*, *L. buchneri*, *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. plantarum*, *L. fermentum*, *L. lactis*, *P. acidilactici* and *P. pentosaceus*. Jin et al. [141] investigated LAB population in Korean traditional rice wine *takju*, and by 16S rRNA gene sequencing, the isolates were identified as

Lactobacillus paracasei, *L. arizonensis*, *L. plantarum*, *L. harbinensis*, *L. parabuchneri*, *L. brevis* and *L. hilgardii*. Doi et al [142] also analyzed the microflora in fermented rice bran product and concluded that *L. johnsonii*-related strains predominate in the product. In India, Tamang et al [143] identified LAB strains isolated from *hamei* and *marcha* starters for rice beer fermentation used in Manipur and Sikkim, respectively. The strains were identified as *P. pentosaceus*, *L. plantarum* and *L. brevis*.

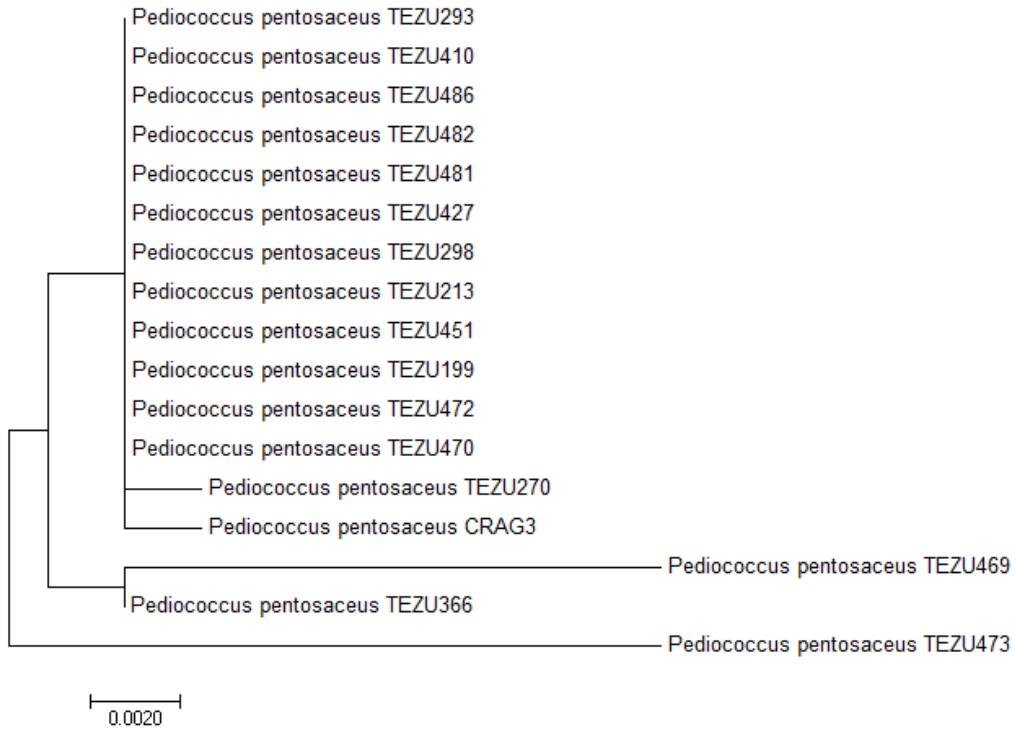

Fig 4.12 Phylogenetic tree of the LAB strains obtained by molecular phylogenetic analysis through maximum likelihood method

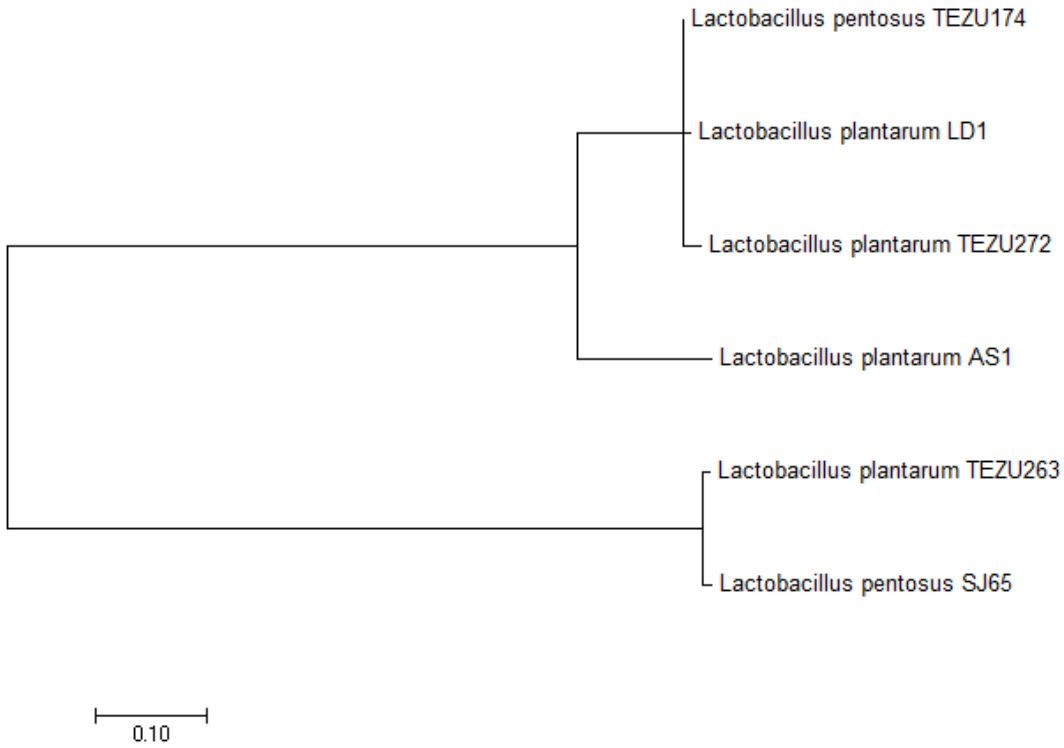

Fig 4.13 Phylogenetic tree of the LAB strains obtained by molecular phylogenetic analysis through maximum likelihood method

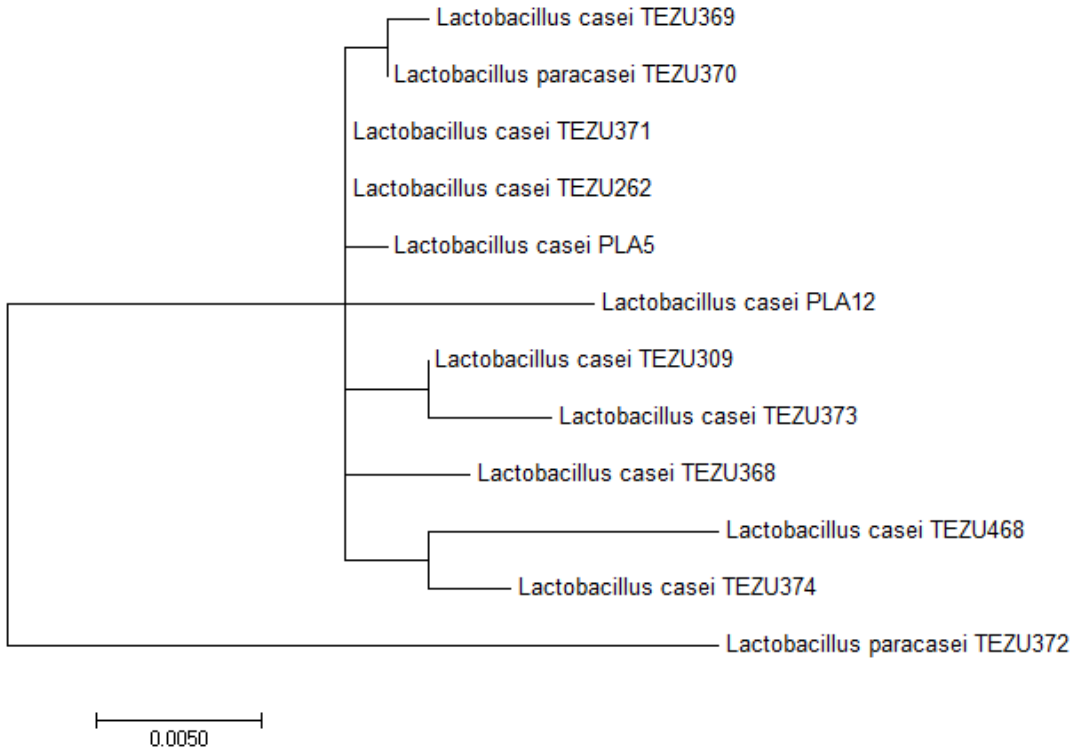

Fig 4.14 Phylogenetic tree of the LAB strains obtained by molecular phylogenetic analysis through maximum likelihood method

The phylogenetic trees (Fig 4.12, 4.13 and 4.14) were constructed based on 500 samplings in which the Neighbor-Join and BioNJ algorithms were applied to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. The topology with superior log likelihood value was selected to obtain the initial trees for the heuristic search. Close association was seen between *P. pentosaceus* TU427 and *P. pentosaceus* TU481, which were again related to the rest of the *P. pentosaceus* strains in the next branch. Relation was also seen between *L. pentosus* TU174 and *L. plantarum* TU272. The remaining *L. casei* strains were also closely related.

4.3.3.2 General characteristics of the LAB strains

The general characteristics of the isolated LAB strains are shown in Table 4.13. All the strains were found to be positive for the production of catalase. The catalase produced by LAB strains hydrolyses hydrogen peroxide which is produced by oxidizing lactate. This is significant in fermented products since hydrogen peroxide can increase rancidity and the discoloration of the final product and hence interfere with the organoleptic properties [144]. It was observed that none of the strains produced gas from glucose. The production of gas from glucose is, a characteristic feature of the obligately heterofermentative lactobacilli that ferment hexoses to lactic acid, acetic acid and/or ethanol and carbon dioxide [145]. The production of ammonia from arginine was found to be negative in all the strains. The dergedation of L-arginine leads to the formation of ornithine and ammonia. The screening of LAB for the ability to degrade arginine is importance in their selection as starter cultures since degredation of arginine is also an indicative of the LAB's ability to form ethyl carbamate (a carcinogen found in fermented foods and alcoholic beverages) precursors, viz., citrulline and carbamyl phosphate [146]. None of the LAB strains showed gelatin hydrolysis activity. Hydrolysis of gelatin is an indicative of the proteolytic activity of LAB. Even though LAB have only weak proteolytic action on myofibrillar proteins, however, certain strains actively contribute to the hydrolysis of the sarcoplasmic proteins and decomposition of peptides into amino acids. The amino acids also act as precursors of favor compounds in the final product. Hence screening for gelatine hydrolysis is important for selection of probiotic LAB strains [144]. The haemolytic activity was also absent in all the strains. Testing for haemolytic activity is a safety prerequisite for the selection of probiotic LAB strain. Absence of haemolytic activity is an indicative that the strain is non-pathogenic [147].

