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Identification of Yeast and Mould Isolated from *murcha* in Nepal for Rice Wine Production

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HIGHLIGHTS

- Two novel yeast strains were identified by sequencing for rice wine production.
- Rice wine (low alcohol by volume) was prepared from local raw materials of Nepal.
- Yeast and mould isolated from Parbat *murcha* showed potential fermentative characters.

Abstract: Rice wine is an alcoholic beverage made by simultaneous saccharification and fermentation by using mould and yeast, respectively. In Nepal, traditional starter culture locally known as *murcha* has been used for fermenting locally available raw materials such as millet, rice, wheat, etc. The quality of alcoholic beverages always varies due to the lack of process standardization in terms of culture and process. Here, isolation and screening of mould and yeast were done from the *murcha* collected from different districts of Nepal and used in the production of rice wine. The performance of mould was tested for saccharifying capacity and yeast for sugar, alcohol, pH tolerances and alcohol production. Most potent yeast isolates were identified by the molecular tool using 18s universal primer. Seven mould isolates from *murcha* were tested for saccharification by halo zone on starch media, microscopic observation, liquefication and dinitro salicylic acid test for identification of yeast and mould. All yeast isolates were also compared with commercial yeast (*Saccharomyces bayanus SN9*). Yeasts isolates from Parbat and Dolakha district *murcha* showed 99% identity with *Saccharomyces boulardii* and *Saccharomyces cerevisiae* respectively during basic local

alignment search tool. Among eight isolates, *murcha* from Parbat sample showed better performance in terms of alcohol production. Yeast and mould from Parbat district were used for the fermentation of rice wine. During fermentation change in acidity, ˚Brix and pH respectively 1.0-2.6 g/L, 1-5 and 4.1-3.4. After pasteurized rice wine showed 5% abv, 1.5 g/L succinic acid, 0.27 g/L amino acid, 5.8 ˚Brix, 4.7 pH, 0.56 g/100 mL glucose with no detected methanol (g/100 L) while non pasteurized rice wine showed 5% abv, 1.3 g/L succinic acid, 0.34 g/L amino acid, 6 ˚Brix, 4.65 pH, 0.60 g/100 mL glucose with no detected methanol (g/100 L). *Murcha* sample collected from Parbat has highest potentail to produce sake campare to other sample collected from different district of Nepal.

Keywords: *Murcha*; saccharification; fermentation; tolerance tests; rice wine.

INTRODUCTION

Fermentation is used to produce a desirable value-added product by utilizing different microorganisms namely yeast, mould and bacteria [1]. During the natural fermentation, functional microorganisms which modify the substrates such as plants and animal origin into consumable products such as fermented foods and alcoholic liquor [2]. In Africa, nutrient beers and wines are obtained from fermentation of the commodities like maize, millet, bananas, honey, palm, bamboo saps, and many fruits. The most popular products include kaffir beer and palm wine. Fermentation helps to preserve by means of acidification or alcohol production from fruits besides alternation, improvement and enhancement of bioactive compounds and organoleptic properties of fruits and other foods [3]. Thereby, fermentation technology minimizes the post-harvest and production losses which in turn helps to generate more benefits as well as enhancement in consumption and export of increased fruits products.

Two-third of the world's population most important staple foods is rice [4]. Nowadays, research in the production of rice-based products has shown continued growth [5]. Rice constitutes 90% of starch (on dry weight basis) which is main constituent for the fermentation of rice wine as well [2]. Temperate and warm region of Nepal is famous to grow 20 different varieties of rice. These varieties of rice are mainly consumed as a staple food by Nepalese population [6]. Biomass formation, saccharification, alcoholic and acetic acid fermentation can be considering the utilization of rice in different ways. *Sujen is* an indigenous popular local rice beer of Assam, India [7]. Additionally, *shochu* is a distilled rice-based beverages and popular alcoholic drink distilled from sake [8]. In Nepal, the rice wine commonly known as bhaati jaanr. It is a staple food beverage with high calorie mild alcoholic commonly consumed in the Eastern Himalayan regions of Nepal, India and Bhutan. Locally rice wine is produced by using local starter culture (*murcha*) and steam rice all over the Nepal [9].

Beer, Chyang, Bouza, Pito are few rice-based alcoholic beverages consumed worldwide. These beverages provide several health-promoting benefits such as anti-oxidant, anti-hypertensive, and anti-diabetes activities due to presence of vitamins, minerals, proteins, organic acids and other nutritional components [6]. Further, dealcoholized rice wine can cause the death of gastric cancer cells in mice was found by Korean Food Research Institute [10].

Several traditional fermentation methods from Asia and Africa have been upgraded to high technology production system because of continual efforts on research and development [11]. These exercises can be used to upgrade Nepalese traditional food and beverages as well. The defined fermented starter to carry out fermentation not only ensure safety of the product but also imparts suitable flavour in the product [12]. In countries like Japan, China and Malaysia, rice wine has been successfully commercialized for large scale production. But in Nepal, it is still produced at limited household scale for their own consumption. Currently, there are more than a dozen foreign brands of wines and beers capturing more than 30% of the Nepalese market. However, in the last five years, wine consumption has grown significantly and around one lakh bottles of Nepalese wines are on demand monthly in the market (bossnepal.com/wines-nepal/). Despite this high market demand, raw materials are import from other countries. If substrates (malt, yeast and rice) used for rice-wine production could be made available locally, the overflow of a huge Nepalis currency could be reduced significantly, thereby, causing benefits to both local industries and farmers.

Hence, using local starter culture there is change of getting most potent yeast and mould for producing unique alcoholic beverages. Instead of using *murcha* as a fermenting starter it would be more beneficial to develop the pure starter culture of fermenting yeast isolating from *murcha* as a source to maintain quality of these products. The pure starter culture can also be commercialized instead of *murcha*. Therefore, this research aims to develop a pure starter culture from the existing traditional starter culture with high fermenting

capacity and high saccharification capacity. In addition, the study focuses on the improvement and standardization of starter culture for rice wine production in Nepal.

MATERIAL AND METHODS

Raw Materials

Traditional starter cultures (*murcha*) were collected from different districts of Nepal in polyethene pouches, placed in icebox during transportation and stored at refrigeration condition in laboratory of Central Department of Biotechnology, Tribhuvan University, Kathmandu till further use. These murcha cultures were coded based on their places of collection as Lalitpur (LM), Palpa (PM), Sunsari (SM), Dolakha (DM), Parbat (ParM), Ramechhap (RM), Bardia (BM), and Commercial yeast (CY) for convenience in this research work. Paddy (*Taichin*) was procured from the local market of Bhaktapur while the commercial yeast (*Saccharomyces bayanus*, SN9) was imported from Mangrove Jack's, UK. Yeast extract peptone dextrose agar (YEPDA), Peptone beef extract starch agar (PBSA) and formalin were purchased from HiMedia, Mumbai, India. Triton-X (2%), sodium dodeylsulphate (2%), NaCl (0.1 M), EDTA (1 mM), Tris-HCl of pH 8, phenol chloroform isoamylalcohol (PCI) and Tris-EDTA(TE) were purchased from Thermo fisher-scientific, USA while Tris aetate-EDTA(TAE) purchased from Promega, Madison, WI, USA.

