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Research Article

Production of Junar (*Citrus sinensis*) Wine Using Selected Fermentative Yeast from Indigenous Starter Murcha

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Abstract

Junar is a citrus fruit of Nepal, which contains a good source of minerals, vitamins and soluble fiber conferring various health benefits. It is mainly found in the mid-hill region of the country. During its peak harvest time, significant amounts are wasted due to fluctuating humidity, insufficient storage facilities, problematic transit, and microbial diseases. The alternative to this is to convert junars into wine. Similarly, for the improvement of flavor, aroma and health benefit parameters of junar wine, screening and isolation of novel yeast strain is beneficial. Therefore, this study aimed to isolate new wine yeast strains from Nepalese indigenous starter named murcha and compare its efficiency for wine preparation. The study was carried out in the food microbiology laboratory of National College, Lainchaur from November 2021 to May 2022. Seven murcha samples (Chamati, Lubu, Panchthar, Pharping, Sankhu, Thali, and Thecho) were used for isolation of potent wine yeasts. Pour plating, sugar fermentation and 18S rRNA gene sequencing were performed for its identification. The junar juice fermentation was carried out for 21 days. The proximate analysis of junar and physio-chemical and sensory parameters of wines were analyzed. From 7 murcha samples, 14 fermentative yeasts and 7 wine yeasts were isolated. Among them, two isolates *Saccharomyces cerevisiae* Ch1 and *Saccharomyces spp* Pn4 were screened to be ethanol and acid tolerant strains. The resulting juice yield, moisture content, pH, total soluble solid, titrable acidity and vitamin C content were 56.09%, 90.72%, 4.01, 11.2˚Brix, 0.92% and 34.37%, respectively. Moreover, the ethanol, methanol, total phenolic, total flavonoid content and antioxidant activity were: 8.3% and 7.8%, 0.019% and 0.013%, 484.11 mg GAE/ml and 480.78 mg GAE/ml, 110.12 mg CAE/ml and 125.72 mg CAE/ml and 22.61% and 21.17%, in terms of Ch1 and Pn4 wine accordingly. Based on sensory quality, Ch1 wine was superior to Pn4 wine. Thus, this study concluded that *S. cerevisiae* isolated from traditional starter murcha was significant for the junar wine production.

INTRODUCTION

Wine is an ancient alcoholic drink, generally made using the grape as a substrate (Yeshaneh, 2024). Recently, non-grapefruits have also gained much attention for wine making due to their unique aroma and bouquet, organoleptic qualities and additional health benefits. Many fruits like banana, blueberry, cherry, coconut, jamun, mango, oranges, papaya, peach, pineapple, plum, strawberries, watermelon are reported to be used as a substrate for wine making (Ogodo *et al*., 2015).

Orange is one of the most widely cultivated (the tropical and subtropical areas of world) and consumed fruit differing in nutritional parameters (Kaini, 2013). Among oranges, *Citrus sinensis* is a sweet orange variety having light orange or dark orange skin color (Iglesias *et al*., 2007). It along with *C. aurantium* (sour orange) accounts for 50% global citrus production (USDA, 2018). In Nepal, sweet orange is commonly known as junar. Junar is found mainly in the mid-hill region of the country. Generally, junar contains 88.5% of moisture, 0.05% of fat, 0.3% of ash, 0.7% of crude protein, 11˚Brix of total soluble solid, 3.8 pH, 0.51% of acidity, 8.36% of total sugars, 1.8% of reducing sugars, 6.56% of non-reducing sugars (Shravan *et al*., 2018). However, the pulp and juice content were reported to be widely varied: pulp content: 51.1-69.7% and juice content: 26.2-30% (Paudyal and Subedi, 2008).

With the current understanding of wine fermentation, the focuses are now made on enhancement of the flavor diversity, health benefits and appeal of wines either by controlled fermentation with novel yeast strains or/and using different non-grape fruits (Csernus *et al*., 2013; Machamangalath *et al*., 2016). Several studies have already described the fermentation of non-grape fruits with various new strains of yeast. These new yeast strains are isolated from different sources like traditional starter culture, fruits itself, and breading (Tsuyoshi *et al*., 2005; Nasir *et al*., 2017). It is apparent from these studies that the profile of flavonoids content, phenolic compound content, antioxidant properties, aroma and flavor substances differ widely depending upon the types of fruits, types of strain used (Kelebek *et al*., 2009; Pandeya *et al*., 2018; Mascarin *et al*., 2023).

Murcha, an indigenous starter culture of Nepal, rich in diverse fermentative yeasts, has been used by various ethnic groups such as Newar, Tamang, Sherpa, Rai, Limbu, Gurung, Magar, and Tharu community to produce traditional alcoholic beverages (Karmacharya *et al*., 2024). It has been extensively studied for cereal-based alcoholic beverage production, particularly rice and millet wines (Pakuwal and Manandhar, 2020). However, there is a lack of research on its application in fruit-based fermentations, especially high-acid citrus fruits like junar. There are no reports regarding studies related to ethanol yield, sugar utilization, phenolic content changes, acidity changes when using indigenous murcha yeasts in citrus juice i.e., junar.

Junar is a seasonal fruit which is consumed fresh, but during its peak harvest time, significant amounts are wasted due to insufficient storage facilities, lack of good market value, problematic transit, microbial diseases (Adhikari and G. C., 2022). This problem can be reduced by converting the ripe junars or its juice into wine. Hence, this study aims to utilize indigenous yeast culture from traditional starter murcha for the fermentation of junar juice. This study marks the first known attempt to modify this conventional starter culture for a high-acid, citrus-based substrate by using murcha-isolated native yeasts to ferment junar juice. Through the isolation, characterization, and use of native yeast strains, the study investigates their function in giving junar wine a unique sensory identity in addition to assessing their fermentation efficiency and ethanol production.

MATERIALS AND METHODS

The samples were transported to the food microbiology laboratory of National College, Lainchaur and processed accordingly.

Sample collection, transportation and processing:

Junar samples were collected from Kalimati market and transported to the laboratory. Similarly, 7 murcha samples (Chamati, Lubu, Panchthar, Pharping, Sankhu, Thali, and Thecho) were collected in a zip-lock bag and transported to the college laboratory.

Isolation and identification of yeast from murcha

The yeast strains were isolated using a pour plate method on yeast malt extract agar (YMA) (Pudasaini, 2012). The samples were grinded using sterilized mortar and pestle. One gram of finely crushed sample was mixed in 10 ml of sterile diluent and labelled as first dilution, 10^{-1}. The serial dilution was made to 10^{-6}. One ml of sample from each dilution was inoculated into a YMA plate. The plates were then incubated at 28°C for 48 hours. Identification of yeast was preliminarily done on the basis of cultural morphology and microscopic examination by Gram staining.