Table 4.13 General characteristics of the strains

Strain name	Catalase	Gas production	Ammonia production	Gelatin hydrolysis	Haemolysis activity
L. casei TEZU309	+	-	-	-	-
L. pentosus TEZU174	+	-	-	-	-
L. casei TEZU468	+	-	-	-	-
P. pentosaceus TEZU469	+	-	-	-	-
P. pentosaceus TEZU470	+	-	-	-	-
P. pentosaceus TEZU472	+	-	-	-	-
P. pentosaceus TEZU473	+	-	-	-	-
P. pentosaceus TEZU199	+	-	-	-	-
P. pentosaceus TEZU366	+	-	-	-	-
P. pentosaceus TEZU451	+	-	-	-	-
P. pentosaceus TEZU213	+	-	-	-	-
P. pentosaceus TEZU298	+	-	-	-	-
P. pentosaceus TEZU270	+	-	-	-	-
L. plantarum TEZU272	+	-	-	-	-
P. pentosaceus TEZU427	+	-	-	-	-
P. pentosaceus TEZU481	+	-	-	-	-
P. pentosaceus TEZU482	+	-	-	-	-
P. pentosaceus TEZU486	+	-	-	-	-
L. casei TEZU262	+	-	-	-	-
L. plantarum TEZU263	+	-	-	-	-
P. pentosaceus TEZU293	+	-	-	-	-
P. pentosaceus TEZU410	+	-	-	-	-
L. casei TEZU368	+	-	-	-	-
L. casei TEZU369	+	-	-	-	-
L. paracasei TEZU370	+	-	-	-	-
L. casei TEZU371	+	-	-	-	-
L. paracasei TEZU372	+	-	-	-	-
L. casei TEZU373	+	-	-	-	-
L. casei TEZU374	+	-	-	-	-

Note: "+": positive result; "-": negative result

4.3.3.3 Growth a different pH, NaCl concentration and temperature

The growth characteristics of the strains at different pH, NaCl concentration and temperature are shown in Table 4.14.

In general, the optimum ranges of external pH required for growth and survival of bacteria are between 4 and 8. In an unbalanced pH state, the normal cellular components are not synthesized and the cell does not divide and grow. In conditions of low pH growth may have stopped, but LAB cells may survive and still be metabolically active. In adverse conditions energy-requiring proton pump are used by the bacteria to either pump protons out of the cell (in low pH) or into the cell (in high pH) [148]. It was observed that all the strains

could grow at pH 7. However, at pH 9.6, *L. casei* TEZU309, *L. pentosus* TEZU174 and *L .plantarum* TEZU272 could not grow. At the low pH of 3.9, *L. casei* TEZU262, *L. casei* TEZU368, *P. pentosaceus* TEZU366, *L. casei* TEZU369, *L. paracasei* TEZU370 and *L. casei* TEZU371 displayed proper growth, *P. pentosaceus* TEZU469, *P. pentosaceus* TEZU473, *L. plantarum* TEZU272, *P. pentosaceus* TEZU427, *P. pentosaceus* TEZU482, *L. paracasei* TEZU372 and *L. casei* TEZU374 showed some growth, while the rest of the strains could not grow.

Most of the strains except *L. casei* TEZU309 could grow at 6.50 % NaCl, whereas at 10 % NaCl *L. pentosus* TEZU174, *L. pentosus* TEZU174, *P. pentosaceus* TEZU213, *L. plantarum* TEZU272, *P. pentosaceus* TEZU481and *P. pentosaceus* TEZU410 could not grow. At the highest concentration of 18 % NaCl only the strains *P. pentosaceus* TEZU427, *L. casei* TEZU369 and *L. paracasei* TEZU370 exhibited some growth. Osmotic stress causes a reduction in the amount of water available to a microorganism. LAB have minimum water activity (a_w) limits, below which they cannot grow. The limiting value of a_w depends on the type of solute viz. salts like NaCl, KCl etc. and sugars like glucose, sucrose etc. In case of a hyperosmotic shock (low a_w outside the cell) resulting in a loss of turgor pressure, the bacteria responds by osmoregulation. The bacteria also raise the levels of internal solute resulting in an increase in internal osmotic pressure. They may also cause certain changes in the membrane phospholipid and fatty acids [148].

At 10 ºC, most of the strains showed poor growth, and some of the strains could not grow. At 15 ºC only the strains *P. pentosaceus* TEZU410 and *P. pentosaceus* TEZU410 could not grow. Whereas, at 45 °C the strains *L. casei* TEZU309, *L. casei* TEZU468, *P. pentosaceus* TEZU451, *P. pentosaceus* TEZU473, *P. pentosaceus* TEZU366, *P. pentosaceus* TEZU298 and *P. pentosaceus* TEZU293 displayed proper growth. Growth of LAB below the optimum growth temperature results in growth cessation and changes in metabolic products and due to delays in enzyme activity and metabolic regulatory processes. Membrane leakage occurs when fluid components become gel-like and prevents the proteins from functioning correctly. The tolerant strains adapt to this situation by increasing the proportion of unsaturated fatty acids, thereby allowing the membrane to retain fluidity and prevent gel formation [148].

Table 4.14 Growth characteristics of the strains at different pH, NaCl concentration and temperature

Strain number	Growth at different pH			Growth at different salt (NaCl) concentration			Growth at different temperature		
	3.9	9.6	7	6.50%	10%	18%	10 °C	15 °C	45 °C
L. casei TEZU309	-	-	++	-	+(w)	-	+(w)	+	++
L. pentosus TEZU174	-	-	++	+	-	-	-	+	-
L. casei TEZU468	-	++	++	+(w)	+(w)	-	+(w)	++	++
P. pentosaceus TEZU469	+(w)	++	++	+(w)	+(w)	-	+(w)	+	+
P. pentosaceus TEZU470	-	++	++	+(w)	+(w)	-	+(w)	+	+(w)
P. pentosaceus TEZU472	-	++	++	+(w)	+(w)	-	+(w)	++	+(w)
P. pentosaceus TEZU473	+(w)	++	++	+(w)	+(w)	-	+(w)	++	++
P. pentosaceus TEZU199	-	++	++	++	+	-	-	+	-
P. pentosaceus TEZU366	+	++	++	+(w)	+(w)	-	+(w)	++	++
P. pentosaceus TEZU451	-	+	++	+(w)	+(w)	-	+(w)	++	++
P. pentosaceus TEZU213	-	++	++	++	-	-	-	+	-
P. pentosaceus TEZU298	-	+	++	-	-	-	+(w)	+	++
P. pentosaceus TEZU270	-	-	++	+(w)	-	-	-	+(w)	-
L. plantarum TEZU272	+(w)	-	++	++	-	-	-	+(w)	-
P. pentosaceus TEZU427	+(w)	++	++	++	+(w)	+(w)	+(w)	+(w)	+
P. pentosaceus TEZU481	-	+	++	+(w)	-	-	+(w)	++	+
P. pentosaceus TEZU482	+(w)	+	++	+(w)	+(w)	-	+(w)	++	+
P. pentosaceus TEZU486	-	+	++	++	++	-	-	-	+(w)
L. casei TEZU262	+	++	++	++	+	-	-	+(w)	-
L. plantarum TEZU263	-	++	++	+	+	-	+(w)	+	+
P. pentosaceus TEZU293	-	++	++	+(w)	+(w)	-	+	++	++
P. pentosaceus TEZU410	-	+	++	+	-	-	-	-	-
L. casei TEZU368	++	++	++	+(w)	-	-	+(w)	+	+
L. casei TEZU369	+	++	++	+(w)	-	+(w)	+(w)	+(w)	+(w)
L. paracasei TEZU370	+	++	++	+	-	+(w)	+(w)	+	+(w)
L. casei TEZU371	+	++	++	+(w)	+(w)	-	+(w)	+	+(w)
L. paracasei TEZU372	+(w)	++	++	+	+(w)	-	+(w)	+	++
L. casei TEZU373	-	++	++	+	+(w)	-	+(w)	+	-
L. casei TEZU374	+(w)	++	++	+	+(w)	-	+(w)	+	++

Note: "++": Good growth; "+": Growth; "+(w)":Poor growth; "-":No growth

4.3.3.4 Utilization of various sugars by the LAB strains

The results for carbohydrate fermentation by the LAB strains are show in Table 4.15. All the isolated LAB strains were found to utilize glucose, arabinose and maltose. Xylose and trehalose were not utilized by *L. plantarum* TEZU272, whereas, galactose was not utilized by *P. pentosaceus* TEZU427. Cellobiose was not utilized *L. casei* TEZU309, *P. pentosaceus* TEZU451 and *L. casei* TEZU374. Mannose, on the other hand was not utilized by *L. casei* TEZU309, *L. casei* TEZU374 and *P. pentosaceus* TEZU482. The utilization of sucrose and salicin varied among all the strains. Lactose was however not utilized by *L. casei* TEZU309, *L. casei* TEZU468, *P. pentosaceus* TEZU451, *P. pentosaceus* TEZU427, *P. pentosaceus* TEZU482. Raffinose, sorbitol and dulcitol were scarcely utilized by all the LAB strains.