Preparation of stock culture

Murcha samples were grinded by mortar pestle and mixed with sterile 500µl distilled water. Ten folds serial dilution was done in sterile distilled water. From the dilution, 0.1 mL mixture was pipetted and spread onto YEPDA plates under a biosafety hood. At 28˚C plates were incubated for 1-2 days. Similarly, moulds were isolated on PBSA plates and representative mould spores from each *murcha* sample were sub-cultured and stored at refrigeration condition (4˚C) till further use. On the other hand, yeast colonies that appeared on YEPDA plates were sub-cultured and the number estimated as colony-forming unit per gram (cfu/gm) of fresh weight. The representative colony of each *murcha* sample was selected on YEPD agar, purified and preserved at -20˚C in YEPD broth mix with 20% (v/v) glycerol for further identification. Similar procedure was used for the stock preparation of commercial dry yeast.

Morphological characterization

Yeast colonies that appeared on YEPDA were sub-cultured from stock culture at 28˚C for one day and number estimated in terms of cfu (colony forming unit)/gm of fresh weight. Yeast smear was prepared from one day growth yeast culture by simple staining on a grease free slide and heat fixed to coagulates the yeast proteins causing them to stick to the slide. Then placed on the staining tray and flooded with methylene blue for 20-60 seconds. The smear was gently washed to remove the excess stain. The prepared slide was then air dried and examined under oil immersion. For mould, cello-tape method was used, for which small piece of clear tape was cut and gently touched to the mould colony with sticky surface so that mycelial fragments and some spore were stacked to the tape. Sticky tape culture was examined under a microscope with a drop of methylene blue [13].

Chemical test of yeast and mould

Sugar utilization, urea hydrolysis and starch hydrolysis

One day culture growth from stock culture was used to prepare YEP with different type of sugar such as sucrose, D-glucose and starch along with agar. The growth was observed after 1-2 days at 28˚C [14]. And color change was observed for the hydrolysis of urea in 2 days YEPD broth at 28˚C [15]. For mould, halo zone was observed by naked eye when three days culture from stock was inoculated on 1% starch plate.

Saccharification test

Dinitrosalicylic acid (DNS) method was applied for determination of reducing sugar [16] for which mould strains were first grown in PBSA broth for 2 days to obtain the stock culture. Then different strains of mould isolates were cultured in conical flask containing steam rice sealed by cotton plug and incubated at 28˚C for 3 days. Liquification was observed in the conical flask. From this flask, 1 mL of sample was taken in test tube and 3 mL of DNS reagent was added to it. The sample was then incubated in a boiling water-bath for 5 min

after which it was cooled to room temperature and absorbance reading recorded at 540 nm using a spectrophotometer (51119500, Thermo fisher scientific, USA)

The concentration of sugar in the sample was calculated by drawing standard graph of glucose. For this, stock solution of glucose (10 mg/mL) was prepared followed by different dilutions namely 50, 100, 200, 400, 600, 800, 1000 and 1200 µg/mL. The standard curve was obtained and then the concentration in the sample was estimated by plotting observed absorbance at 540 nm.

Selection criteria for yeast on YEPD broth

Different yeast isolates were used for different tolerance tests using day 1 culture broth from stock culture on fresh YEPDA media at 28˚C [14].

Osmotic tolerance test

YEPD broths containing various concentrations of glucose (20, 25, 30, 35, 40, 45 and 50%) were prepared in test tube from different yeast cultures from different districts of Nepal sealed by cotton plug and incubated at 28˚C for 24 h. The growth of yeasts strains in various concentrations of glucose was compared to that of the control by spectrophotometer reading at 600 nm.

Ethanol tolerance test

YEPD broths containing various percentages of absolute alcohol (10, 11, 12, 13, 14, 15 and 16%) were prepared in test tube from different yeast cultures, sealed by cotton plug and incubated at 28˚C for 24h. The growth of yeasts strains in various concentrations of absolute alcohol was compared to that of the control by spectrophotometer reading at 600 nm.

pH tolerance test

YEPD broths with varying pH levels (3, 3.5, 4, 4.5, 5 and 5.5) were prepared in test tube for different yeast cultures, sealed by cotton plug and incubated at 28˚C for 24 h. The growth of yeasts strains at various pH levels was compared to that of the control by spectrophotometer reading at 600 nm.

Alcohol production test

YEPD broths containing 20% of glucose were prepared in test tube from different yeast cultures, sealed by cotton plug and incubated at 28˚C. The alcohol produced by yeast strains was measured everyday up to 14^{th} day of fermentation by using ERMA hand refractometer at 20°C. Further to test alcohol production by dichromate oxidase method, 1 mL of TBP (tri-n-butyl phosphate) and 1 mL of sample solution were mixed in Eppendorf tube and vortexed vigorously for few seconds. After phase separation, 750 µL of dichromate reagent was added and further vortexed till phase separation. Then upper phase (solid) was discarded, and lower phase (Blue green color liquid) was used for optical density measurement at 595nm as per the method described by Caputi [17].

The concentration of ethanol in the unknown sample was calculated using a standard curve method. For this, stock solution of ethanol (10 mg/mL) was prepared in distilled water followed by dilutions to obtains various concentration of ethanol (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mg/mL). The optical density of these dilutions was measured and the values were plotted to get standard curve and concentration of unknown sample was calculated by plotting the values on standard curve.

Testing CO_2 production by yeast and mould in steam rice

Yeast cultures were first grown in YEPD broth for 24 h to obtain from the stock culture and mould isolates were grown in PBS broth for 48 h. Then different isolates of yeast and mould cultures were put in conical flask containing steam rice, sealed by paraffin tape with small balloon on top and incubated at 28˚C for 5 day. CO_2 production were observed in the balloon.

Molecular characterization

Extraction of gDNA from isolated yeast strain

High quality of gDNA for PCR amplification was extracted from all isolated yeast strains as per the method described by Garibay-Orijel [18]. Firstly, 5 mL overnight broth culture of different *murcha* sample from stock culture was used for harvesting cells by centrifugation at 12000 rpm for 1 min at room temperature. To the pellet collected from centrifugation, 230µL of DNA lysis buffer (2% Triton-X, 2% sodium dodeylsulphate, 0.1M NaCl, 1mM EDTA, Tris-HCl of pH 8) was added and mixed vigorously. Then, acidified 8-10 glass beads (0.2-0.5 mm diameter) and 200 µL PCI were added to the solution in the eppendorf tube, capped properly and vortexed for 30 sec followed by centrifugation at 12000 rpm for 5 min and incubation on ice for 30 min after mixing supernatant (aq. layer) with 600µL of isopropanol. After incubation, centrifugation (@ 12000 rpm for 12 min) was repeated followed by washing with 70% ethanol and dried at room temperature. The dried pellet was re-suspended in 300-500µL TE with RNase and finally stored at -20°C for further use. Gel Electrophoresis of the genomic DNA was performed in 0.8% Agarose gel prepared in 1X TAE. Ethidium bromide was used as the staining agent and visualization was performed under UV-transilluminator.