Screening of fermentative yeast strains

Glucose fermentation test was used to screen the fermentative yeast among the isolated yeast strains. The yeast isolated were inoculated in YM broth with inverted Durham's tube. The tube was incubated at 28°C for 48-72 hours. The changes (alcoholic smell, CO_2 production) inside the tube were observed. The strain that produced adequate CO_2 gas and a good odor was regarded as fermentative yeast.

Differentiation of wine and wild yeast strains

Best fermentative and flavor producing yeast strains were isolated and further subjected to select appropriate strain for junar wine fermentation. This test was based on the reduction test of 2,3,5 triphenyl tetrazolium chloride (TTC). The isolated yeast strains were spread in the wine yeast differential medium (WYDM). Then the plates were incubated at 28°C for 48 hours. The formation of red colonies in the media was observed (Pudasaini, 2012).

Sugar fermentation test of wine yeast strains

Sugar fermentation test was carried out in YMB (Yeast Extract Malt Extract Broth) which composed of 1% sugars (Glucose, Fructose Sucrose, Maltose, Lactose, Xylose, Galactose, Raffinose, Cellobiose and Arabinose) and indicator phenol red (Tsuyoshi *et al*., 2005). The isolates were inoculated separately on YMB and these tubes were incubated at 28°C for 7 days. The changes in color of media were observed (red to yellow).

Screening of ethanol and acid tolerant yeast strains

A loopful of yeast inoculum was inoculated in sterile YM broth and incubated at 28°C overnight. 300µl of overnight culture was inoculated in 10 ml of YM broth supplemented with ethanol at different concentrations (10%, 12.5%, 15%, 17.5% and 20%). Similarly, 300µl of overnight culture was inoculated in 10 ml of YM broth of different pH (2.5, 3.0, 3.5 and 4.0) respectively. All the tubes were then incubated at 28°C for 48 hours and the growth (optical density) was measured at OD_{600} with a UV spectrophotometer (Zhong *et al*., 2020).

Confirmative identification of ethanol and acid tolerant strain by 18S rRNA gene sequencing

Two potent ethanol and acid tolerant strains were sent to the laboratory of Macrogen Inc., Seoul, South Korea for 18S rRNA gene sequencing. According to it, the genomic DNA was isolated using the Prep Man Ultra reagent. 18s rRNA was amplified using universal primer NS1(5'-GTA GTC ATA TGC TTG TCT C-3') and NS24(5'-TCC GCA GGT TCA CCT ACG GA-3'). PCR was performed in a 20 µl total volume reaction containing 1X Taq PCR buffer, 0.5 pmol primers, 200 µM dNTPs, 2ng DNA template, 0.025 U/µl KOMA Taq polymerase. The amplified PCR products were purified using the Montage PCR clean up kit. The purified PCR product was then sequenced using the primer pair NS1(5'-GTA GTC ATA TGC TTG TCT C-3') and NS24(5'-TCC GCA GGT TCA CCT ACG GA-3'). The obtained sequencing results were analyzed using the Bio edit tool.

Junar fermentation

The fermentation of junar was done using protocol described by Patharkar *et al*. (2017). Briefly, the good quality junar fruits were collected and washed properly. The fruits were then peeled and squeezed to extract the juice. The juice was filtered through clean muslin cloth and was pasteurized at 90°C for 5 minutes. The TSS of pasteurized juice was adjusted to 24° Brix with the addition of table sugar. 50 ppm potassium metabisulphite (KMS) was added. Junar juice was fermented with the inoculated starter culture at a room temperature of <20°C. During the course of fermentation, the change in pH, acidity, TSS, reducing sugar and viable yeast load were monitored at an interval of 48 hours. When there was no change in the TSS of the fermentation medium, the fermentation was terminated. The sediment and wine were separated from the jar by siphoning. It was clarified using a 0.05% of bentonite solution, which was followed by chilling. The clarified juice was filled in the glass bottle with head space. Then pasteurization of the filled bottles was done at 72°C for 15 secs and it was cooled at room temperature. All the bottles were well labelled with their respective code. The junar wine was matured for about 3 months in a cool and dark place for flavor development.

Chemical analysis of junar juice and junar wine

Proximate analysis of junar juice like juice yield, moisture content, pH, TSS, acidity, reducing sugar and vitamin C content were carried out. Pour plating in YMA was done to check the growth pattern of yeast.

Juice yield

Randomly selected junars were weighted, squeezed in manual cone and cup extractor and filtered through 1 mm sieves (Schvab *et al*., 2015). The volume obtained were measured and the percentage of juice yield were calculated as:

$$\text{Juice yield} = \frac{\text{Volume(ml)} * 100}{\text{Weight(g)}}$$

Moisture content

The moisture content of junar juice was determined by using the hot-air oven method given by KC and Rai (2007). Three clean and dried petri dishes were taken. About 10 grams of junar sample was weighed in each dish by difference. The samples were placed in a hot air oven set at 103±2°C. The weight of the plate in every hour was noted until two consecutive weights differ only by ±5mg. Before each weighing, the dish was cooled in a dessicator.

$$\text{Moisture content (\%)} = \frac{\text{Initial wt.-Final wt.} * 100}{\text{Volume of sample}}$$

Determination of pH

For the determination of pH, the pH meter was calibrated with buffers of pH 4.0 and 7.0. The pH meter was washed with distilled water several times to remove the buffer. The pH was determined by dipping the electrode of the pH meter in the sample. About 10 ml of sample (junar juice and wine) was taken in a clean conical flask. Then a glass electrode was directly dipped in the wine sample and the readings were taken (AOAC, 1990).

Total soluble solid (TSS)

The change in TSS during fermentation was determined with the help of a refractometer. Firstly, the refractometer was rinsed with the distilled water to adjust the brix. Then the surface of the refractometer was wiped with the clean blotting paper. One drop of sample was placed on the surface of the refractometer with a micropipette. The reading was taken by looking into the eyepiece against the direct sunlight (Kirk and Sawyer, 1999).

Titrable acidity

The acidity was measured in terms of citric acid. Ten ml of the wine sample was taken in a beaker by using a sterile pipette. Then 2-3 drops of indicator phenolphthalein solution were added. It was titrated with freshly prepared 0.1 N NaOH solution. The NaOH solution was first standardized with 0.1 N oxalic acid. The end point was determined by the appearance of pink color (AOAC, 1990). The volume of NaOH consumed was noted and acidity was determined using following formula:

$$\text{Titrable acidity} = \frac{\text{Titer*Normality of alkali*Eq. wt. of citric acid*100}}{\text{Volume of sample taken (ml)*1000}}$$

The equivalent weight of citric acid is 64.04.

Reducing sugars

Reducing sugars were determined by Lane and Eynon methods as described by KC and Rai (2007).