Based on the results of the above tests, three strains of *Lactobacillus casei*, one strain of *Lactobacillus pentosus*, one strain of *Lactobacillus plantarum* and eight strains of *Pediococcus pentosaceus* were chosen for further studies

Table 4.15 Carbohydrate fermentation profile of the strains

Strain number	Lactose	Maltose	Sucrose	Xylose	Mannose	Trehalose	Galactose	Raffinose	Arabinose	Salicin	Sorbitol	Dulcitol	Glucose	Cellobiose
L. casei TEZU309	-	+	-	+(w)	-	++	+(w)	-	+(w)	-	-	++	++	-
L. pentosus TEZU174	+(w)	++	++	+	++	+(w)	++	-	++	++	+(w)	-	++	++
L. casei TEZU468	-	+(w)	-	+(w)	++	++	-	-	+(w)	-	-	-	++	+(w)
P. pentosaceus TEZU469	-	-	-	+	++	++	-	-	+	-	-	++	++	+(w)
P. pentosaceus TEZU470	-	-	-	+	++	++	+	-	+	+(w)	-	++	++	+(w)
P. pentosaceus TEZU472	-	+(w)	-	+	++	++	++	-	+	-	+	-	++	-
P. pentosaceus TEZU473	-	+	-	+(w)	++	-	-	-	+(w)	-	-	-	++	+(w)
P. pentosaceus TEZU199	+(w)	+	-	++	++	++	++	-	++	++	-	-	++	++
P. pentosaceus TEZU366	-	++	-	+	++	++	++	-	+(w)	-	-	++	++	+(w)
P. pentosaceus TEZU451	-	++	-	+(w)	-	++	++	-	+(w)	-	-	-	++	+(w)
P. pentosaceus TEZU213	+(w)	+	-	++	++	++	++	-	++	++	-	-	++	++
P. pentosaceus TEZU298	-	+	-	+(w)	+	++	+	-	+(w)	-	-	++	++	+(w)
P. pentosaceus TEZU270	+(w)	++	++	-	+	-	+(w)	-	-	-	-	-	++	++
L. plantarum TEZU272	++	++	++	-	+(w)	-	+(w)	-	+(w)	-	-	-	++	++
P. pentosaceus TEZU427	-	++	-	++	++	++	+	-	++	-	+	-	++	++
P. pentosaceus TEZU481	-	++	-	+(w)	-	++	++	-	+(w)	+(w)	-	++	++	+(w)
P. pentosaceus TEZU482	-	++	-	+	++	++	+	-	+(w)	-	-	-	++	-
P. pentosaceus TEZU486	-	++	-	+	++	++	+(w)	-	+(w)	-	-	++	++	+(w)
L. casei TEZU262	++	++	+(w)	++	++	++	++	-	++	++	+(w)	-	++	++
L. plantarum TEZU263	++	++	++	+	++	-	+(w)	-	-	-	-	-	++	++
P. pentosaceus TEZU293	-	++	-	+	-	++	++	-	+(w)	+(w)	-	++	++	+(w)
P. pentosaceus TEZU410	++	++	++	++	++	++	+	+	++	-	+	+	++	+
L. casei TEZU368	+(w)	++	-	+	++	++	++	-	+	+	+(w)	++	++	+(w)
L. casei TEZU369	-	-	-	+	++	++	-	-	+	++	-	++	++	+(w)
L. paracasei TEZU370	++	++	-	+(w)	-	++	++	-	+(w)	-	+	-	++	+(w)
L. casei TEZU371	++	++	-	+	-	++	++	-	+(w)	-	+	-	++	-
L. paracasei TEZU372	+(w)	++	-	+(w)	++	++	++	-	+(w)	+	-	++	++	+(w)
L. casei TEZU373	+(w)	-	-	+(w)	-	++	++	-	+(w)	-	-	-	++	-
L. casei TEZU374	+(w)	++	-	+(w)	-	++	++	-	+(w)	-	-	-	++	-

4.3.3.5 Antibotic susceptibility of the LAB strains

The results for antibiotic resistance test of the LAB strains are shown in Table 4.16. It was observed that all the studied LAB strains were susceptible towards the tested antibiotics in varying degrees. However none of the strains were found to be affected by kanamycin, while nine of the strains were also non susceptible by vancomycin. The antibiotic resistance of LAB strains is important to be assessed in order to limit the transmission of antibiotic resistance genes to unrelated pathogenic or opportunistic bacteria. Some LAB strains such as *L. sakei*, *L. curvatus* and *L. plantarum* involved in meat fermentation have been reported to show antibiotic resistance, which may be due to intrinsic factors or genetic determinants such as chloramphenicol acetyltransferase (*cat*-TC), erythromycin[*erm*(B)] and tetracycline [*tet*(M), *tet*(S)] resistance genes [144].

4.3.3.6 Antibiosis activity of the LAB strains

The results for antibiosis activity of the LAB strains are shown in Table 4.17. It was observed that all the LAB strains were highly antagonist against both *E. coli* and *S. aureus*. In both the cases, the zones of inhibition were above 30 mm, and some of the strains also exbihited zones of inhibition up to 40 mm. In case of *A. niger*, *P.pentosaceus* TEZU486 and *L.casei* TEZU374 showed the highest inhibition of growth, while all the other strains exhibited zones below 3 mm. Probiotic strains show antagonism against pathogenic bacteria either via antimicrobial substance production or competitive exclusion, and thus have an impact on the colonic flora. They may produce bacteriocins, low molecular weight metabolites such as hydrogen peroxide, lactic and acetic acid, certain aroma compounds and secondary metabolites. Probiotics have been found to exhibit inhibitory spectrum against many harmful organism like *Salmonella*, *Escherichia coli*, *Clostridium* and *Helicobacter* [149]. The antimicrobial activity sown against the potentially pathogenic Gram-negative and Gram-positive bacteria also indicated that these strains can reduce the number of undesired microorganisms in fermented food products and make them fit for human consumption.

Table 4.16 Antibiotic resistance test of the strains

Strain	Zone of inhibition (mm)											
	AM10	C30	CIP5	E15	P10	GM50	K30	RA30	S25	TE10	VA10	LZD30
L. casei TEZU309	29	32	12	34	30	18	0	27	0	18	10	30
L. pentosus TEZU174	34	35	14	35	35	22	0	30	16	27	0	37
L. casei TEZU468	26	37	12	34	30	24	0	28	13	27	0	37
P. pentosaceus TEZU199	24	32	11	31	26	15	0	29	0	23	0	34
P. pentosaceus TEZU451	28	35	13	32	26	20	0	31	11	26	10	35
P. pentosaceus TEZU213	22	24	12	32	30	21	0	30	10	24	0	35
L .plantarum TEZU272	36	38	17	37	35	26	0	32	13	28	0	39
P. pentosaceus TEZU427	22	32	12	32	24	19	0	25	10	26	0	32
P. pentosaceus TEZU481	23	32	10	30	25	19	0	28	11	23	0	34
P. pentosaceus TEZU482	24	32	10	31	28	19	0	28	10	24	10	33
P. pentosaceus TEZU486	24	32	11	31	28	18	0	28	10	22	10	34
*L. casei*TEZU262	25	28	33	30	31	24	0	31	16	33	0	38
P. pentosaceus TEZU410	24	36	12	32	30	20	0	28	10	25	13	35
L. casei TEZU374	23	28	17	30	31	19	0	29	12	29	0	31

Note: AM10: Ampicillin (10 mcg); C30 - Chloramphenicol (30 mcg); CIP5: Ciprofloxacin (5 mcg); E15: Erythromycin (15 mcg); P10: Penicillin (10 mcg); GM50: Gentamycin (50 mcg); K30: Kanamycin (30 mcg); RA30: Rifampicin (30 mcg); S25: Streptomycin (25 mcg); TE10: Tetracycline (10 mcg); VA10: Vancomycin (10 mcg); LZD30: Linezolid (30 mcg)

Table 4.17 Antibiosis activity of the strains

Strain	Zone of inhibition (mm±SD)		
	E. coli	*S. aureus*	*A. niger*
L. casei TEZU309	32±1.56	34±1.44	2.87±0.31
L. pentosus TEZU174	40±2.22	40±1.63	0.87±0.42
L.casei TEZU468	39±2.36	36±1.19	2.80±0.20
P. pentosaceus TEZU199	30±1.14	40±2.18	1.13±0.42
P. pentosaceus TEZU451	40±2.22	34±1.54	0.67±0.31
P. pentosaceus TEZU213	31±1.41	40±1.56	2.07±0.42
L .plantarum TEZU272	34±1.15	40+2.51	1.40±0.40
P.pentosaceus TEZU427	33±1.06	40±2.78	2.07±0.70
P.pentosaceus TEZU481	31±2.42	39±2.72	3.40±0.20
P.pentosaceus TEZU482	35±1.86	35±1.52	2.80±0.20
P.pentosaceus TEZU486	34±1.22	39±1.94	4.33±0.12
*L. casei*TEZU262	40±2.56	38±1.93	1.47±0.42
P.pentosaceus TEZU410	32±1.16	40±2.64	0.00±0.00
L.casei TEZU374	34±1.31	34±1.57	4.20±0.60

Note: Values are mean (n=3) ± standard deviation (SD)

4.3.3.7 Antioxidant activity of the LAB strains

ROS are produced during passage of food through the GI tract, leading to oxidative damage which forms part in the pathogenesis of cancer, cirrhosis, atherosclerosis and other chronic diseases [150]. The antioxidative activity probiotic LAB decreases the risk of accumulation of ROS in the GI tract. The antioxidant mechanisms include ROS scavenging, metal ion chelation, enzyme inhibition, reduction activity and inhibition of ascorbate autoxidation [151]. The important components of their defence against ROS damage are the antioxidative enzymes, reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidise (GSHPx) and catalase (CAT) [152]. DPPH free radical accepts electrons or hydrogen atoms from antioxidant substances and converts them into irreversible stable molecules which can be detected spectrophotometrically [153]. The results for antioxidant activities of the LAB strains are shown in Table 4.18. In the test for resistance to H_2O_2, good results were shown by the strains *P. pentosaceus* TEZU481, *P. pentosaceus* TEZU482 and *L. casei* TEZU374, which maintained OD of above 1 even at concentration of 1.0 mM H_2O_2. High •OH radical scavenging activity was shown by *L. casei* TEZU468 (48.96 %), *P. pentosaceus* TEZU451 (49.07 %), *P. pentosaceus* TEZU481 (55.41 %), *P. pentosaceus* TEZU482 (50.04 %) and *L. casei* TEZU374 (60.54 %). In the DPPH radical scavenging activity also it was observed that that strains *L. casei* TEZU468, *P. pentosaceus* TEZU451, *P.*

pentosaceus TEZU481, *P. pentosaceus* TEZU482, *P. pentosaceus* TEZU486 and *L. casei* TEZU374 exhibited activity above 50 %.