PCR amplification of gDNA

The gDNA was amplified using 18S rRNA primers. The sequences of forward primer was 5‘GGTCTTGTAATTGGAATGAG3’ and for reverse was 5‘CTTCCGTCAATTCCTTTAAG3‘. The PCR mixture was prepared in PCR tubes (master mixture -10µL, $MgCl_2$-0.6µL; forward primer-1µL, reverse primer-1µL) and nuclease free water 6.4µL), centrifuged at 12000 rpm for 2 min) and 1µL template was added in each tube. After that PCR mixture were kept in PCR machine previously set a condition. After completion of PCR cycle, gDNA was run in 1% gel-electrophoresis at 50 V for 1 hour and then it was visualized under UV. For sequencing purpose, the sample was sent to Xcelris Labs Pvt. Ltd., Gujarat, India.

Sequence editing and alignment

The chromatograms obtained for each region were set as base using PHRED quality score [19]. To determine the quality of generated sequence traces, the original forward and reverse raw sequences were assembled and edited in Sequencer v. 4.1.4 (GeneCodes Corporation, USA). Sequences were assembled based on the parameters minimum match 70% and minimum overlap 20%. Each Contig was viewed and manually edited (removal of gaps and dealing with ambiguous nucleotides). The aligned sequences were also edited by comparing with the reference sequence by closely inspecting the peaks of chromatograms of forward and reverse sequence. The assembled consensus contigs were exported in text format and imported in Bioedit v.7. All candidate barcode sequences were aligned by Cluster W, (multiple sequence alignment tools) in Bioedit using default parameters. Both primer ends were delineated from the alignment matrix. Primer excluded barcode sequences were exported for further analysis.

Phylogeny inference

Phylogeny tree was reconstructed by Neighbor-joining (NJ) in MEGA v.7.0.14. NJ tree was constructed using P-distance as genetic measure and setting negative branch length to zero with uniform distribution rates applied. Typically, 1000 replicates of bootstrap were used to estimate tree reliability. Based on the following scale: BS 50-74% (weak bootstrap support) and 75-100% for strong support node support was estimated. The efficiency of tree resolution was considered successful only when the clades have at least ≥50% bootstrap value. The sequence was submitted in the GeneBank database and accession number was obtained.

Rice wine production from selected yeast and mould isolates

Based on the above discussed selection criteria, both yeast and mould isolates of *murcha* sample that showed greater potential compared to others were used for the production of rice wine. The inoculum for fermentation was prepared in YEPD broth for 1d and PBS broth for 2d from stock culture at 28˚C of yeast and mould, respectively. Koji mould is one of the most essential constituents for the preparation of rice wine.

Fermentation of rice wine

For fermentation of rice wine, standard ratio of water, koji and steam rice (13:2:8) were placed in the fermentation tank. The addition into fermentation tank was done over four days followed by three steps. The amount of steamed rice and *koji* placed in the tank on the first day was equal to one-sixth of total mass. Seed mash (*shubo*) was also added on the first day and then allowed yeast to multiply. A quantity equal to two-sixths of the total mass was placed in tank on the third day and the enduring three-sixths added on the fourth day. The temperature of the mix was kept at 23±1˚C. The fermentation process took two weeks, surrendering an alcohol content of around 17-20%. After the completion of the fermentation process, the *moromi* was filtered with cheesecloth by low-temperature treatment; this precipitates out as sediment, and further filtration was done by using the three-layer filter to obtain a clear liquid to remove the undissolved rice and yeast, leaving behind the new sake and two-layer charcoal for decoloring, flavor adjustment and control of the aging process**.**

Chemical analysis of rice wine

Determination of ethanol content

The method described by Sake and Sochu maker association was adopted to determine ethanol content in the prepared rice wine. The sake obtained during rice wine making process was heated in a beaker to slowly boil close to half of its starting volume. By adding distilled water level of the sake was made to the pre-boil level. The pre-boil amount was made close to 250 mL and the specific gravity was measured with a specific gravity bottle and calculated using the equation (1):

$$\text{Specific gravity (SG)} = \frac{(\text{measured substance mass/volume})}{(\text{distilled water mass/volume})} \quad \text{.....(1)}$$

The refractive index (given in Brix equivalents) with a refractometer was obtained and these values were fed into the equation (2) to obtain ethanol content (%ABV).

$$\text{Ethanol (\%ABV)} = 1.646 \times \text{RI} - 2.703\,(145 - 145 / \text{SG}) - 1.794 \quad \text{.....(2)}$$

Where, SG = specific gravity; RI = Refractive index

Total soluble solid (TSS), pH and total titratable acidity

ERMA hand refractometer at 20°C was used in the sample to determine total soluble solid and the triplicate readings noted in °Brix. For pH estimation, 15 mL of sample was taken from storage tank of sake and pH was noted by applying the pH meter (Analog model, Corion Research, USA). Standard solutions of pH 4.0, 7.0 and 10.0 were used to calibrate the pH meter before taking the readings. By following the method described by Sake and Sochu maker Association a total titratable acidity was determined. 10 mL of the sample was taken in 100 mL volumetric flask and distilled water was used to make up volume. For determining the total titratable acidity, titration was done by using 10 mL sample in a 100 mL conical flask containing one or two drops of phenolphthalein as an indicator and titrated against 0.26 M NaOH solution. The result was expressed in term of titratable acidity as g succinic/L

Amino acid

10 mL sample was taken in a beaker and few drops of phenolphthalein were added. The burette was loaded with 10 mL (reading, R1) of NaOH and was titrated drop by drop from the burette to the sample until colour changed to a light pink for at least 30 sec (this reading noted as R2). The difference between R1 and R2 was used for neutralizing the sample. Further, to 10 mL of this neutralized sample, 10 mL of the neutralized formalin was added in a 100 mL beaker. Reloaded the burette with NaOH and titrated this 20 mL sample until the colour changed to a light pink and persisted for at least 30 sec. The volume of NaOH used was noted and the quantity of amino acid present in the sample was determined as described by *Sake* and *Sochu* maker Association of Japan.