Sample Preparation

Exactly 25 ml of sample was taken in clean beaker and neutralized with dil. NaOH solution using phenolphthalein indicator and it was transferred to the volumetric flask of 250 ml. The Carrez solution I and II 10/10 ml was added to the sample and shaked for 1 min. The volume was made up to 150 ml by adding distilled water and the mixture was then filtered through the filter paper to get a clear extract.

Standardization of Fehling's solution

The Fehling's A and B of 5-5ml was pipetted out in porcelain dish and mixed properly by addition of 50 ml of water to it, the Fehling's solution was heated to boiling condition and rapidly titrated with standard dextrose from burette until the color of the Fehling's solution almost vanishes. After it, about 3 drops of methylene blue was added to it, the solution turned to blue color again. The solution was boiled and titrated against dextrose solution until blue color vanishes, then the solution turned to brick red precipitate. The titer was noted, and it was taken as a trial titer.

Similarly, Fehling's A and B of 5-5ml was pipetted out in porcelain dish and 50 ml of water was added to it. The standard dextrose was added to the Fehling's solution without heating to represent 80% of trial titer. Then the mixture was boiled for 2 min and three drops of methylene blue indicator was added to it and it was titrated with standard dextrose to colorless end point without removing the flask from the burner and the actual titer was noted.

The same process of standardization of Fehling's solution (trial and actual) were repeated using the sample filtrate in place of standard dextrose.

Calculation

$$\text{Fehling Factor} = \frac{\text{Actual titer} \times \text{mg reducing sugar per ml}}{1000}$$

$$\%\ \text{Reducing sugar} = \frac{\text{Fehling factor} \times \text{Dilution} \times 100}{\text{Aliquot titer} \times \text{Sample volume}}$$

L-Ascorbic acid content

The ascorbic acid content of junar wine samples was determined by 2,6 Dichlorophenol indophenol titration method as described by Ranganna (2009).

Yeast count during fermentation

1 ml of sample was pipetted out from the fermentation jar at the interval of two days till the end of fermentation. It was serially diluted up to 10^{-8} dilution using sterile peptone water. 0.1 ml of sample from each 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} dilution was spread plated on a YMA plate and incubated at 28°C for 48 hours. Then, colony forming units were enumerated.

Quality evaluation of Junar wine

Ethanol content

The ethanol content of the samples was measured by a specific gravity method (KC and Rai, 2007).

Methanol content

Methanol content of the orange wine samples was calculated by colorimetric method as described by Kirk and Sawyer (1999).

Determination of antioxidant activity (AA)

DPPH radical-scavenging assay

50µl of diluted wine samples (5-fold dilution) and ethanol (standard) was pipetted out in a separate well of 96 well plates (Moreno-Arribas and Polo, 2009). 800µl of DPPH solution in methanol (0.004% w/v) was added to each well. The mixtures were incubated in the dark at room temperature for 30 min. Absorbance was measured at 515 nm. The antioxidant activity was calculated using the formula:

$$\%\text{Discoloration} = \frac{A_c - A_s * 100}{A_C}$$

where A_C was the control sample absorbance mean value and A_S, the sample absorbance mean value.

Total phenolic content

The total phenolic content in wine was measured using the Folin-Ciocalteu method (Mitrevska *et al*., 2020). Briefly, 20 µl of the sample and standard was pipetted out in 96 well plates. 780 µl of distilled water and 50 µl of Folin-Ciocalteu reagent was added to each well. After 1 min, 150 µl of 20% (w/v) Na_2CO_3 was added. The mixtures were then incubated in the dark at room temperature for 1 hour. After incubation, absorbance was measured at 750 nm. Gallic acid (concentration within a range of 31.25-500 mg/L) was used as standard.

Total flavonoid content

Total flavonoid content was determined by using the aluminium chloride assay (Mitrevska *et al*., 2020). 1 ml of sample and standard was pipetted out in a conical flask and mixed with 4 ml of distilled water and 0.3 ml of 5% (w/v) $NaNO_2$. The mixture was then mixed thoroughly for 5 minutes. 0.3 ml of 10% (w/v) $AlCl_3$ was added, followed by 2 ml of 1 M NaOH. The contents of the volumetric flask were immediately diluted with distilled water to a volume of 10 ml. Absorbance was measured at 510 nm. Catechin (concentration within the range of 20-100 mg/L) was used as a standard.

Sensory evaluation

The sensory evaluation was carried out using a 9 points hedonic rating described by Ranganna (2009). Different parameters like color, smell, taste, mouth feel and overall acceptability were examined.

RESULTS

The isolated yeasts were found to be creamy white in color, circular in shape and smooth in consistency. However, the shape, margin and elevation of colonies largely varied. The size varied from 1 mm to 3 mm and the elevation varied from flat, convex to raised. On the microscopic examination, all of the cells were Gram positive, oval shaped and budding (Table 1).

Table 1: Cultural and morphological characteristics of yeast isolates in YMA plate

Isolates	Size	Shape	Color	Consistency	Sliminess	Margin	Elevation
Ch1	2mm	Circular	White	Soft	Viscous	Entire	Convex
Ch2	3mm	Circular	Creamy white	Soft	Viscous	Entire	Raised
Lb3	3mm	Circular	Pale yellow	Soft	Viscous	Entire	Raised
Lb4	2mm	Circular	Pale yellow	Soft	Viscous	Entire	Raised
Pn1	1.5mm	Circular	Creamy white	Soft	Viscous	Entire	Raised
Pn4	1.5mm	Circular	Creamy white	Soft	Viscous	Entire	Raised
Ph2	1mm	Circular	White	Soft	Dry	Entire	Flat
Ph5	1mm	Circular	White	Soft	Dry	Entire	Flat
Ta2	2mm	Circular	White	Soft	Viscous	Entire	Convex
Ta6	2mm	Circular	White	Soft	Viscous	Entire	Raised
Te1	1mm	Circular	White	Soft	Viscous	Entire	Convex
Te2	1mm	Circular	Pale yellow	Soft	Viscous	Entire	Convex
Sn2	1.5mm	Circular	Pale yellow	Soft	Viscous	Entire	Convex
Sn3	1.5mm	Circular	Pale yellow	Soft	Viscous	Entire	Convex

Screening of fermentative yeast from isolated yeasts

Among 32 isolates, 14 yeast isolates (Ch1, Ch2, Lb3, Lb4, Pn1, Pn4, Ph2, Ph5, Sn2, Sn3, Ta2, Ta6, Te1 and Te2) were found to be fermentative i.e., both CO_2 gas and alcohol producer. 7 yeast isolates (Lb1, Lb2, Pn2, Pn3, Te3, Ch3, Ch4) produced only alcohol and 7 yeast isolates (Pn6, Te4, Te5, Te6, Ta3, Ta4, Ta5) were only gas producers. 4 yeast isolates (Lb5, Pn5, Sn4, Ph1) were found to be non-fermentative.