The antioxidative activity of LAB isolated from Korean healthy infant feces and kimchi was studied by Ji et al. [169] and found effective scavenging activity for DPPH and ABTS radicals. Reports of DPPH free radical scaveninging by LAB also includes that of *Lactobacillus plantarum*7FM10 isolated from the traditional Japanese food narezushi [154] and that of *Lactobacillus brevis* BJ20 in a fermented sea tangle solution [155]. High survival time of two antioxidative strains *Lactobacillus fermentum*, E-3 and E-18 isolated from intestinal microflora of a healthy child in the presence of ROS like hydrogen peroxide, superoxide anions and hydroxyl radicals was found by Kullisaar et al. [152]. Li et al. [45] also found that the strain *L. plantarum* C88 isolated from traditional Chinese fermented food showed high *in vitro* scavenging activity against DPPH free radicals at a dose of 10^{10} CFU/ml with inhibition rate of 53.05 %. They also concluded that the cell-surface proteins or polysaccharides of this strain were involved in the antioxidant activity.

4.3.3.8 Tolerance of the LAB strains to acid

The transit time for any bacteria in the human GIT can be from less than 1 h to 4 h depending on various conditions. Therefore their survival in the gastric juice depends on their ability to tolerate low pH. Hence, strains of LAB intended for probiotic purposes should be screened for tolerance to low pH [144]. Weak acids affect the cells' ability to maintain pH homeostasis, thereby disrupting substrate transport and inhibiting metabolic pathways. The tolerance may be developed when the microbes are exposed to a mild concentration of a weak acid, rendering them resistant to a stronger dose [148]. Acid tolerance of the LAB strains at two different pH levels are shown in Table 4.19. At the pH of 2.5 high resistance was shown by the strains *L. pentosus* TEZU174, *P. pentosaceus* TEZU199, *P. pentosaceus* TEZU199, *L. plantarum* TEZU272, *L. casei* TEZU262, *P. pentosaceus* TEZU410 and *L.casei* TEZU374. *L. pentosus* TEZU174 could maintain a count of 4 log CFU even till 48 h. At the pH of 1.5, *L. pentosus* TEZU174 and *P. pentosaceus* TEZU199 could survive till the 4th hour with counts of 5 log CFU.

Table 4.18 Antioxidant activities of the strains

Strain	Resistance to H_2O_2 (OD at 600 nm)				•OH radical scavenging (%)	DPPH radical scavenging (%)
	0.0 mM	0.4 mM	0.7 mM	1.0 mM		
L. casei TEZU309	2.22±0.09	1.56±0.17	1.08±0.12	0.09±0.02	33.64±1.22	42.41±0.80
L. pentosus TEZU174	2.23±0.12	1.47±0.16	1.11±0.18	0.17±0.03	24.56±1.87	34.83±1.90
L. casei TEZU468	2.22±0.20	1.84±0.08	1.07±0.14	0.62±0.08	48.96±2.05	59.12±1.38
P. pentosaceus TEZU199	2.25±0.14	0.60±0.05	0.33±0.08	0.14±0.04	6.54±0.24	8.98±2.29
P. pentosaceus TEZU451	2.21±0.19	1.50±0.11	1.11±0.10	0.29±0.04	49.07±1.60	56.90±0.48
P. pentosaceus TEZU213	2.24±0.23	0.82±0.13	0.53±0.07	0.38±0.02	16.64±1.33	28.45±1.27
L .plantarum TEZU272	2.21±0.19	1.37±0.19	0.98±0.06	0.60±0.07	28.47±1.10	32.83±2.53
P. pentosaceus TEZU427	2.23±0.16	1.40±0.06	1.04±0.10	0.03±0.01	19.70±1.16	37.85±0.56
P. pentosaceus TEZU481	2.24±0.07	1.94±0.14	1.60±0.13	1.51±0.08	55.41±2.04	70.18±2.13
P. pentosaceus TEZU482	2.23±0.42	1.90±0.20	1.67±0.13	1.73±0.08	50.04±1.87	71.62±0.92
P. pentosaceus TEZU486	2.21±0.09	1.27±0.12	0.85±0.08	0.22±0.07	34.01±2.24	51.64±0.35
L. casei TEZU262	2.22±0.36	0.97±0.17	0.59±0.07	0.27±0.05	10.23±0.64	12.66±1.01
P. pentosaceus TEZU410	2.25±0.22	1.09±0.12	0.88±0.04	0.51±0.03	19.65±0.30	22.56±0.47
L. casei TEZU374	2.24±0.10	1.93±0.18	1.68±0.19	1.79±0.08	60.54±1.57	76.74±4.07

Note: Values are mean (n=3) ± standard deviation (SD)

Table 4.19 Acid tolerance of strains at two different pH levels

Strain	Change in count (log CFU) with time															
	pH 2.5											pH 1.5				
	0 h	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	24 h	48 h	0 h	1 h	2 h	3 h	4 h
L. casei TEZU309	9.30	8.90	8.43	8.26	5.95	5.00	5.00	5.00	5.00	0.00	0.00	9.30	7.00	0.00	0.00	0.00
L. pentosus TEZU174	8.30	8.03	7.94	7.92	7.77	7.67	7.57	7.08	6.60	5.78	4.00	8.30	7.00	6.48	5.16	5.00
L. casei TEZU468	9.48	6.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.48	0.00	0.00	0.00	0. 00
P. pentosaceus TEZU199	8.60	7.99	7.45	7.20	7.15	7.18	6.60	5.78	5.60	0.00	0.00	8.60	7.85	6.56	5.64	5.00
P. pentosaceus TEZU451	9.48	7.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.48	0.00	0.00	0.00	0.00
P. pentosaceus TEZU213	8.45	8.39	8.22	8.16	8.04	7.84	7.75	7.70	7.48	0.00	0.00	8.45	0.00	0.00	0.00	0.00
L .plantarum TEZU272	9.60	9.60	9.48	9.48	9.47	9.40	9.40	9.08	9.04	0.00	0.00	9.60	8.25	7.36	7.00	0.00
P. pentosaceus TEZU427	9.60	9.30	6.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.60	0.00	0.00	0.00	0.00
P. pentosaceus TEZU481	9.04	5.48	5.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.04	0.00	0.00	0.00	0.00
P. pentosaceus TEZU482	9.30	7.90	7.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.30	0.00	0.00	0.00	0.00
P. pentosaceus TEZU486	7.48	7.00	6.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.48	0.00	0.00	0.00	0.00
L. casei TEZU262	9.07	9.02	8.59	8.54	8.22	7.95	7.52	7.11	7.08	0.00	0.00	9.07	6.29	5.00	0.00	0.00
P. pentosaceus TEZU410	9.60	9.40	9.30	8.04	7.60	7.30	7.30	7.00	7.00	4.30	0.00	9.60	9.60	7.11	7.00	0.00
L. casei TEZU374	9.60	9.60	9.48	9.48	9.45	9.40	9.30	9.30	9.00	5.08	0.00	9.60	0.00	0.00	0.00	0.00

4.3.3.9 Tolerance of the LAB strains to bile salts

After surviving the acidic conditions of the stomach, the ingested bacteria should be able to tolerate the detergent-like emulsifying effect of the bile salts released into the duodenum. The may achieve it by hydrolysing them with bile salt hydrolase enzymes (BSHs), thereby decreasing their solubility [144]. The results for tolerance of the LAB strains to bile salt are shown in Table 4.20. It was seen that most of LAB strains were highly tolerant against bile salt. Except, *L. casei* TEZU262 and *P. pentosaceus* TEZU410 all the strains could maintain a count of above 7 log CFU up to a final concentration of 15 % bile salt.

Table 4.20 Tolerance of the strains to bile salt

Strain	Count (log CFU) in different bile concentrations			
	8%	10%	12%	14%
L. casei TEZU309	7.80	7.56	7.36	6.95
L. pentosus TEZU174	7.89	7.83	7.83	7.81
L. casei TEZU468	7.86	7.79	7.77	7.52
P. pentosaceus TEZU199	7.28	7.26	6.95	6.70
P. pentosaceus TEZU451	7.70	7.67	7.15	6.78
P. pentosaceus TEZU213	7.30	7.04	6.90	6.90
L. plantarum TEZU272	7.77	7.76	7.72	7.70
P. pentosaceus TEZU427	7.91	7.83	7.70	7.28
P. pentosaceus TEZU481	8.06	7.90	7.60	7.11
P. pentosaceus TEZU482	7.99	7.86	7.53	7.30
P. pentosaceus TEZU486	7.86	7.83	7.48	7.23
*L. casei*TEZU262	3.00	0.00	0.00	0.00
P. pentosaceus TEZU410	7.95	4.73	4.64	4.41
L. casei TEZU374	7.40	7.26	7.20	7.20

4.3.3.10 Cell Aggregation and microbial adhesion to solvents (MATS)

The adhesion ability of LAB such as hydrophobicity and the production of exopolysaccharides are involved in the modulation of host immune response. The MATS technique is used because it makes it helps in determining not only hydrophobicity but also the electron donor or electron receptor character of the cell surface, which is another method to explore adhesion [156]. Adherence of LAB to mucosal surfaces and intestinal epithelium is related to the cell surface characteristics. Whereas, autoaggregation is the ability of clumping of the cells of the same strain to form multicellular aggregates [157]. In order to exert beneficial

probiotic effects, LAB need to achieve an adequate mass through aggregation. Autoaggregation correlates adhesion, which is a complex process involving non-specific (hydrophobicity) and specific ligand-receptor mechanisms [158]. The surface properties of LAB play an important role in the adhesion of the bacteria to the gastrointestinal epithelium which is considered to be a prerequisite for the exclusion of enteropathogenic bacteria or immunomodulation of the host [159]. The peptidoglycan layer of the cell wall of LAB is covered by a variety of substances like lipoteichoic acids, neutral and acidic polysaccharides, and surface proteins [159] and their adhesive properties include different features like passive forces, electrostatic interactions and hydrophobic steric forces [157].

The results for cellular aggregation of the strains are shown in Table 4.21. It was observed that *L. casei* TEZU309 and L. *casei* TEZU262 could obtain 100 % aggregation within 5 h of incubation. *L. pentosus* TEZU174 and *L .plantarum* TEZU272 could attain 100 % aggregation within the 8^{th} hour, while the rest of the strains attained the same on the 19^{th} hour. Adhesion of the strains to different solvents after 1 h incubation is shown in Table 4.22. It was observed that the percentage of adhesion for all the strains was more in ethyl acetate, followed by chloroform and xylene. The highest percentage of adhesion was shown by *L. casei* TEZU309, *L. pentosus* TEZU174, *P .pentosaceus* TEZU427, *L. casei*TEZU262 and *L.casei* TEZU374. The results hence indicate that the isolated strains have moderate adhesion capacity [156]. Certain strains also showed high adherence scores for chloroform, which is a monopolar solvent. This may be due to the basic properties (Lewis base) of the bacterial cell surface which is again related to the presence of a carboxylic group [156]. In similar studies, Collado et al. [158] studied the cell surface properties of dadih lactic acid bacteria strains for adhesion to hydrocarbons and aggregation abilities and found significant differences in cell surface properties among the tested natural LAB strains.