Methanol content

Methanol content of sake was determined by spectrophotometric method. 50 mL sample was taken from storage tank and distillation was done, 40 mL of distillate was collected and further 1 mL of distillate from 40 mL distillate and 5 mL distilled water mixed properly. In three different 50 mL stoppered test tubes 1 mL of

diluted sample, 1 mL of distilled water (for blank) and 1 mL of methanol (for standard) were taken and stored in ice-cold water. 30 min left after adding 2 mL of $KMnO_4$ (potassium permanganate) reagent to each test tube. After that the solution was decolorized by adding a small amount of sodium bisulphite, then added 1 mL of chromotropic acid, mixed well and slowly added 15 mL of sulphuric acid by swirling followed by storage in hot water bath at 80˚C for 20 min. Once the colour changed from violet to red, the mixture was cooled and the absorbance was recorded at 575 nm using spectrophotometer and the equation (3) was used to calculate the % of methanol.

$$\text{Methanol}\left(\%,\frac{\text{V}}{\text{V}}\right)=\frac{\text{Sample OD}-\text{Blank OD}}{\text{Standard OD}}\times 0.025\times \text{dilution factor}\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

RESULTS AND DISCUSSION

Morphological characterization of yeast and mould

Different fermentative yeasts isolated from *murcha* sample from seven different places in Nepal were normally round and oval while the *murcha* from Bardia was flat in shape. *Murcha* from different places were coloured differently due to different flour (such as wheat, corn, millet, rice etc) used in their preparation. The isolated strains grown on YEPDA media were found to have smooth surface with creamy/creamy white and yellowish white circular margin (Figure 1B). Budding of yeasts were clearly observed under microscopic examination (Figure 1C). Similar observations were reported by Yadav [20]. Similarly, mould were isolated from the PBSA media based on their ability to grow on it. They were further isolated based on morphological character. All of mould isolates showed filamentous and mucoid with hard surface. Due to morphological character and starch hydrolysis test on PBSA media, the fungus was considered as *Rhizopus* Figure 2(A). They were further isolated based on morphological character. All of mould isolates showed filamentous and mucoid with hard surface. Staining of mould isolates showed Sporangiophore with sporangium Figure 2(B). The maximum number of the yeast's colonies were obtained from the *murcha* sample prepared from rice flour collected from Parbat yeast isolate at 10^8 dilution factor. It was white in color and marketed for the preparation of *Jand.*

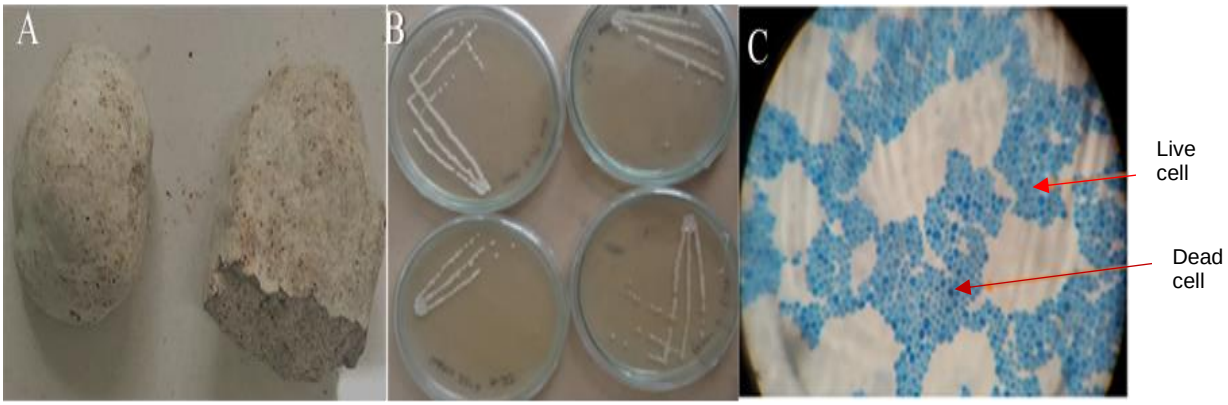

Figure 1. Starter culture A) *Murcha*; B) Colonies of yeast on YEPDA plates and C) Microscopic observation of yeast cell.

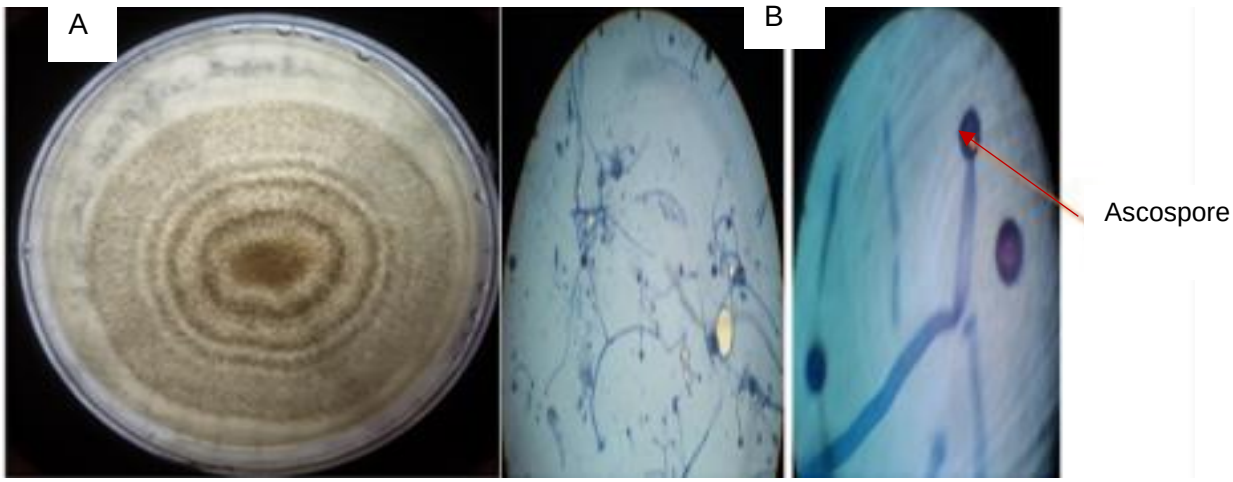

Figure 2. A) Mold growth appeared and B) Microscopic observation of mold on PBSA plate.

Chemical test of yeast and mould

Carbohydrate and urea utilization test

Table 1. Carbohydrate and urea of different isolates of yeast

S.N	Sample code	D-Glucose	Sucrose	Starch	Urea hydrolysis test at 24 h of incubation	Urea hydrolysis test at 21 days of incubation
1.	LY	+	+	-	-	+
2.	PY	+	+	-	-	+
3.	SY	+	+	-	-	+
4.	DY	+	+	-	-	+
5.	ParY	+	+	-	-	-
6.	RY	+	-	-	-	+
7.	BY	+	+	-	-	+
8.	CY	+	+	-	-	-

Note: + indicates positive test; - indicates negative test

All yeast isolates utilize glucose and sucrose as carbon source except Ramechhap yeast isolates (Table 1). Ramechhap yeast isolates cannot utilize sucrose and none of them utilized starch correspond to the Koschwanez [21]. All yeast cannot break down sucrose due to absence of invertase. On the other hand, mutant opportunistic yeasts are not able to break down the sucrose which is costly than glucose and fructose [21]. All yeast isolates did not utilize urea till 1 day of incubation, but it was utilized by 21 days of incubation except the parbat yeast and commercial yeast (Table 1). In the urea hydrolysis test, isolates showed negative results with 24 h of incubation (Table 1) and similar results were reported by Yadav [20]. But after 3 weeks of incubation, they showed positive results, except for Parbat yeast and commercial yeast.

Starch hydrolysis test and saccharification test

All isolates of mould showed halo zone in the PBSA media similar result was reported by Tamang [22]. Maximum halo zone was showed by mould isolated from Parbat *murcha*. *Murcha* is a source of amylase enzyme. Presence of amylase activity in *murcha,* hydrolysed starch in medium caused halo zone when iodine float.