Differentiation of wine and wild yeast

Among tested yeast isolates, 50% yeast isolates (Ch1, Ch2, Lb3, Pn1, Pn4, Ta6 and Te2) were found to be wine yeast (red colored colonies) and 50% isolates (Lb4, Ph2, Ph5, Ta2, Te1, Sn2 and Sn3) were found to be wild yeast (pink colonies).

Sugar fermentation test for identification of yeasts

5 yeast isolates were identified as *Saccharomyces spp* (ferment arabinose, dextrose, fructose, galactose, maltose, raffinose, sucrose), 1 isolate as *Endomycopsis spp* (ferment arabinose, cellulose, dextrose, fructose, galactose, maltose, raffinose) and the remaining as *Pichia spp* (ferment dextrose, fructose, galactose, and xylose) as shown in Table 2.

Table 2: Fermentation of different sugars by selected wine yeast isolates

Isolate code	Ara	Cel	Dex	Fru	Gal	Lac	Mal	Raf	Suc	Xyl	Probable yeast
Ch1	+	-	+	+	+	-	+	+	+	-	*Sachharomyces spp*
Ch2	+	-	+	+	+	-	-	+	+	w	*Saccharomyces spp*
Lb3	w	+	+	+	+	-	+	+	-	-	*Endomycopsis spp*
Pn1	+	-	w	+	+	-	+	+	+	w	*Saccharomyces spp*
Pn4	+	-	+	+	+	-	+	w	+	-	*Saccharomyces spp*
Ta6	+	-	+	+	+	-	+	+	+	w	*Saccharomyces spp*
Te2	-	-	+	+	+	-	-	-	-	w	*Pichia spp*

(Note: + = fermentation, - = non-fermentation, w = weak fermentation; Sugars: Ara = Arabinose, Cel = Cellobiose, Dex = Dextrose, Fr = Fructose, Gal = galactose, Lac = Lactose, Mal = Maltose, Raf = Raffinose, Suc = Sucrose, Xyl= Xylose)

Ethanol tolerance test

The maximum cell growth obtained at 10% ethanol was dramatically decreased with increase in ethanol concentration to 12.5-20%. The OD (Ch1) of 1.4 obtained at 10% ethanol concentration was decreased by 28.9%, 61.6% and 76.8% at 12.5%, 15% and 20% ethanol respectively. The OD of Pn4 isolate was 1.24 at 10% followed by 0.14 at 20% ethanol concentration. Ch1 isolate showed the highest ethanol tolerance capacity followed by Pn4, compared to all other isolates (Figure 1).

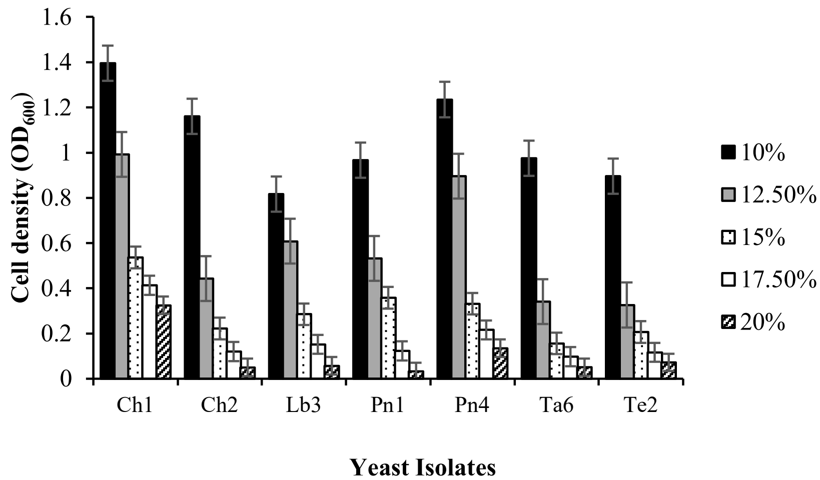


Figure 1: Cell density of selected yeast isolates after 48 hours of incubation in YM broth containing different ethanol concentration i.e., 10%, 12.5%, 15%, 17.5% and 20%

Acid tolerance test

All the isolates, except Ch1, showed reasonable growth at the tested 2.5 pH condition. Ch1 showed the cell growth of 1.75, 1.69, 1.54 OD at pH 4, 3.5 and 3 respectively, which sharply decreased to 0.73 OD at pH 2.5. In contrast, Pn4 showed the highest cell growth of 1.87, 1.65, 1.56 and 1.5 at pH 4, 3.5, 3 and 2.5 with highest acid tolerance capacity (Figure 2).

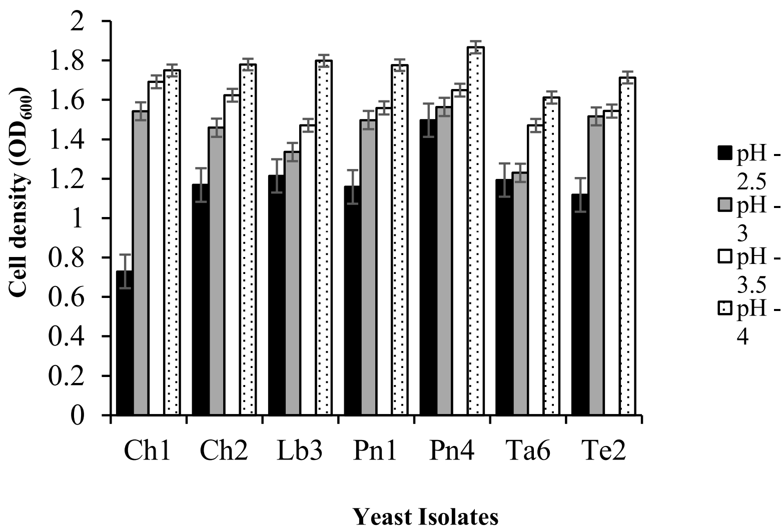


Figure 2: Cell density of selected yeast isolates after incubation of 48 hours in YMB medium at varying pH values i.e., 2.5, 3, 3.5, and 4

Sequencing results:

The quality of the obtained sequencing results was checked and contig was developed. The contig was then blasted using the NCBI blast server. The contig of *Saccharomyces* isolates Ch1 was found to be matched with 18S rRNA sequence of *S. cerevisiae* (Figure 3).