Table 4.21 Cellular aggregation of the strains

Strain	The % aggregation with increasing time										
	0 h	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	9 h	10 h
L. casei TEZU309	0.00	2.50	17.50	70.05	95.84	100.00	100.00	100.00	100.00	100.00	100.00
L. pentosus TEZU174	0.00	17.39	26.09	34.78	36.96	41.30	52.17	82.61	100.00	100.00	100.00
L. casei TEZU468	0.00	1.23	7.58	14.26	24.16	33.11	48.97	58.16	76.25	82.56	100.00
P. pentosaceus TEZU199	0.00	2.68	10.56	19.54	28.44	39.81	51.48	66.93	74.86	87.41	100.00
P. pentosaceus TEZU451	0.00	1.54	8.37	16.24	25.87	40.56	59.87	62.18	77.12	92.21	100.00
P. pentosaceus TEZU213	0.00	1.71	6.09	12.57	20.36	30.09	45.30	54.41	66.38	78.56	100.00
L .plantarum TEZU272	0.00	20.51	30.77	43.59	28.21	46.15	56.41	76.92	100.00	100.00	100.00
P. pentosaceus TEZU427	0.00	1.61	6.71	12.49	28.09	39.48	55.61	60.34	72.48	96.35	100.00
P. pentosaceus TEZU481	0.00	2.56	8.66	17.43	20.56	29.81	40.06	58.33	66.45	78.84	100.00
P. pentosaceus TEZU482	0.00	1.73	9.27	19.04	26.88	39.27	47.39	66.91	68.82	75.62	100.00
P. pentosaceus TEZU486	0.00	1.67	8.44	16.98	21.45	33.22	48.09	50.19	76.41	80.57	100.00
*L. casei*TEZU262	0.00	20.00	30.25	40.00	60.74	100.00	100.00	100.00	100.00	100.00	100.00
P. pentosaceus TEZU410	0.00	6.67	8.89	11.11	11.11	13.33	13.33	20.22	57.78	84.25	100.00
L. casei TEZU374	0.00	7.50	27.50	12.50	22.50	22.50	25.27	37.50	80.60	100.00	100.00

Table 4.22 Adhesion of the strains to different solvents after 1 h incubation

Strain	% adhesion		
	Xylene	Chloroform	Ethyl acetate
L. casei TEZU309	35.66	51.66	72.51
L. pentosus TEZU174	32.79	26.21	24.95
L. casei TEZU468	0.35	12.55	19.52
P. pentosaceus TEZU199	0.66	11.36	17.42
P. pentosaceus TEZU451	1.24	15.42	17.08
P. pentosaceus TEZU213	1.68	18.18	19.64
L .plantarum TEZU272	12.47	20.98	24.79
P .pentosaceus TEZU427	34.20	42.80	96.90
P. pentosaceus TEZU481	1.06	12.17	06.70
P. pentosaceus TEZU482	5.42	13.94	21.07
P. pentosaceus TEZU486	8.16	14.72	24.63
*L. casei*TEZU262	43.76	35.05	45.14
P. pentosaceus TEZU410	1.77	32.33	11.48
L. casei TEZU374	14.36	23.76	37.61

4.3.3.11 Surface associated adhesion proteins of two *Lactobacillus casei* strains

4.3.3.11.1 Adhesion properties with and without the surface proteins

The cellular autoaggregation and adhesion to solvents of the strains were assessed, with and without the surface proteins (removal by LiCl treatment). Cellular aggregation and adhesion to solvents is shown in Table 4.23. *L. casei* TEZU309 attained 100 % aggregation in the 6th hour, while *L. casei* TEZU374 attained 100 % aggregation in the 9th hour. On the other hand, the LiCl treated *L. casei* TEZU309-Li and *L. casei* TEZU374-Li could attain only 14.56 % and 18.59 % autoaggregation respectively till the 10th hour. Adhesion of the strains to different solvents after 1 h incubation is shown in Table 4.24. It was observed that their adhesion to organic solvents were also reduced many folds when the surface proteins were removed. Thus, the strains of LAB showed hydrophobicity. A high degree of hydrophobicity indicates the potential of adhesion to human GIT epithelial cells and colonization, one of the main criteria in the selection of potential probiotic cultures. A percentage of hydrophobicity greater than 70 % is arbitrarily classified as hydrophobic [160].

Table 4.23 Cellular aggregation and adhesion to solvents

Strain	The % aggregation with increasing time						
	0 h	2 h	4 h	6 h	8 h	9 h	10 h
L. casei TEZU309	0.00	17.50	95.84	100.00	100.00	100.00	100.00
L. casei TEZU309 - Li	0.00	2.00	5.56	8.94	10.23	11.21	14.56
L. casei TEZU374	0.00	27.50	22.50	25.27	80.60	100.00	00.00
L. casei TEZU374 - Li	0.00	2.70	6.22	8.59	12.45	16.47	18.59

Table 4.24 Adhesion of the strains to different solvents after 1 h incubation

Strain	% adhesion		
	Xylene	Chloroform	Ethyl acetate
L. casei TEZU309	35.66	51.66	72.51
L. casei TEZU309 - Li	14.26	15.21	17.51
L. caseiTEZU374	14.36	23.76	37.61
L. caseiTEZU374 - Li	3.49	2.54	5.88

4.3.3.11.2 Adherence of the *L. casei* strains to Caco-2 and HT-29 intestinal epithelial cells

In order to exert their beneficial effects, probiotic bacterial cells need to colonise the ileum by adhering to the mucosa. Hence, the LAB strains should be screened for adherence and persistence in the human GIT [144]. Adherence of *L. casei* TEZU309 and *L. casei* TEZU309 - Li cells to CaCo-2 cells are shown in Fig 4.15 and 4.16 respectively. Adherence of *L. casei* TEZU374 and *L. casei* TEZU374 - Li cells to CaCo-2 cells are shown in Fig 4.17 and 4.18 respectively. Fairly good adherence was observed in case of the cells whose surface proteins were not removed. The count of the *L. casei* cells after plating in MRS media after adhesion study is shown in Table 4.25. It was seen that 28.65 % of *L. casei* TEZU309 and 22.74 % of *L. casei* TEZU374 cells adhered to the intestinal CaCo-2 cells after washing. Whereas, only about 2 % of the LiCl treated cells of both the strains could adhere.

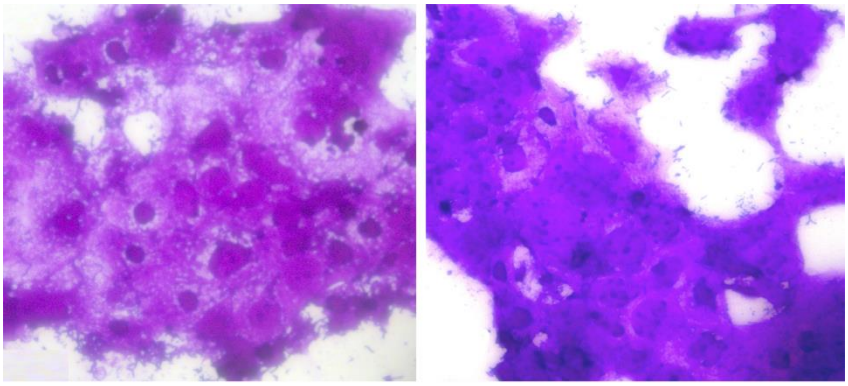

Fig 4.15 Adherence of *L. casei* TEZU309 to CaCo-2 cells

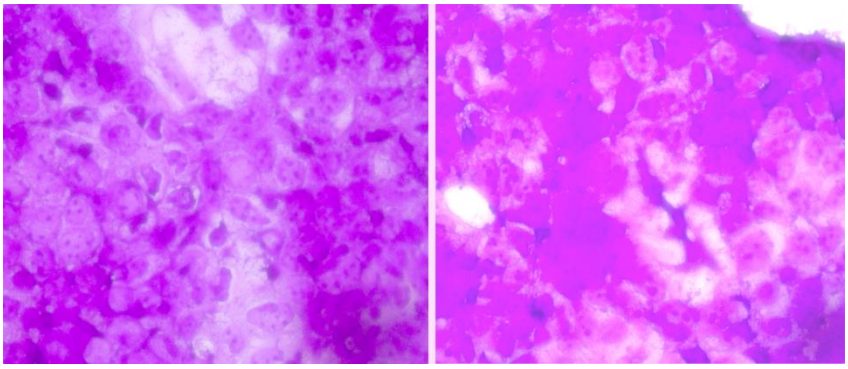

Fig 4.16 Adherence of *L. casei* TEZU309 - Li to CaCo-2 cells

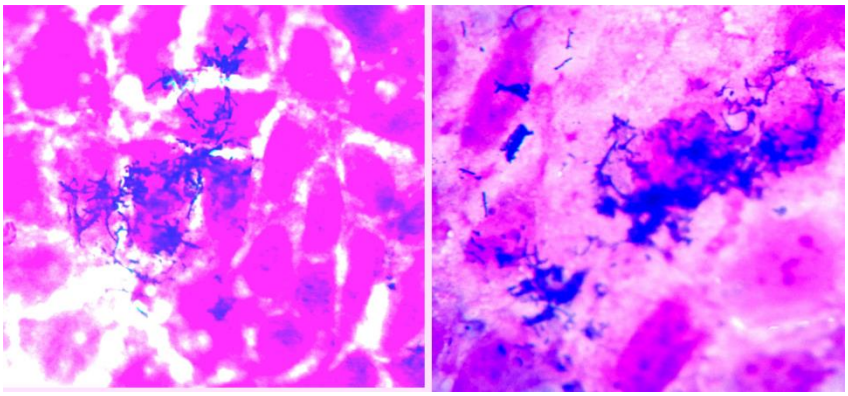

Fig 4.17 Adherence of *L. casei* TEZU374 to CaCo-2 cells

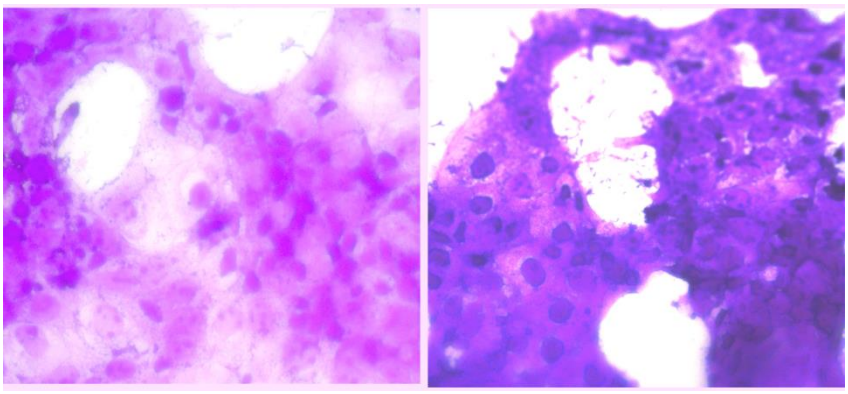

Fig 4.18 Adherence of *L. casei* TEZU374-Li to CaCo-2 cells

Table 4.25 Count of the *L. casei* cells in MRS media after adhesion study

Strain	% adhesion
L. casei TEZU309	28.65%
L. casei TEZU309 - Li	2.11%
L. casei TEZU374	22.74%
L. casei TEZU374 - Li	2.09%