Table 2. Starch hydrolysis test and concentration of reducing sugar produced by different mould isolates.

S.N.	Sample code	Starch hydrolysis	Reducing sugar content (µg/mL)
1	LM	+	335.0
2	PM	+	387.8
3	SM	+	283.3
4	DM	+	296.7
5	ParM	+	417.1
6	RM	+	395.8
7	BM	+	299.2

All the isolated mould were found to exhibit liquification. By DNS test also showed that there was significant increase in fermentable sugar in first day of incubation. Reducing sugar produced during fermentation at 28˚C for 24 h was (0.28-0.41) mg/mL (Table 2), which was less to result reported by Mathew [23]. Time, pH, temperature and source of enzyme determine the enzymatic activity of enzyme. At high temperature and pH value near the limit of the optimum over time, generally enzyme not work properly. Under certain conditions optimum pH should be determined. So that, it is essential to choose an enzyme with a pH range from 4 to 11 [24].

Selection criteria of yeast

Sugar tolerance test of yeast strains

All the eight yeast isolates showed their growth on YEPD broth at 28˚C for 1 day contain 30% dextrose (Figure 3A). Lower sugar concentration was found to enhance the growth of yeast cells, whereas the increase in sugar concentrations inhibited their growth of yeast cells was reported by Osho [25]. Despite the decrease in growth at higher sugar concentration, the yeast cells were found to tolerate up to 40% sugar concentration which was higher than the result reported by Osho [25]. Exposure of yeasts to hyperosmotic environment provokes a positive turgor pressure resulting in a rapid efflux of intracellular water into the medium leading to cell dehydration and sodium specific inhibition of certain proteins [26]. The cytoskeleton collapses due to the rapid efflux of water through the lipid bilayer resulting in the growth arrest of the cell. Cellular reprogramming or adaption is the major defense response under these conditions.

Yeast responds by accumulating intracellular polyols particularly glycerol, trehalose, arabitol, sorbitol and compatible ions [26]. Activity of glyceraldehyde 3-phosphate dehydrogenase (GPD) is known to be increased during sugar and salt stress leading to the increased formation of glycerol which is the major stress protectant. The activity of alcohodehydrogenase is found to be decrease in hyperosmotic condition [27].

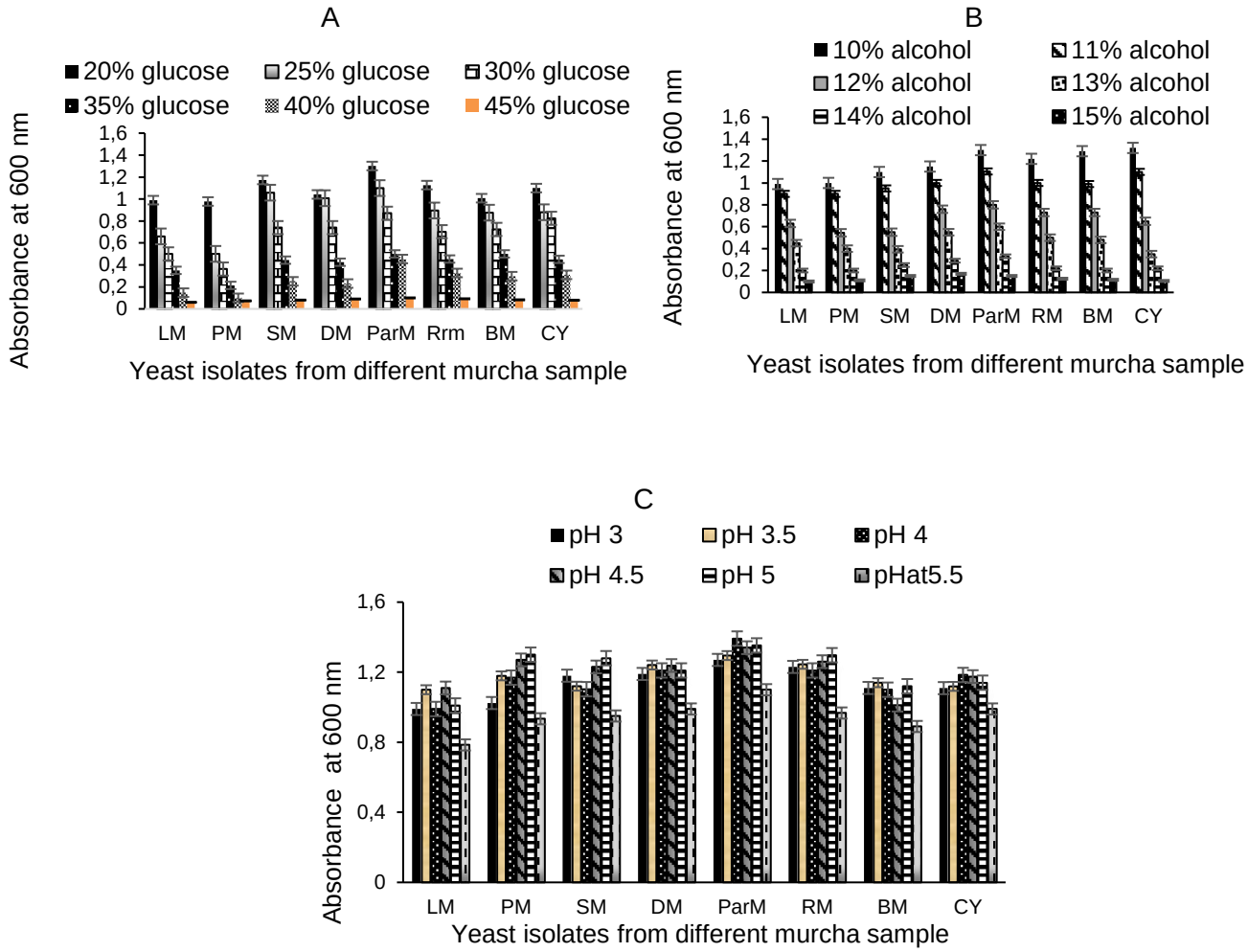

Figure 3. Tolerance of yeast isolates at different concentration of A) glucose B) absolute alcohol C) pH. Vertical bars indicate ± standard error.