Table 3: 18S rRNA sequencing of potent ethanol tolerant strain Ch1

Accession	Description	Length	Start	End	Coverage	Bit	E-value	Match/Total	Pct. (%)
MG101834.1	*Saccharomyces cerevisiae*	1700	22	1684	97	3072	0.0	1663/1663	100.00

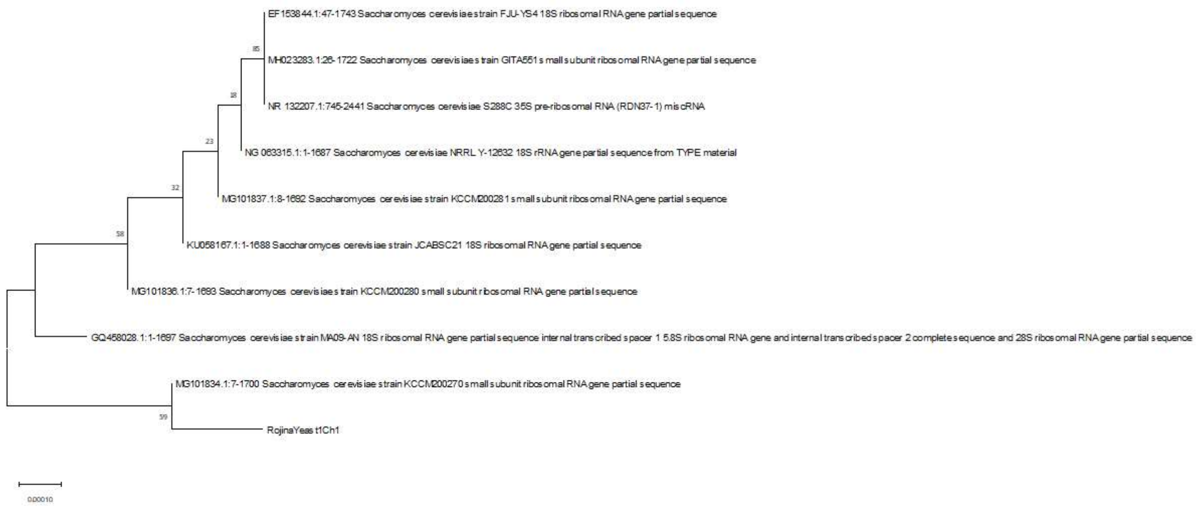


Figure 3: The position of strain *Saccharomyces* isolate Ch1 (RojinaYeast1Ch1) among its closely related organisms can be seen in the phylogenetic tree created using MEGA11 based on 18s rRNA sequences. Numbers in parentheses represent the nucleotide sequences in GenBank.

Table 4: Proximate analysis of junar

S.N.	Distinctive features	Amount
1	Juice yield	56.09%
2	Moisture content	90.726%
3	pH	4.01
4	Total soluble solid	11.2˚Brix
5	Titrable acidity	0.922%
6	Vitamin C content	34.37%

Growth pattern of yeast during fermentation

Approximately 8.6 log CFU/ml and 8.34 log CFU/ml load of *S. cerevisiae*, Ch1 and *S. spp* Pn4 separately, was inoculated for fermentation. The changing pattern in load (both cases) during fermentation was similar. In case of Ch1 fermentation, the load of *S. cerevisiae* Ch1 decreased to 8.45 during 3 days. The load then varied widely till 9 days. Then after the load was gradually decreased and reached 8.16 till the end of fermentation. Similarly, in case of Pn4 fermentation, the load of *Sachharomyces spp* Pn4 decreased from 8.34 to 8.05 till the end (Figure 4).

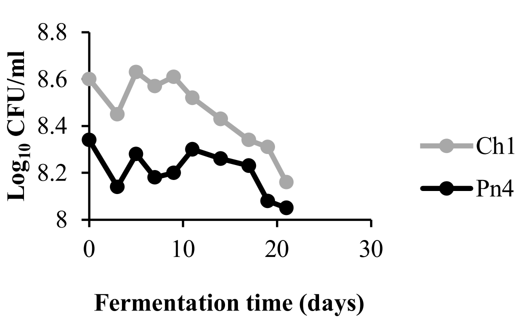


Figure 4: Changes in yeast load count at 2-day intervals during wine fermentation process

Change in pH

The initial pH during Ch1 and Pn4 fermentation was 4.23. The changing pattern of pH during each fermentation was found to be similar. In case of Ch1 fermentation, pH decreased to 3.91 during 9 days of fermentation. Afterwards, the pH remained almost constant at 3.97-3.98. Interestingly, in the case of Pn4, the decrease in pH was very sharp during 9 days of fermentation as compared to Ch1 fermentation. The pH decreased to 3.73 and then remained constant at 3.75-3.81 till the end (Figure 5).

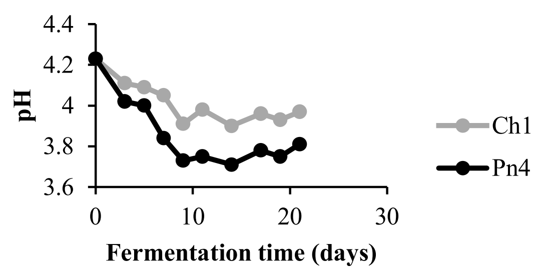


Figure 5: pH variations of the wines during fermentation at 2-day intervals

Change in reducing sugar

The initial reducing sugar content of both fermentations were 3.19%. The reducing sugar percentage then gradually increased and reached to 4.7% and 8.9% in case of Ch1 and Pn4 fermentation respectively. Afterwards, the sugar content eventually decreased and finally reached to 0.34 % (in case of Ch1) and 1.6 % (in case of Pn4) at the end of fermentation. Interestingly, comparing the both fermentations, the increase in sugar content was significantly higher (47.19%) in Pn4 compared to Ch1 fermentation. Similarly, the reduction rate (89.34%) was found to be significantly higher in Ch1 fermentation (Figure 6).

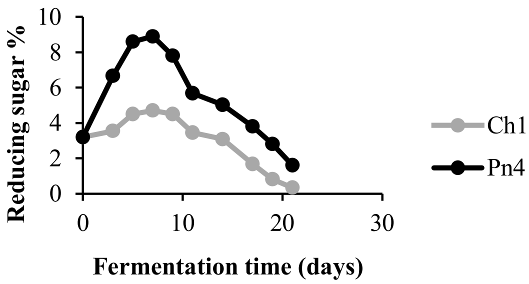


Figure 6: Reducing sugar variations of the wines during fermentation at 2-day intervals

Change in TSS

The initial TSS content of junar juice was maintained at 22 ^{0}Brix. There was a rapid decrease in TSS during 3 days of fermentations (Ch1 and Pn4). Then after, there was slow decrease in TSS (in case of Pn4) throughout the fermentation while decrease in TSS (in case of Ch1) was faster. The final TSS content of Ch1and Pn4 fermentations were 8.6 ^{0}Brix and 9 ^{0}Brix respectively (Figure 7).

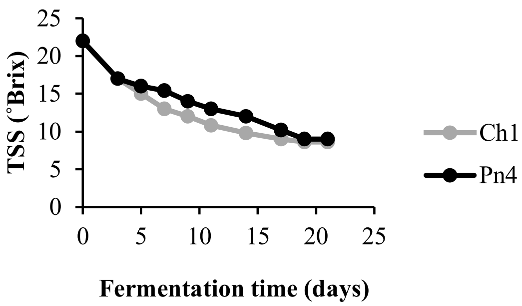


Figure 7: TSS variations of the wines during fermentation at 2-day intervals

Change in titrable acidity

The initial titrable acidity of junar juice was 0.54% as of citric acid. The titrable acidity gradually increased up to 0.85% and 0.96% during 8-9 days of both fermentations. The acidity then varied widely (decrease and increase) till the end of fermentation. It finally reached 0.75% (in case of Ch1) and 0.84 % (in case of Pn4) at the end (Figure 8).