4.3.3.11.3 Imaging of the cell surfaces by transmission electron microscopy (TEM)

The TEM images for *Lactobacillus casei* TEZU309 and *Lactobacillus casei* TEZU374 are shown in Fig 4.19 and 4.20 respectively. Imaging of the cell surfaces by transmission electron microscopy (TEM) revealed the presence of a distinct layer above the cell surface, relating to the S-layer proteins.

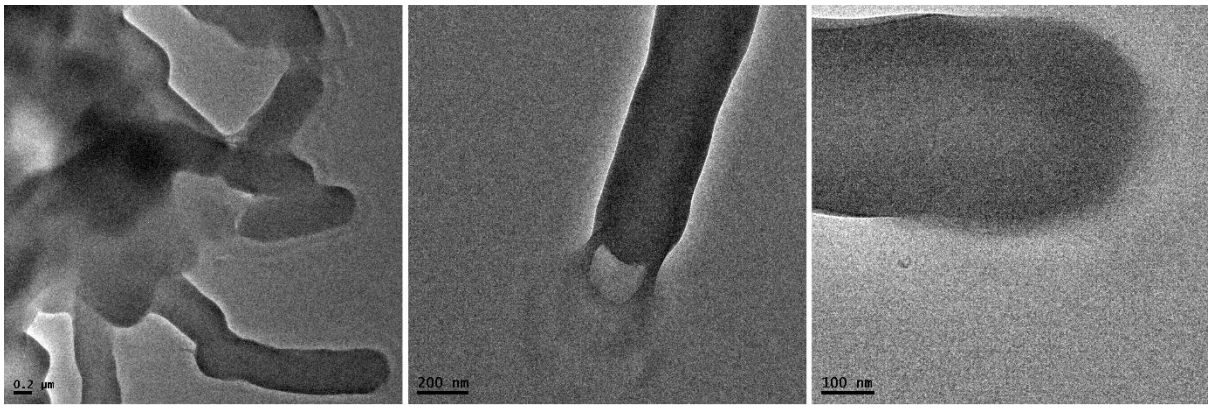

Fig 4.19 TEM image of *L. casei* TEZU309 cells

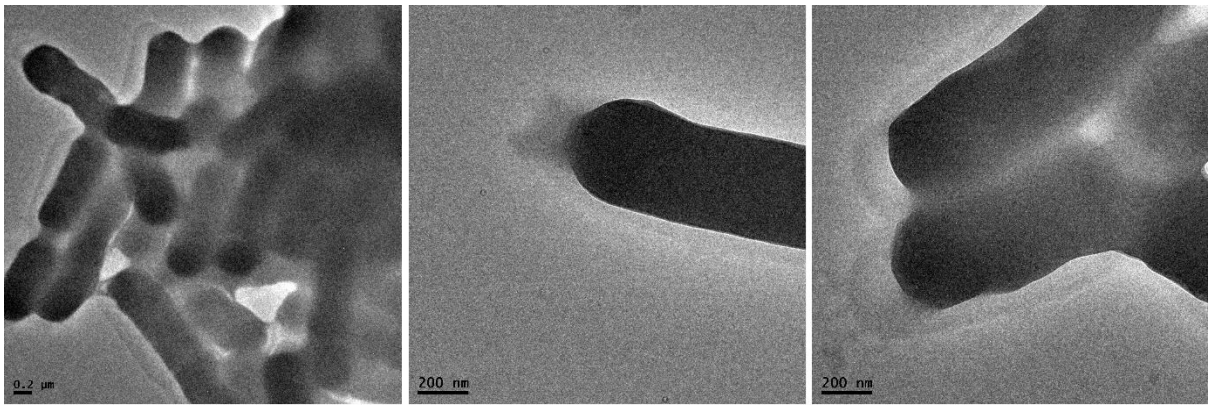

Fig 4.20 TEM image of *L. casei* TEZU374 cells

4.3.3.11.4 Determination of the surfaces associated proteins by SDS-PAGE

SDS-PAGE gel of the surface associated proteins of *L. casei* TEZU309, *L. casei* TEZU309 – Li, *L. casei* TEZU374 and *L. casei* TEZU374 - Li are shown in Fig 4.21. SDS-PAGE revealed the molecular weights of the major surface associated proteins to be of 52 Kda and 51 Kda in *L. casei* TEZU309 and *L. casei* TEZU374 respectively.

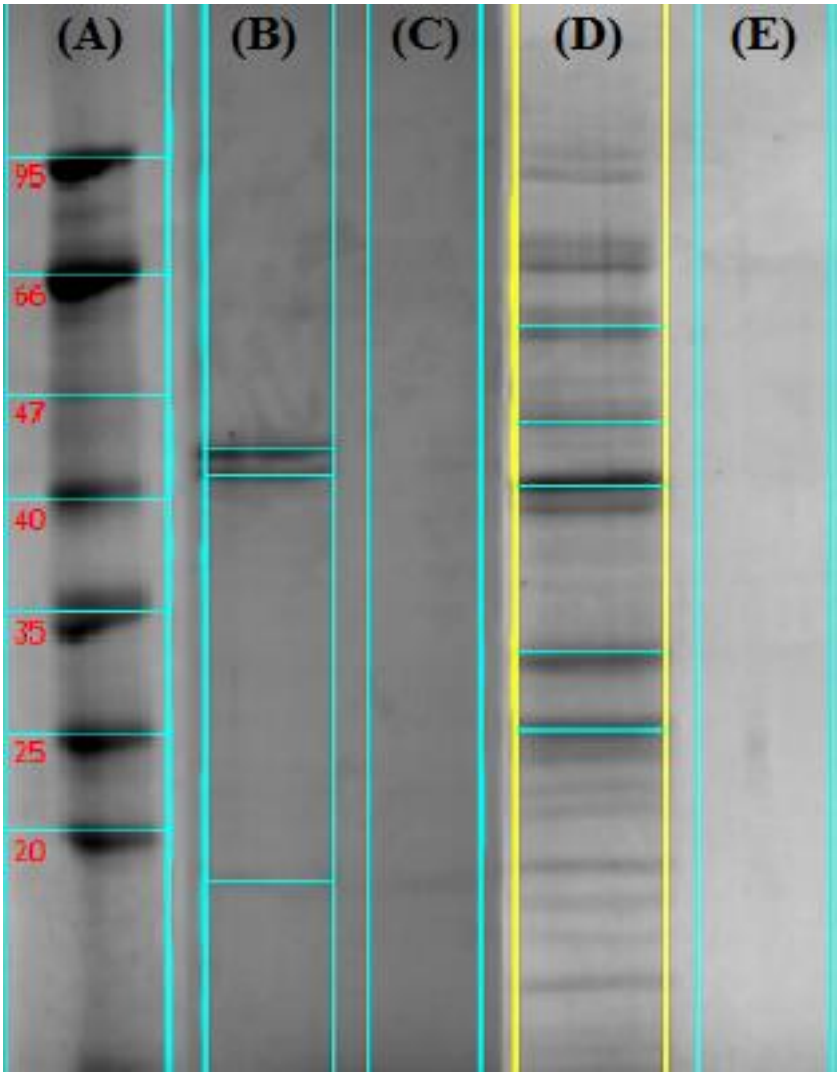

Fig 4.21 SDS-PAGE gel of the surface associated proteins. A – Protein marker, B – *L. casei* TEZU309, C - *L. casei* TEZU309 – Li, D- *L. casei* TEZU374, E - *L. casei* TEZU374 - Li

4.3.3.12 Utilization of different carbohydrates for production of organic acids by the LAB strains

4.3.3.12.1 Production of various organic acids by utilizing D-glucose as carbon source

The HPLC chromatograms for the detection of various organic acids by utilizing D-glucose are shown in Fig 4.23 and the results are shown in Table 4.26. Lactic acid and acetic acid were the major organic acids produced by utilization of glucose by all the strains. *L. casei* TEZU468 produced the highest amount of lactic acid (5863.37 ppm) and acetic acid (14358.46 ppm), oxalic acid and propionic acid were also variably produced by all the strains in concentration ranging from 71.93 ppm to 1695.42 ppm and 392.99 ppm to 1276.73 ppm respectively. Succinic acid in high content (13259.83 ppm) was found to be produced by only one strain i.e *P. pentosaceus* TEZU451. Low content (less than 100 ppm) of butyric acid, maelic acid and formic acid were also produced by most of the strains. The production of tartaric acid and citric acid was scarce among all the strains whereas pyruvic acid was not produced by any. Gluconic acid on the other hand was produced in high amount ranging from 3913.61 ppm to 66992.14 by some of the strains.