Ethanol tolerance test of yeast strains

Eight including commercial yeast strain different yeast strains were found to exhibit remarkable higher ethanol tolerance. All the strains showed significant growth at up to 14% ethanol concentration (Figure 3B) and above this concentration, there was a significant decrease in growth as reported by Xue [28]. Lower concentration of ethanol was found to favour the growth of yeast cells whereas higher concentration of ethanol was found to inhibit and hence decrease growth. The ability of yeast to tolerate and grow in the presence of higher concentration of sugar have also been reported to tolerate to higher concentration of ethanol [29]. Yeast cells treated with higher concentrations of sugar are known to show increased accumulation of trehalose, an important storage compound and a stress protectant. Trehalose was reported to confer the growth under hyper osmotic conditions and also helps to withstand toxic concentration of ethanol [30]. During fermentation, the most common stress that yeast cells encounter is ethanol toxicity [31]. Yeast cells decrease the cell volume and increasing thermal death at low concentration of ethanol, whereas higher concentration of ethanol reduces cell vitality increases the cell death rate [32]. High concentration of ethanol also decreases the fluidity of plasma membrane leading to dissipation of trans-membrane electrochemical potential and subsequently acidify the intracellular and vacuolar conditions. Ethanol at higher concentration has also been shown to denature protein and its dysfunction [30]. Energy surrendering pathway of cells such as glucose transport and metabolism in this case ethanol stress respond by increasing the expression of gene and energy demanding pathway anabolic pathways like cell growth, leading to an initial growth lag period in this case ethanol stress respond by decreasing the expression of gene ex [32].

pH tolerance test of yeast strains

As shown in Figure 3C, all the isolated yeast strains grow on YEPD broth at 28˚C for 1 day contain pH range from 3-5.5. There was a change in growth at different pH values, a similar result reported by Liu [33]. Acidic condition was found to favour the growth of yeasts whereas slightly acidic or near to neutral condition inhibits the growth of yeasts [34]. Beside some special cases such as growth condition of yeast cells, yeast cells show the long lag phase at low initial pH that causes less accumulation of biomass, reducing the consumption rate of total sugar, increasing final content of acetic acid and glycerol, and decreasing final content of ethanol and L- succinic acid [33]. Proteins protruding from the plasma membrane of yeast cell wall affect by external environmental pH especially caused by weak acid. Mostly, pH value affects the yeast growth, fermentation rate and fermentation products [33].

Alcohol production test using hand refractometer and dichromate oxidase method in YEPD broth.

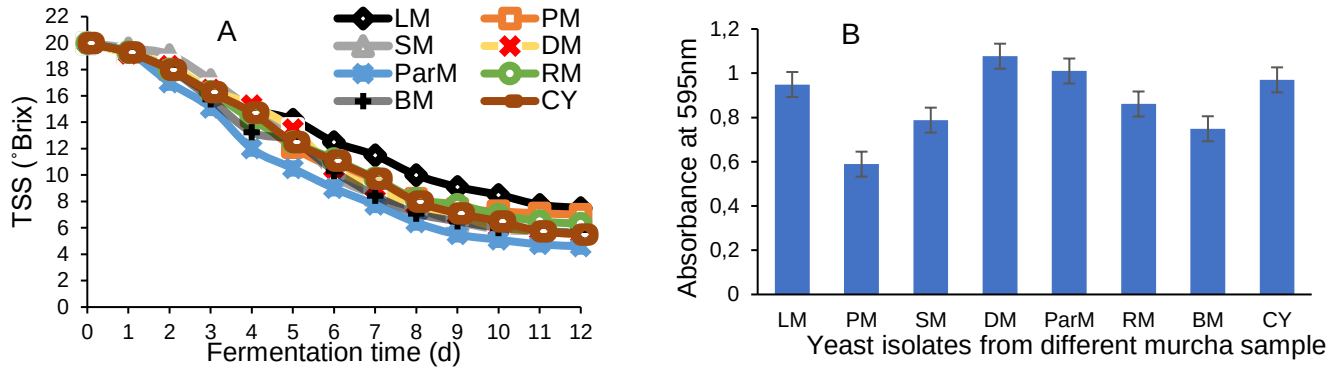

Figure 4. A) Changes in total soluble solid (˚Brix) during fermentation in YEPD broth at 28˚C for 12-day B) ethanol production in YPD broth at 28˚C for 18 h from yeast inoculation and growth was observed O.D. at 595nm. Vertical bar indicates standard error (±0.056), n=2.

By hand refractometer all yeast isolates showed gradual decrease in TSS(˚Brix) up to 12 days. Day 1 showed very less decrease in TSS after day 1, there was continuous decrease in TSS up to day 9. After day 9 there was no change in TSS (Figure 4A), which was similar result reported by Thammasittirong [35]. Maximum sugar fermentation was done by Parbat yeast isolates (4.75˚Brix) while minimum sugar fermentation was done by Lalitpur yeast isolates (7.5˚Brix). This could be because of yeast isolates vary in their fermentation efficiency and sugar conversion ability. Initially, TSS continuously decreased due to faster conversion of sugars into alcohol and low alcohol present in the medium. Later, fermentation decreased due to a high amount of alcohol exerting an effect on the fermentation process by reducing the activity of yeast [36].

A hand refractometer showed no significant decrease in TSS on the first day of fermentation, but the dichromate oxidase method showed that there was the production of ethanol by all yeast isolates after 18 h (Figure 4B). All yeast isolates produced ethanol very first day of fermentation (Table S2). Among them yeast isolated from Dolakha *murcha* showed highest production of ethanol (10.52 mg/mL). Ethanol production start from very first day of fermentation reported by Yadav [21].

PCR amplification results of yeast isolates and molecular identification of potential yeast for fermentation

All isolates of yeast isolated from different *murcha* sample grow in YEPD broth at 28˚C for one day and maintaining OD 0.4 at 600 nm. Genomic DNA and PCR of yeast isolates were done as shown in Figure 5(A). All of yeast isolates showed similar type of DNA bands that lied in between 600 to 700 bp by comparing with ladder (100 bp) of Solis Biodyne. Based on selection criteria of yeast isolates, Parbat yeast isolate showed most potent character among seven yeast isolates. Further analysis of yeast from Dolakha *murcha* Figure 5(B) and Parbat *murcha* Figure 5(C) was done by sequencing.

Molecular characterization of two selected yeast strains

Sequencing was performed for two selected strains, strain DD-CDBT (Dolakha yeast) and EE-CDBT (Parbat yeast). Both were found 99% identical to *Saccharomyces cerevisiae* and *Saccharomyces boulardii* by blast result and phylogenetic tree of Dolakha yeast as shown in Figure 5(B). Phylogenetic tree of Parbat yeast depicted in Figure 5(C).

Saccharomyces cerevisiae is known for its fermenting ability utilizing hexoses. Saccharomyces yeasts are favoured because of their excellent ethanol yield, tolerance of low pH that discourage the growth of spoilage microbes. It has the ability to grow aerobically for efficient cell generation [37]. The optimized condition for *S. cerevisiae* was found to be; pH 4.5, peptone as nitrogen source, 20% glucose condition and temperature of 28˚C. In this study *S. cerevisiae* found to produce ethanol 10.52 mg/mL. The metabolism of *S. cerevisiae* is programmed to catabolize external sugar rapidly to ethanol, irrespective of presence of oxygen [38]. It is thought that this behaviour provides a competitive advantage: *Saccharomyces cerevisiae* is tolerant upto 20% ethanol, while most competing yeast and bacteria are killed at more than 5% ethanol [38]. This behaviour of yeast is important for sake preparation. pH is also key factor that affects growth of yeast and finally in ethanol fermentation as ability of ethanol production ability changes with pH. Below pH 4, the incubation time for maximum ethanol production prolongs and above pH 6, formation of other by-products such as acetic acid and butyric acid dominates. The pH value of 4.0-5.0 is thus regarded as operational limit for ethanol production [39]. The optimum pH in this study was found to be 4.5 for DD-CDBT, which corresponds Liu [33]. Nitrogen is essential for yeast metabolism and growth and considered to be a limiting factor during fermentation process. The nitrogen sources not only increase biomass, but also reduce time required for the completion of fermentation [40].