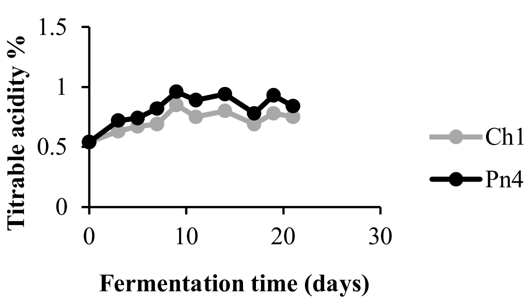


Figure 8: Titrable acidity variations of the wines during fermentation at 2-day intervals

Chemical properties of wine

The vitamin C content, ethanol content, methanol content, antioxidant activity and total phenolic content were found to be almost similar for both Ch1 and Pn4 wines. In contrast, total flavonoid content was found to be higher in Pn4 wine (12.40 %) respectively.

Table 5: Comparison between Ch1 and Pn4 junar wines

Parameters	Junar Wine Ch1	Junar Wine Pn4
Ethanol	8.3%	7.8%
Vitamin C	25%	23.5%
Methanol content	0.019%	0.013%
Antioxidant activity	22.61%	21.17%
Total phenolic content	484.11 mg GAE/L	480.78 mg GAE/L
Total flavonoid content	110.12 mg CE/L	125.72 mg CE/L

Sensory evaluation

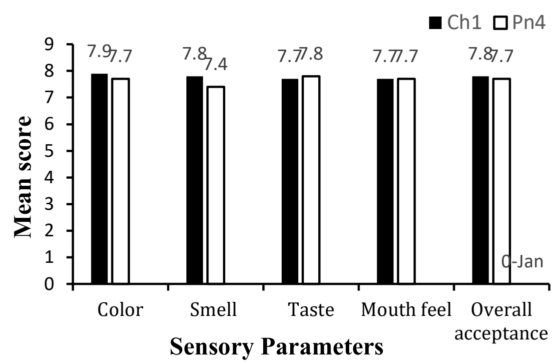
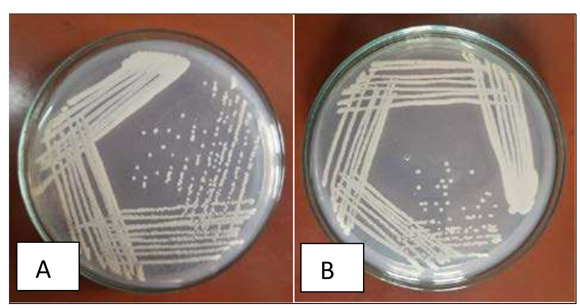


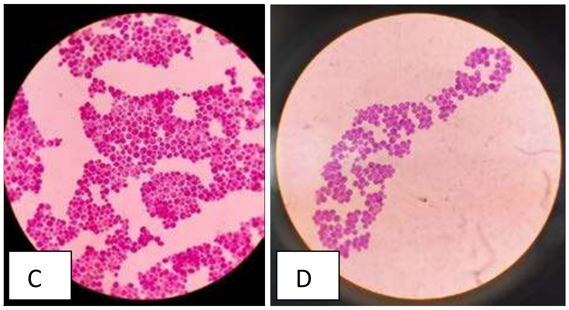
Figure 9: Mean scores for sensory parameters of Ch1 and Pn4 junar wines based on evaluations from 10 panelists

Among the two laboratory prepared wines, junar wine Ch1 was preferred to Pn4. The determined overall acceptability of CH1 wine and Pn4 wine were 7.78 and 7.66 respectively (Figure 9).

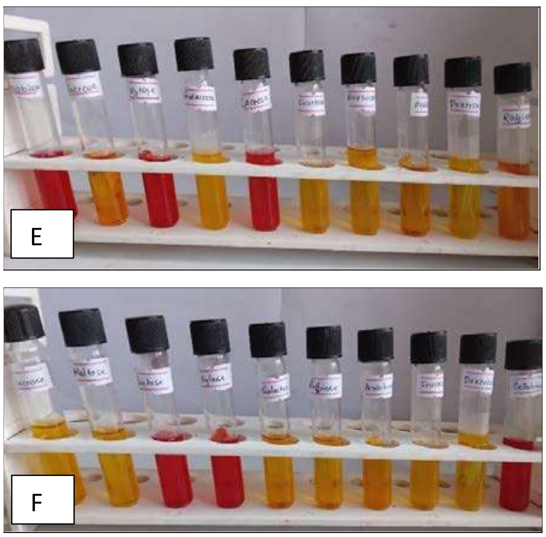
PHOTOGRAPHS



Photograph 1: Colonies of yeasts on YMA after 24-48 hours of incubation at 28°C. **A**- colonies of Ch1 isolate **B**- colonies of Pn4 isolate.



Photograph 2: Microscopic observation of yeast on 100X after Gram staining. **C**- spherical shaped cells of Ch1 isolate **D**- oval shaped cells of Pn4 isolate.



Photograph 3: Results of sugar fermentation test of yeast isolates. **E**- sugar fermentation test of *Saccharomyces cerevisiae* Ch1 **F**- sugar fermentation test of *Saccharomyces spp* Pn4.

Photograph 4: Junar wine prepared in the lab. **G**- Junar wine (Ch1) prepared using *S. cerevisiae* Ch1 **H**- Junar wine (Pn4) prepared using *Saccharomyces spp* Pn4.

DISCUSSION

The use of *S. cerevisiae* as starter culture is the most widespread practice in winemaking (Zhang *et al*., 2023). The key qualities of wines like ethanol concentration, aroma, flavor largely depends upon the juice types and metabolism capability of yeast strain i.e., metabolism of juice sugars and amino acid into ethanol and other aroma compound like terpenes, volatile phenols, acids, ketones, aldehydes. Therefore, the isolation and development of novel wine yeast isolates for wine fermentation always play a key role in commercial production of wine.

In this study, the citrus wine fermentation was studied using the junar fruit and new isolates of *S. cerevisiae* from indigenous starter murcha. Altogether 14 fermentative yeast strains were isolated from 7 murcha samples collected from different places (Chamati, Lubu, Panchthar, Pharping, Sankhu, Thali, and Thecho) of Nepal. Among them, only two wine yeast isolates Ch1 (ethanol tolerant strain) and Pn4 (acid tolerant strain) were identified through 18s rRNA gene sequencing. Only Ch1 isolate was identified as *Sachharomyces cerevisiae*.