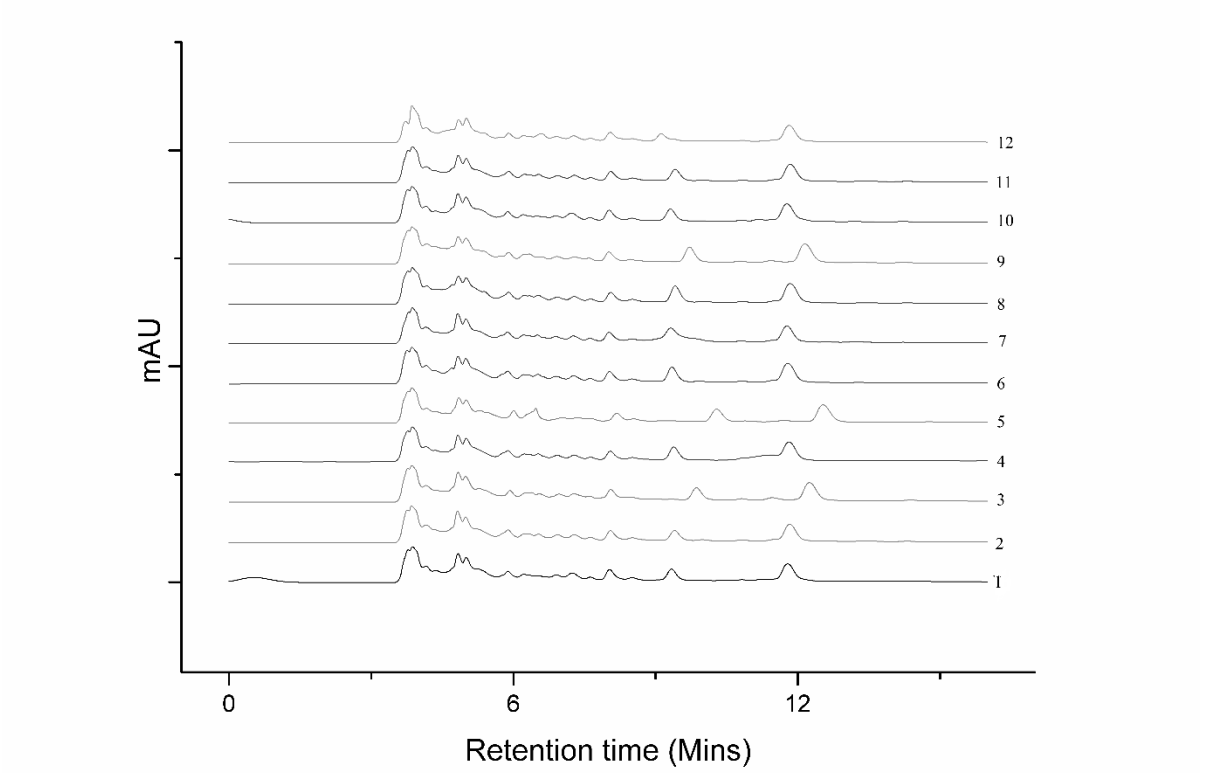

Fig. 4.23 HPLC chromatograms showing the production of different organic acids by utilization of D-glucose

Table 4.26 Production of different organic acids by utilization of glucose

Strain	Concentration of organic acid (ppm) in the culture media											
	Gluconic acid	Oxalic acid	Pyruvic acid	Formic acid	Tartaric acid	Malic acid	Lactic acid	Acetic acid	Citric acid	Succinic acid	Propionic acid	Butyric acid
L. casei TEZU309	66573.38	74.57	--	77.93	--	7.29	5539.49	12282.55	--	--	456.10	30.60
L. pentosus TEZU174	3913.61	1284.45	--	--	--	9.52	5831.28	11310.94	--	--	461.30	55.67
L. casei TEZU468	--	1365.03	--	--	--	17.87	5863.37	14358.46	--	--	1276.73	--
P. pentosaceus TEZU199	4359.58	1226.48	--	41.56	--	14.53	4154.47	10392.92	--	--	398.01	46.41
P. pentosaceus TEZU451	--	1445.65	--	44.77	--	88.50	3827.58	13376.98	73.74	13259.83	872.77	--
P. pentosaceus TEZU213	21207.27	972.49	--	--	39.93	--	4593.77	8853.24	--	--	481.86	41.11
L. plantarum TEZU272	--	1292.71	--	--	148.76	21.20	5787.37	11772.75	--	--	703.13	63.91
P. pentosaceus TEZU427	74325.15	96.33	--	50.73	--	--	6399.71	12608.68	93.98	--	431.46	56.87
P. pentosaceus TEZU482	--	1695.42	--	37.02	--	23.18	5103.78	13576.85	76.92	--	882.65	--
*L. casei*TEZU262	4065.66	1273.94	--	75.27	--	--	4907.99	11622.48	--	--	466.09	26.89
P. pentosaceus TEZU410	66992.14	71.93	--	22.92	--	11.44	5420.08	11496.62	--	--	412.95	42.57
L. casei TEZU374	--	1078.78	--	--	--	--	5567.58	10157.41	86.89	--	392.99	19.33

4.3.3.12.2 Production of various organic acids by utilizing maltose as carbon source

The HPLC chromatograms for the detection of various organic acids by utilizing maltose are shown in Fig 4.24 and the results are shown in Table 4.27. Lactic acid, acetic acid and succinic acid were the major organic acids produced by all the strains by utilizing maltose except *L. pentosaceus* TEZU174 and *L. casei* TEZU468 which did not produce acetic acid. *L. casei* TEZU468 was the highest producer of lactic acid (7868.37 ppm), followed by *P. pentosaceus* TEZU451 (5633.02 ppm) and *L. casei* TEZU309 (5592.89 ppm), *L. casei* TEZU374 was the highest producer of acetic acid (15753.03 ppm) followed by *P. pentosaceus* TEZU482 (12911.40 ppm) and *P. pentosaceus* TEZU451 (12246.54 ppm). The production of succinic ranged from 30031.07 ppm in *L. casei* TEZU262 to 2194.02 ppm in *L. pentosus* TEZU174. Tartaric acid and maelic acid were produced in concentration below 500 ppm by most of the strains. *L. pentosus* TEZU174 was the highest producer of formic acid (4732.16 ppm) while *L. casei* TEZU468 was the highest producer of citric acid (1176.96 ppm). Oxalic acid was produced by all the strains in concentration ranging from 3.076 ppm to 1052.6 ppm (*L. plantarum* TEZU272), while pyruvic acid and butyric acid were not produced by any. Both gluconic acid propionic acid were produced in concentration above 3000 ppm by some of the strains where *L. casei* TEZU468 and *P. pentosaceus* TEZU482 were highest producer of gluconic acid and propionic acid respectively.

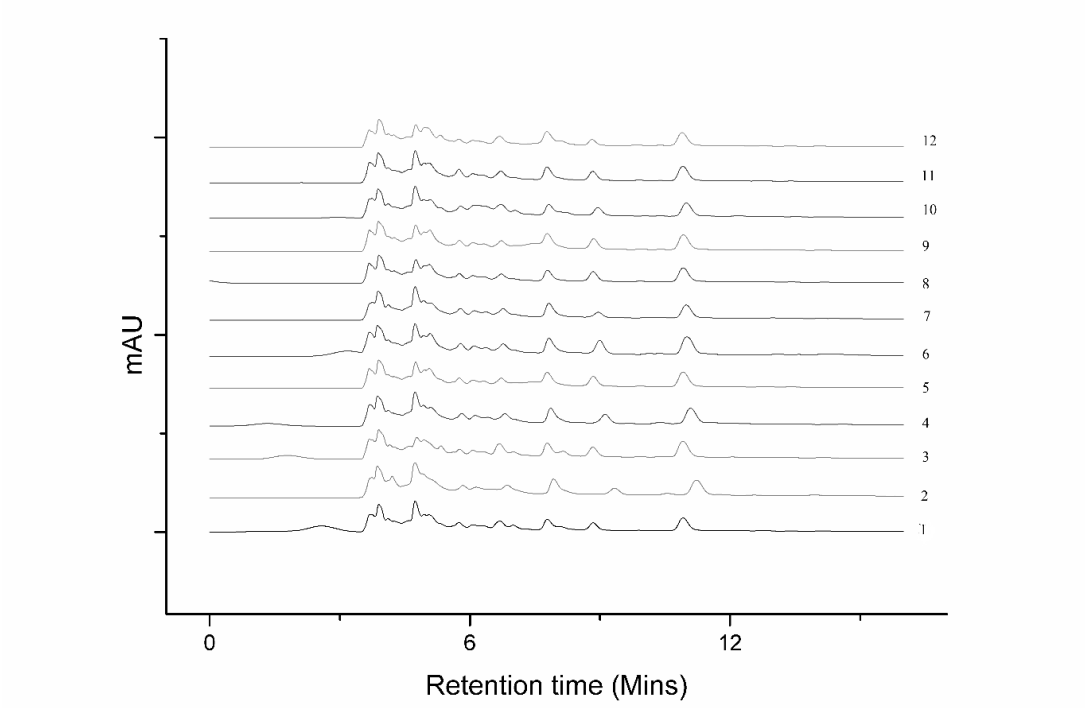

Fig. 4.24 HPLC chromatograms showing the production of different organic acids by utilization of maltose

Table 4.27 Production of different organic acids by utilization of maltose

Strain	Concentration of organic acid (ppm) in the culture media											
	Gluconic acid	Oxalic acid	Pyruvic acid	Formic acid	Tartaric acid	Malic acid	Lactic acid	Acetic acid	Citric acid	Succinic acid	Propionic acid	Butyric acid
L. casei TEZU309	49291.14	99.10	--	47.00	68.45	165.23	5592.89	623.91	--	12719.55	5339.92	--
L. pentosus TEZU174	3994.40	769.38	--	4732.16	--	--	72.67	--	--	2194.20	--	--
L. casei TEZU468	42557.14	3.076	--	--	--	414.22	7868.37	--	1176.96	3473.47	5044.04	--
P. pentosaceus TEZU199	--	907.38	--	--	57.63	--	120.10	666.43	--	18614.58	--	--
P. pentosaceus TEZU451	--	804.00	--	61.91	18.35	237.56	5633.02	12246.54	--	15337.96	11059.44	--
P. pentosaceus TEZU213	--	791.74	--	--	36.54	--	87.83	9595.38	--	2693.74	--	--
L. plantarum TEZU272	3617.72	1052.56	--	--	127.43	--	110.57	481.90	--	17829.19	--	--
P. pentosaceus TEZU427	--	749.99	--	118.06	19.09	225.07	4772.30	9972.73	--	4674.79	4212.78	--
P. pentosaceus TEZU482	--	891.29	--	122.47	12.56	222.65	80.79	12911.40	--	16354.63	13516.80	--
L. casei TEZU262	5499.80	1008.41	--	32.79	79.94	--	62.70	757.59	73.33	30031.07	--	--
P. pentosaceus TEZU410	--	872.60	--	--	37.12	225.07	70.90	12035.87	--	13951.40	5417.43	--
L. casei TEZU374	--	746.66	--	104.15	37.77	260.70	4667.60	15753.03	303.13	4451.47	7291. 84	--