Optimized condition for *S. boulardii* found to pH 4, peptone as nitrogen source, 20% glucose condition and temperature of 28˚C. In this study *S. boulardii* found to produce maximum ethanol 9.72 mg/mL. *S. boulardii* have unique features which allow it to survive in the most utmost situations [41]. It has hindering abilities on pro-inflammatory cytokines due to secretion of variety of proteases also has been known to secrete a variety of proteases. *S. boulardii* can grow in aerobic as well as anaerobic condition. It characterizes as fermentation over respiration 98 to 2% [42]. Both the yeast strains showed maximum growth at 20% sugar concentrations. Industrial ethanol production by using this strain of yeast is beneficial due to high sugar tolerance and ethanol tolerance [43]. The optimal sugar concentration of 20% for strains conferring ethanol tolerance of between 14-18% is like that of Osho [26]. An accession number was obtained from GenBank, MK253787 and MK253281 for yeast isolates isolated from Dolakha and Parbat *murcha*.

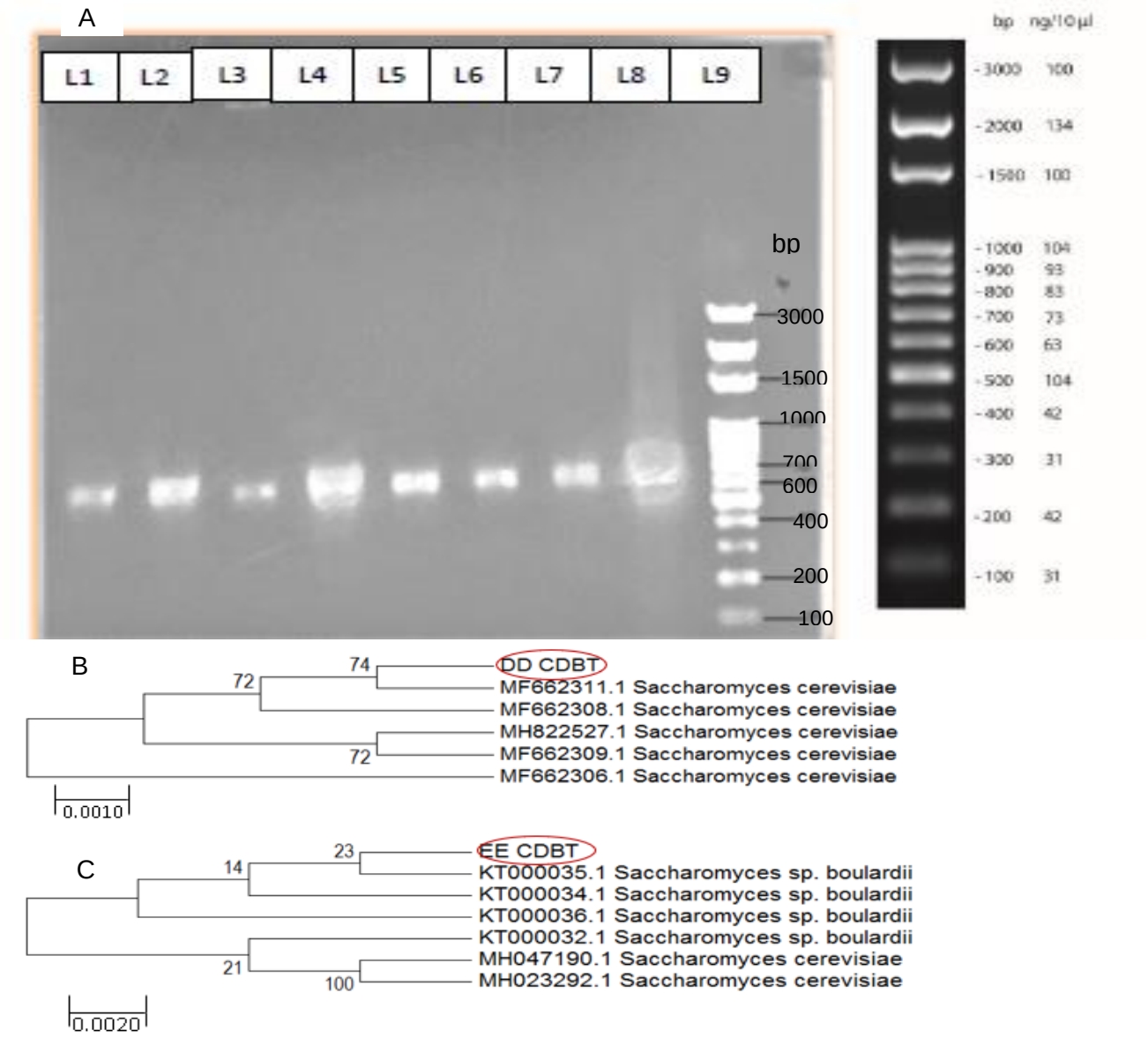

Figure 5. Molecular identification of yeast isolates A) PCR products of yeasts isolates from different *murcha* samplewith ladder of 100 bp (Solis Biodyne) and Phylogenetic tree of yeast isolate of B) Dolakha *murcha* and C) Parbat *murcha* Note: L1= Lalitpur yeast; L2= Palpa yeast; L3= Sunsari yeast; L4= Dolakha yeast; L5= Parbat yeast; L6= Ramechhap yeast; L7= Bardia yeast; L8 =Commercial yeast; L9= Ladder.

Analysis of rice wine production from selected yeast and mould isolate

Changes in ˚Brix, pH and acidity during fermentation

As shown in Figure 6A there was a continuous increase in TSS (˚Brix) of wine from the initial day of fermentation up to day 4 and a decrease on day 5. After day 5, there were no change in TSS. Continuous increase in TSS may be due to continuous inoculation of yeast, *mould* and steam rice in fermentation tank during day 1, day 3 and day 4 of fermentation. Another reason may be due to less activity of yeast strain to produce ethanol as compared to fermentable sugar produced by mould. After day 5 (ca 5˚Brix), there was almost no fermentation that might be because of ethanol concentration or very less power of saccharification by mould. The final change in TSS stops at 5 ˚Brix during rice wine preparation was also reported by Chay [43].

The initial pH of fermentative mash was 4.5, so no acid added to maintain the pH of sake.The acidic nature of koji might be caused by the growth of Lactic Acid Bacteria (LAB) during preparation and storage of *koji.* There was a decrease in pH up to day 4 of fermentation (Figure 6A) since fermentation caused the production of ethanol and carbon dioxide. CO_2 is one of the factors that declines the pH of fermentative broth and the production of an organic acid such as succinic acid, pyruvic acid [44]. After day 9 of fermentation,

there was a slight increase in pH, that was the autophagy nature of yeast cells [45]. Change in pH from 4.75 to 3.7 up to last day of fermentation, which showed similar result during the preparation of rice wine reported by Chen [45].