S. cerevisiae Ch1 and *Saccharomyces spp* Pn4 isolates were used for junar wine preparation. In this study, the ethanol concentration of Ch1 and Pn4 wine were 8.3% and 7.8%, respectively. Patharkar and Rathod (2019) also reported the similar ethanol content in citrus wine. In contrast, Xu *et al* (2013) reported slightly higher, 8.3-13.9%, ethanol content in citrus wine. The difference in ethanol concentration between wines in this and other studies could be mainly due to the different fermentation capability of the yeast isolates used (Duarte *et al*., 2010).

The methanol content is an important indicator in determining the quality of wine. Methyl alcohol is considered as one of the harmful by-products of the alcoholic fermentation and can deteriorate the optical nerve thereby causing blindness (Ribereau-Gayon *et al*., 2006). The methanol content of junar wines were found to be 0.019% in case of Ch1 wine and 0.013% in case of Pn4 wine. Additionally, The BAFT limit of methanol content in wine is less than 1,000 mg/L (0.1%) (Zoeckein *et al*., 1999).

In this study, the ascorbic acid content of Ch1 and Pn4 junar wine were 25% and 23.5% respectively. Schvab *et al* (2015) reported the similar result i.e., 25.52%. In contrast, Patharkar *et al*. (2017) reported 14.4-21.7% higher percentage of ascorbic acid concentration in citrus wine as compared to this study. The ascorbic acid content was found to decrease in wine as compared to its juice. This may have resulted due to its degradation during pasteurization of the juice (Schvab *et al*., 2015).

Regarding the flavonoid content, Pn4 wine had higher flavonoid content than Ch1 wine. The flavonoid content of Ch1 and Pn4 wine were 110.12 mg CE/L and 125.72 mg CE/L. Zhu *et al* (2019) also reported similar flavonoid content in junar wine (119.1 mg/L). Kelebek *et al* (2009) reported higher flavonoid content, 14.3%-21.5% in their prepared wine (133.8±9.96 mg/LThe flavonoid content of Ch1 and Pn4 wine were higher compared to wine prepared from other non-grape fruits and grape fruits (Pandeya *et al*., 2018). This difference in flavonoid content might be due to the differences in the varieties of substrate used.

The phenolic content of Ch1 and Pn4 wine were 484 mg GAE/L and 480.78 mg GAE/L. Mascarin *et al*. (2023) also reported similar phenolic content, 459.4 mg GAE/L. In contrast, Kelebek *et al*. (2009) reported lower phenolic content (163-177 mg GAE/L) compared to this study. Similarly, Barreto *et al*. (2023) also reported lower phenolic content (154.17-165.47 mg GAE/L) in his study. The observed difference in phenolic content between our and other studies could be related to the metabolic capacity of yeast microbes, and pH in the fermentation environment (Zhang *et al*., 2023).

The antioxidant activities of both Ch1 and Pn4 wine were almost similar i.e., 22.61% and 22.17% respectively. However, Schvab *et al*. (2015) reported higher antioxidant activities, 33.02-53.79% in their prepared wine. Similarly, the reported flavonoid content of wine was higher compared to wine prepared from other non-grape fruits and grape fruits (Pandeya *et al*., 2018). The difference in antioxidant activities could be mainly related to variation in the phenolics and flavonoids content. Higher the phenolics and flavonoids content of fruits, higher will be the antioxidant properties (Lebedev *et al*., 2022).

A limitation of this study is that junar wine fermentation using commercial wine yeast was not conducted, which would have allowed a direct comparison with wines produced using *murcha*-derived yeasts. According to this study, indigenous starter murcha could be a suitable source for the isolation of *Saccharomyces cerevisiae* and Ch1 could be a potential isolate for junar wine fermentation. The junar wine prepared in the laboratory was organoleptically acceptable. Moreover, Junar wine being preferable through sensory analysis, ensures that junar wine can be produced, consumed and commercialized.

CONCLUSIONS

Wine yeast (*S. cerevisiae*) derived from Nepalese indigenous starter murcha was used for the junar wine production. The Ch1 and Pn4 junar wine had ethanol content as 8.3% and 7.8% respectively. The wine produced was acceptable, as well as meeting all the standards required by a good wine in terms of physiochemical and sensory attributes of color, flavor, taste, aroma, and overall acceptability for junar wine. This study concluded that wine yeast from murcha was good strain for the production of the junar wine. This study, which is the first scientific attempt to use murcha yeasts in the production of junar wine, not only advances our understanding of citrus wine enology but also emphasizes the significance of protecting and valuing Nepal's natural microbial resources.

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CRediT author

RM: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing- Original Draft, Writing- Review & Editing, Visualization, Supervision, Project administration, Funding acquisition; **TBK:** Conceptualization, Writing- Review & Editing, Supervision; **SL:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Resources, Writing- Review & Editing, Visualization, Project administration

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Adhikari A., and G.C. Bigyan (2022). Identification of Problems of Sweet Orange A Case Study from Pyuthan Municipality, Pyuthan. International Journal of Applied Sciences and Biotechnology 10(2), 134-139. DOI: https://doi.org/10.3126/ijasbt.v10i2.46190

AOAC, (1990). Official methods of analysis. 18th edition. Association of official analytical chemists, Washington DC.

Barreto, S. M. A., Silva, A. B. M., Dutra, M. C. P., Bastos, D. C., Carvalho, A. J. B. A, Viana, A. C., Narain, N., and Lima, S. (2023). Effect of commercial yeasts (*S. cerevisiae*) on fermentation metabolites, phenolic compounds and bio accessibility of Brazilian fermented oranges. Food Chemistry 408(135121), 1-9.

Csernus, O., Pomazi, A., and Magyar, I. (2014). Isolation, characterization and selection of wine yeast strains in Etyek-Buda wine district, Hungary. Acta alimentaria 43(3), 489-500.

Duarte, W. F., Dias, D. R., Oliveira, M. J., Teixeira, J. A., Silva, J. D., and Schwan, R. F. (2010). Characterization of different fruit wines made from Cocoa, Cupuassu, Gabiroba, Jaboticaba and Umbu. Food Science and Technology 30:1-9.

Iglesias, D. J., Cercós, M., Colmenero-Flores, J. M., Naranjo, M. A., Ríos, G., Carrera, E., Ruiz-Rivero, O., Lliso, I., Morillon, R., Tadeo, F. R., and Talon, M.

(2007). Physiology of citrus fruiting. Brazillian Journal of Plant Physiology 19(4), 333–362.

Jackson, R. S. (2000). Wine science: principles, practice and perception, Academic Press, California, USA pp 144-427.

Kaini, B. R. (2013). Sweet orange production and post-harvest management. Central Junar Cooperative Federation, Tinkune, Kathmandu.