4.3.3.12.3 Production of various organic acids by utilizing lactose as carbon source

The HPLC chromatograms for the detection of various organic acids by utilizing lactose are shown in Fig 4.25 and the results are shown in Table 4.28. The lactic acid was the major organic acid produced by all the strains, except *L. casei* TEZU374 which produced low content (294.20 ppm). Its highest content was found in *L. pentosus* TEZU174 (23173.61 ppm), while in all the others it was above 4500 ppm. Except *L. pentosus* TEZU174, tartaric acid, lactic acid, acetic acid, citric acid and oxalic acid were produced by all the strains. Tartaric acid ranged from 83.35 ppm in *P. pentosaceus* TEZU427 to 7374.98 ppm in *P. pentosaceus* TEZU482. *P. pentosaceus* TEZU410 (14359.07 ppm) and *L. casei* TEZU374 (14179.62 ppm) were the highest producer of acetic acid. Critic acid was found highest in *L. casei* TEZU374 (4275.12 ppm) while in all the others it was below 4000 ppm. The content of succinic acid was also high and ranged from 245.09 ppm to 13867.91 ppm. The content of gluconic acid, formic acid, malic acid propionic acid and butyric acid was low and varied among the strains. Pyruvic acid (680.56 ppm) and propionic acid (11.669 ppm) were produced only by *L. pentosus* TEZU174 and *L. casei* TEZU309 respectively.

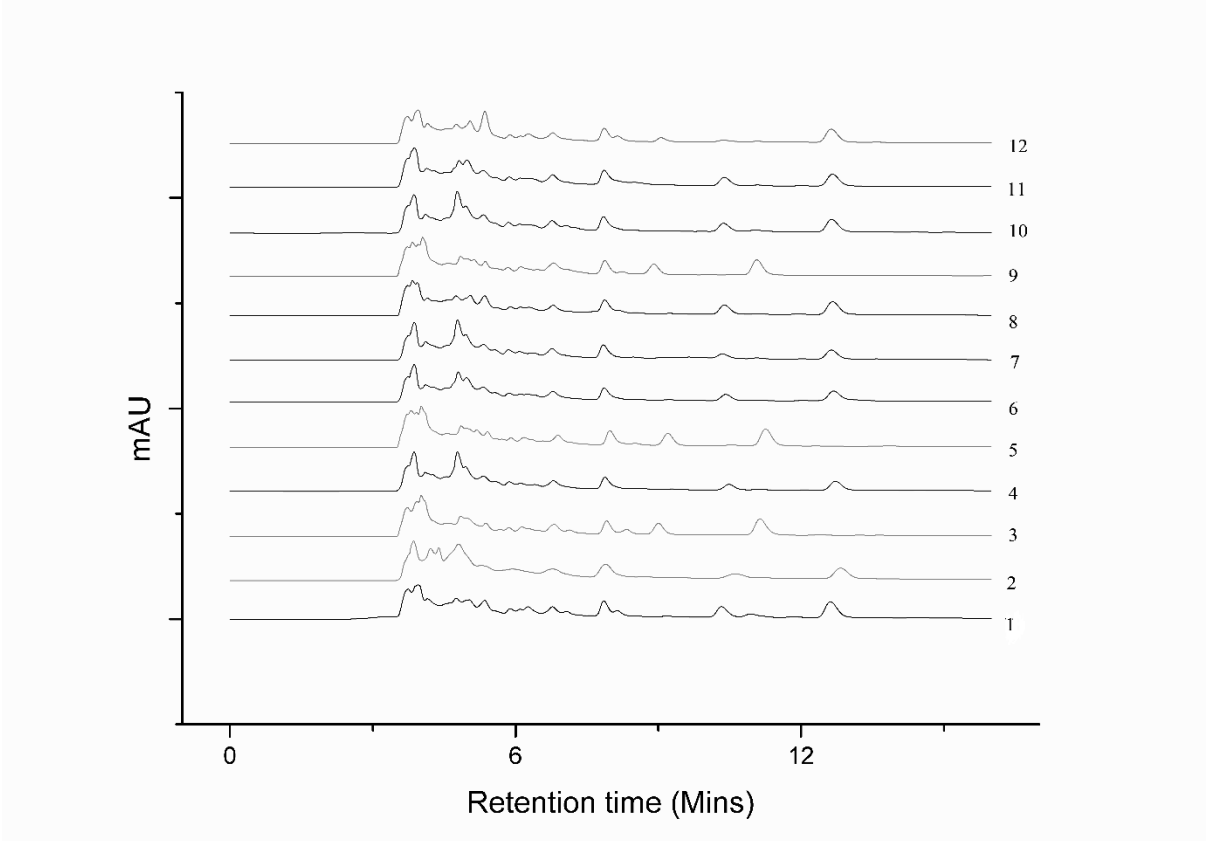

Fig. 4.25 HPLC chromatograms showing the production of different organic acids by utilization of lactose

Table 4.28 Production of different organic acids by utilization of lactose

Strain	Concentration of organic acid (ppm) in the culture media											
	Gluconic acid	Oxalic acid	Pyruvic acid	Formic acid	Tartaric acid	Malic acid	Lactic acid	Acetic acid	Citric acid	Succinic acid	Propionic acid	Butyric acid
L. casei TEZU309	--	458.06	--	--	4675.56	--	4581.88	6275.51	3030.58	356.74	11.669	80.72
L. pentosus TEZU174	--	752.44	680.56	3103.9	--	--	23173.61	--	--	--	--	--
L. casei TEZU468	1151.30	371.54	--	--	5768.89	310.05	7493.29	275.63	1423.76	13867.91	--	--
P. pentosaceus TEZU199	--	683.09	--	54.70	2369.2	122.01	13195.00	401.35	347.87	704.34	--	--
P. pentosaceus TEZU451	265.44	797.72	--	--	4343.51	235.52	8545.42	174.93	3188.19	--	--	--
P. pentosaceus TEZU213	--	672.43	--	56.89	2219.62	129.92	11952.85	843.03	317.10	11577.88		259.25
L. plantarum TEZU272	--	1011.04	--	--	318.17	--	14094.44	462.59	316.032	780.69	--	160.08
P. pentosaceus TEZU427	1470.34	1127.38	--	26.70	83.35	79.958	4659.78	6494.70	3323.10	4849.00	--	--
P. pentosaceus TEZU482	--	23.23	--	--	7374.98	356.45	7453.35	83.35	2198.61	13828.60	--	--
*L. casei*TEZU262	--	707.46	--	22.91	2290.92	--	14843.74	557.28	450.80	14661.57	--	88.92
P. pentosaceus TEZU410	--	824.19	--	56.28	824.19	158.59	5552.99	14359.07	415.22	14642.07	--	71.03
L. casei TEZU374	--	353.23	--	--	4469.65	157.05	294.20	14179.62	4275.12	245.09	--	--

4.4 Conclusion

For the first time the fungi responsible for converting rice starch to fermentable sugars in the age old process of rice beer production in Assam, India have been established. Study on the effectiveness in the production of α-amylase and glucoamylase by *A. rouxii* TU460 and *R. oryzae* TU465 have shown good potential as compared to established saccharifying organism viz. *A. oryzae*. Moreover, *A. rouxii* TU460 was found to be a more promising producer of extracellular glucoamylase. The safety of these two strains in terms of non-production of mycotoxin is an indication of safer and promising sources of these enzymes and these strains can be used in the food processing industries. These enzymes may be considered as a potentially strong candidate for the starch processing industry. Further characterization of these enzymes may include detailed proteomic investigation and study of the secretome.

The yeast strains isolated from the rice beer and starter cakes used various tribes from were Assam were identified. *Saccharomyces cerevisiae* was the most common species, while *Wickerhamomyces anomalus* and *Pichia membranifaciens* strains were also found. The study on their phylogenetic relationship and the carbon sources utilization patterns evolutionary may help in elucidating and establishing the history of carbon metabolism. The study revealed some basic physiological properties of the strains. Most of the strains were also found to be highly tolerant to ethanol and were good producers of ethanol. Along with ethanol, phenylethyl alcohol was also produced by most of the strains. The isolated strains by further be utilized for the production of beers and wines on an industrial scale, and also for the production of other fermented products.

Ten strains of *Lactobacillus casei*, one strain of *Lactobacillus pentosus*, two strain of *Lactobacillus plantarum* and sixteen strains of *Pediococcus pentosaceus* were identified. All the strains were found to be negative for gelatinase and haemolysis activities. The strains showed growth at low pH, *P. pentosaceus* TEZU427 could grow at high osmotic pressure while at 45 °C *L. casei* TEZU309, *L. casei* TEZU468 and *P. pentosaceus* TEZU451 displayed proper growth. The strains were susceptible towards most of the studied antibiotics and they were also antagonist against both *E. coli* and *S. aureus*. All the strains also showed good antioxidant activity and they were tolerant to acid and bile and showed cell surface hydrophobicity. Thus the strains of LAB isolated form rice beer prepared in Assam has probiotic properties and this study may provide wider application area and higher flexibility of LAB strains to be used in starter cultures. These results indicated that the surface associated 52 Kda protein in *L. casei* TEZU309 and 51 Kda protein *L. casei* TEZU374 are responsible for adhesion properties of the

studied LAB strains. It was also observed that *L. casei* TEZU309 possessed relatively better adhesion properties as compared to *L. casei* TEZU374. This study showed that the strains of LAB isolated from rice can utilize D-glucose, maltose and lactose for the production of various organic acids. It was seen that lactic acid and acetic acid were the major organic acids produced by utilization of glucose by all the strains. Lactic acid, acetic acid and succinic acid were the major organic acids produced by all the strains by utilizing maltose except *L. pentosaceus* TEZU174 and *L. casei* TEZU468 which did not produce acetic acid. Lactic acid was the major organic acid produced by all the strains by utilizing lactose as carbon source, except *L. casei* TEZU374 which produced low content. These results suggests the possibility of utilizing these strains for the industrial production of selective organic acids by altering the carbon source.

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