There was an increase in acidity after day 1 of fermentation up to day 9 shown in Figure 6B. This may be due to the formation of different organic acid and CO_2 formation during fermentation. Excessive carbon dioxide evolution in the initial and mid-period of the fermentation process caused the formation of carbonic ions in the must and led to increase in acidity. After day 9, there was no change in acidity due to decrease in fermentation. Whereas, at the end of fermentation, CO_2 evolution decreased thus lowed acidity, besides the production of some other organic acid during fermentation [46]. Bhatane [36] suggested that by sake yeasts, simple sugar generated from starch glucose was almost completely converted to ethanol whereas laboratory yeasts were less efficient for glucose conversion into ethanol. Titratable acidity in terms of succinic acid produced during the fermentation of sake showed a continuous increase in pH from day 1 (1.062 g/L) to day 9 (2.98 g/L). After day 9 of fermentation, there was no change in acidity (Figure 6B).

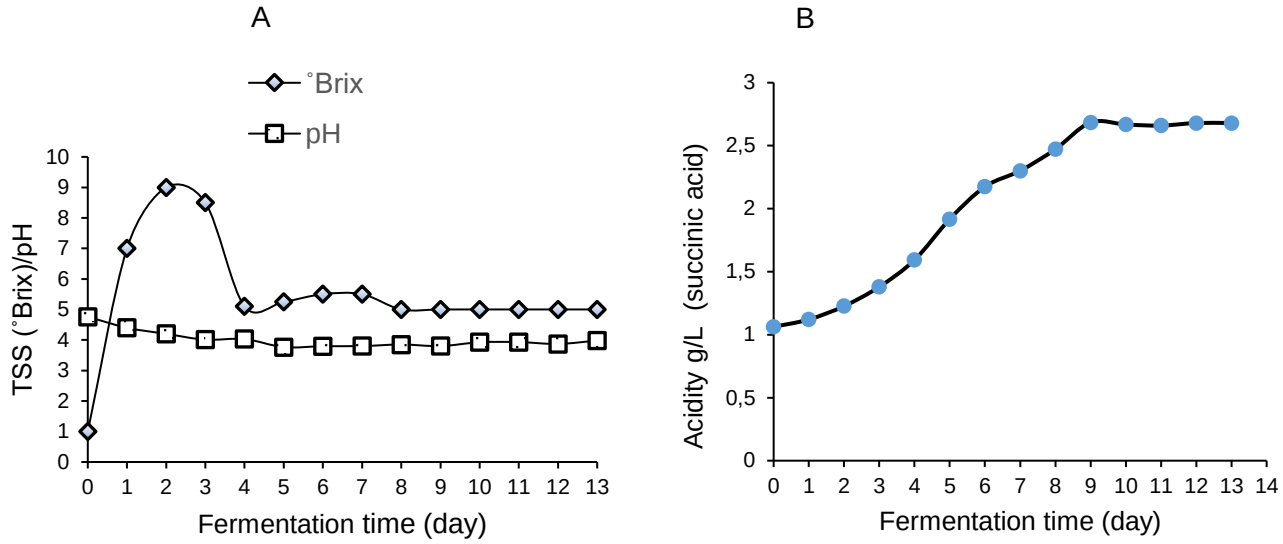

Figure 6. Change A) ˚Brix and pH B) acidity during fermentation of rice mash at room temperature (24±2˚C). During 13 days of fermentation of rice mash at room temperature (24±2˚C).

Comparative study of Lab prepare sake with commercial sake

Final product of rice wine (sake) with different chemical composition were calculated (Table 3).

Table 3. Comparative study of Prepare sake with commercial sake.

S.N.	Particular	Non-pasteurized lab sake	Pasteurized lab sake	Commercial sake	Sake*
1.	Alcohol (%abv)	5	5	18	13-17
2.	pH	4.65	4.7	4.1	4.2-4.7
3.	Succinic acid (g/L)	1.3	1.5	1.1	0.2-0.5
4.	˚Brix	6	5.8	9.5	6.3-11.3
5.	Glucose (g/100 mL)	0.60	0.56	2.5	0.5-2.4
6.	Glycine (g/L)	0.34	0.27	0.27	n.f
7.	Methanol (g/100 L abs. alc.)	_	_	_	n.f

*standard value of sake given by Japan *sake* and *sochu* maker association.
'-' indicates not detection n.f. indicates not found.

As shown in Table 3 different chemical parameter were calculated. But ethanol (%abv) and ˚Brix were not within the value of commercial sake. That may be following reasons:

1. Generally *murcha* contain *Rhizopus*, so during *mould* preparation *Rhizopus* was used instead of *Aspergillus, Aspergillus* is used sake koji prepration in Japan.
2. Rice variety differ from sake rice.
3. Less polishing of rice.
4. Fluctuation of fermentation temperature.

The enzyme, glucoamylase produced by *Rhizopus oryzae* found to be better than *Apergillus oryzae.* However, alpha amylase and protease produced by *Rhizopus oryzae* was very less compared to *Aspergillus oryzae* whereas same species of some fungal isolates produced different level of enzyme for all determined enzyme activity [47].

Quality of rice wine determine different factors such as varieties and fermentation methods of rice and it causes significant differences in different values such as pH, titratable acidity, total soluble solids, alcohol contents, amino-N, protein and reducing sugar and finally in overall sensory acceptance [44]. There are different temperature values in fermentation broth are responsible for optimum production of different chemicals like ethanol and glycerol at 23°C, maltose (90 g/L) is obtained at 18°C, and lactic acid at 33°C. Temperature in fermentation mash of Chinese rice wine plays a meaningful role in the ethanol production, acid flavor contents, and sugar contents [48]. Rice wine made by polishing 70 and 60% showed higher alcohol and better physico-chemical properties than rice polished at 80% and 90% for rice wine production [49]. Low alcohol contains rice wine can be produced by yeast and mould isolated from murcha collected from Parbat district. However, further research is required to identify the strain of mould and the sensory profile of rice wine due to lack of equipment during this research.

CONCLUSIONS

Potential yeast strains can be obtained from locally available starter culture (*murcha*). Two novel yeast strains were identified by sequencing. They were MK253787 and MK253281 for yeast isolates isolated from Dolakha and Parbat m*urcha*. Yeast and mould isolated from *murcha* sample collected from Parbat had significant tolerance of alcohol, sugar, pH, and highest alcohol production, as well as liquification of steam rice among all *murcha* sample including commercial wine yeast (*Saccharomyces bayanus* SN9) and there was generally the presence of *Rhizopus* species that had less capacity of saccharification of starch. By using this yeast and mould, low-calorie sake wine could be produced with no significant difference in the physio-chemical properties of pasteurized and non-pasteurized sake within a short period of storage. Since a low amount of ethanol was produced during the fermentation of rice wine using yeast and mould isolated from a local starter culture (*murcha*). Hence, future research work could focus on using *Aspergillus oryzae* instead of *Rhizopus* species and the polishing ratio of rice could also be increased to obtain more alcohol content along with a better flavour profile.

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