Karmacharya, J., Shrestha P., Karki T. B., and Panta, O. P. (2024). Isolation and Identification of Yeasts in Murcha, a Rice Wine Starter Culture from Nepal. International Journal of Food Science 4188578, 1-8.

KC, J. B., and Rai, B. K. (2007). Basic food analysis handbook, 1st edition. pp 64-89.

Kelebek, H., Selli, S., Canbas, A., and Cabaroglu, T. (2009). HPLC determination of organic acids, sugars, phenolic compositions and antioxidant capacity of orange juice and orange wine made from a Turkish cv. Kozan. Microchemical Journal 91(2), 187–192.

Kirk, R. S., and Sawyer, R. (1999). Pearson's composition and analysis of foods. 9th edition, Addison Wesley Longman Ltd., England 608-640.

Lebedev, V. G., Lebedeva, T. N., Vidyagina, E. O., Sorokopudov, V. N., Popova, A. A., Shestibratov, K. A. (2022). Relationship between Phenolic Compounds and Antioxidant Activity in Berries and Leaves of Raspberry Genotypes and Their Genotyping by SSR Markers. *Antioxidants*. 11(10), 1961.

Machamangalath, R., Arekar, C., and Lele, S. S. (2016). Exotic tropical fruit wines from *Garcinia indica* and *Musa acuminata*. Jourhal of Institute of Brewing 122(4), 745–753.

Mascarin, L. G., Franco, F. W., Dornelles, R. C., Figueredo, K. C., Santos, R. O., Bauermann, L. F., Emanuelli, T., Somacal, S., and Sauter, C. K. (2023). Effect of adding *Matricaria recutita* L., *Cymbopogon citratus*, or *Mentha piperita* L. extracts to fermented orange beverage: sensory evaluation, physicochemical characterization and prediction of toxic risks and biological activity in silico. Multidisciplinary Digital Publishing Institute 12(243), 1-14.

Mitrevska, K., Grigorakis, S., Loupassaki, S., and Calokerinos, A. C. (2020). Antioxidant activity and polyphenolic content of North Macedonian wines. Applied Sciences 10, 1-11.

Moreno-Arribas, M. V., and Polo, M. C. (2009). Wine chemistry and biochemistry. Springer science business media, LLC 3-26.

Nasir, A., Rahman, S. S., Hossain, M. M. and Choudhary. N. (2017). Isolation of Saccharomyces cerevisiae from pine apple and orange and study of metal's effectiveness on ethanol production. European Journal of Microbiology and Immunology 7(1), 76-91.

Ogodo, A. C., Ugbogu, O. C., Ugbogu, A. E., and Ezeonu, C. S. (2015). Production of mixed fruit (pawpaw, banana, and watermelon) wine using *Saccharomyces cerevisiae* isolated from palm wine. SpringerPlus, 4(1), 1–11.

Pakuwal, E., and Manandhar, P. (2020). Production of rice based alcoholic beverages and their quality evaluation. Journal of Food Science and Technology Nepal, Vol. 12, 38 – 49.

Pandeya, A., Rayamajhi, S., Pokhrel, P., and Giri, B. (2018). Evaluation of secondary metabolites, antioxidant activity, and color parameters of Nepali wines. Food Science and Nutrition 6(8), 2252–2263.

Patharkar, S. R., and Rathod, M. S. (2019). Optimization of mixed culture fermentation conditions for the development of orange (*Citrus reticulate* Blanco) wine. International Journal of Chemical Studies 7(1), 1176-1180.

Patharkar, S. R., Shendge, S. N. and Khapre, A. P. (2017). Study of physio-chemical parameters of orange (Citrus reticulate Blanco) for the development of orange wine. Journal of Applied and Natural Science 9(4), 2305-2308.

Paudyal, K. P., and Subedi, H. (2008). Selection of sweet orange (*Citrus sinensis* Osbeck) varieties for production period expansion. Nepalese Horticulture 6, 66-74.

Pudasaini, S. (2012). Development of pure culture fermentation of rice wine and millet wine (jaand). M.Sc. Dissertation Submitted to Central Department of Microbiology, Tribhuvan University, Nepal.

Ranganna, S. (2009). Handbook of analysis and quality control for fruits and vegetable products. 2nd edition. Tata McGraw-Hill publishing com. Ltd, New Delhi pp 80-106.

Ribereau-Gayon, P., Glories, Y., Maujean, A., and Dubourdieu, D. (2006). Handbook of enology: Volume 2 (Second ed.). The chemistry of wine stabilization and treatment. https://doi.org/10.1002/0470010398

Schvab, M. del C., Ferreyra, M. M., Davies, C. V., Stefani, A., Cayetano, M.C., Gerard, L. M., and Gonzalez, R. F. (2015). Effects of orange winemaking variables on antioxidant activity and bioactive compounds. Food Science and Technology 35(3), 407–413.

Shravan, R., Shere, D. M., and Joshi, M. (2018). Study of physico-chemical characteristics of sweet orange (*Citrus sinensis*) fruit. Journal pharmacognosy and phytochemistry 7(6), 1687-1689.

Tsuyoshi, N., Fudou, R., Yamanaka, S., Kozaki, M., Tamang, N., Thapa, S., and Tamang, J. P. (2005). Identification of yeast strains isolated from marcha in Sikkim, a microbial starter for amylolytic fermentation. International Journal of Food Microbiology 99(2), 135–146.

USDA (2018). https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf

Xu, W., Wang, J., and Zhang, D. (2013). Isolation of *Saccharomyces cerevisiae* GJ03 from the spontaneous fermentation of orange wine and application in the course of orange wine brewing. Advanced Materials Research 690-693, 1345-134.

Yeshaneh, A. (2024). Production and optimization of wine from mixed (Banana and Watermelon) fruits using Sacchromys Crevice. International Journal of Food Science and Technology 9(3), 46-57.

Zhang, Y., Ye, H., Zou, Y., He, Z., Xu, B., Wang, S., Peng, C., Zhou, X., Zhang, Q., Xiang, W., Cai, T., and Tang, J. (2023). Flavor Characteristics of Navel Orange Wine Fermented by *Saccharomyces cerevisiae* SC-125 and Angel Yeast SY. Fermentation 9, 872.

Zhong, W., Chen, T., Yang, H., and Li, E. (2020). Isolation and selection of non- saccharomyces yeasts being capable of degrading citric acid and evaluation of its effect on kiwifruit wine fermentation. Multidisciplinary Digital Publishing Institute 6(25), 5–9.

Zhu, X., Tan, A., Fan, J., LiZ, Zhou q., Yi, X., Li, G., Guo, L., Huang, L., and Wang, H. (2019). Optimization of process conditions and quality analysis of fermented wine with sweet orange pulp residue. Food and Fermentation Industries 45(21), 207-214.

Zoecklein, B. W., Fugelsang, K. C., Gump, B. H. and Nury, F. S. (1999). Wine analysis and production. Springer science and business media: New York 107-108.